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STUDY ON ANTIOXIDANT POTENTIAL, FTIR AND GC-MS ANALYSIS OF *CRYPTOSTEGIA GRANDIFLORA (Roxb.) R.Br*

A short term project work submitted to

St. Mary's College(Autonomous) affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY

in partial fulfilment of the requirements for the Degree of

Bachelor of Science in **BOTANY**

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CERTIFICATE

It is certified that this short term project work entitled "**STUDY ON ANTIOXIDANT POTENTIAL, FTIR, AND GC-MS ANALYSIS OF *CRYPTOSTEGIA GRANDIFLORA (Roxb.) R.Br*** submitted to **St.Mary's College (Autonomous)** affiliated to **MANONMANIAM SUNDARANAR UNIVERSITY** in partial fulfilment of the requirements for the degree of Bachelor of Science in Botany, and is a record of work done in the Department of Botany, St. Mary's College (Autonomous), Thoothukudi during the year 2020-2021 by the following students.

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It is our humble attempt to present this **"STUDY ON ANTIOXIDANT POTENTIAL, FTIR, AND GC-MS ANALYSIS OF *CRYPTOSTEGIA GRANDIFLORA (Roxb.) R.Br.*"** First and foremost our sincere gratitude belongs to **Dr. Sr. A.S.J.LUCIA ROSE M.Sc., B.Ed., M.Phil., PGDCA, Ph.D.** principal, St.Mary's College (Autonomous) for providing an opportunity to do this project.

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INTRODUCTION

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India is a tropical country with the rich diversity of medicinal plant resources. Of the 17,000 flowering plants in India nearly 8000 are identified as medicinal plants. Medicinal plants play an important role in supporting health care in India. According to World Health Organisation (WHO) (2002) 80% of the rural population in developing countries rely on locally available medicinal plants for their primary health care. Medicinal plants are important therapeutic aid for the alleviation of ailments of humankind. These plants are used in herbal medicine, cosmetology and nutraceuticals. People are becoming more aware of medicinal plants and many of them utilize these therapeutic interventions and their products for maintaining health and for preventing diseases with an eco-friendly touch. Medicinal plants are potentially renewable. For conservation and sustainable use of medicinal plants, a long-term integrated, scientific plan needs to be adopted.

Historically, plants (fruits, vegetables, medicinal herbs, etc.) have provided a good source of a wide variety of compounds, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids and some other secondary metabolites, which are rich in valuable bioactivities like antioxidant, anti-inflammatory, antitumor, antimutagenic, anti-carcinogenic, antibacterial, or antiviral activities. (Maridass and Britto, 2008).

Antioxidants are capable of preventing oxidative damage, the wide use of natural antioxidants as a replacement of conventional synthetic antioxidants in food and food supplements has been employed, owing to the fact that natural products are considered to be a promising and safe source (Mandal *et al.*, 2011). Moreover, these

that this plant decoction is consumed to treat nervous disorders (Britto JDe and Mahesh R, 2007). This plant species is also reported to possess various biological activities like antioxidant (De Freitas Cleverson DT*et al*, 2010) antitumour (Doskotch RW*et al*, 1972) antiviral (Vijayan K and Srivastava PP, Awasthi AK, 2004) and control schistosomiasis (Adewunmi C, 1984). The aqueous solution of ethanol extract of aerial parts exerted significant hypoglycemic action in normal rabbits (Sharma AL*et al, 1967*, Sharma M and Shukla S, 1977) and the latex derived from this plant has proteolytic and bacteriolytic activity and possess relevant enzymatic activities against pathogenic related proteins (Pant R and Srivastava SC, 1966, Cleverson D *et al*., 2010).

Scope and Objectives

- Collection of leaf and stem from *Cryptostegia grandiflora* for extracts preparation.
- To quantitatively analyze and compare the total phenolics, flavonoids, vitamin C and vitamin E content of leaves and stem of *Cryptostegia grandiflora*using spectrophotometric methods.
- To identify and compare the functional group of leaves and stem of *Cryptostegia grandiflora* by Fourier transform infrared spectroscopy (FTIR) analysis.
- To assess the antioxidant potential of *Cryptostegia grandiflora* using aqueous extract against DPPH radical scavenging activity.
- To identify the bioactive compounds of the methanol extract of leaf and stem of *Cryptostegia grandiflora* using GC-MS analysis.

LITERATURE REVIEW

LITERATURE REVIEW

Antioxidant activity

Antioxidant compounds in food play an important role as a health protecting factor. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Natural antioxidants can also be replaced by commercially available, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are quite unsafe to use and is restricted due to their carcinogenic effect (Velioglu *et al.*, 1998). Natural antioxidants or phytochemical antioxidants are the secondary metabolites of plants (Walton and Brown, 1999). Carotenoids, flavonoids, cinnamic acids, folic acid, ascorbic acid, tocopherols, tocotrienols *etc.*, are some of the antioxidants produced by this plant for their sustenance. Beta-carotene, ascorbic acid and alpha tocopherol are the widely used as antioxidants (McCall and Frei, 1999).

Flavonoids are polyphenolic compounds, which are ingredients of many vegetables and fruits. They are classified into flavanols, flavanones, flavones, isoflavones, catechins, anthocyanins, proanthocyanidins, etc. [Huy *et al.*, 2008]. They are among the most bioactive plant secondary metabolites which outperform well-known antioxidants.

Natural antioxidants are known to exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-thrombic and vasodilatory activities. Antioxidant activity gives rise to anti-carcinogenicity, anti-immunogenicity and anti-aging activity [Gulcin *et al.*, 2010].

Flavonoids serve as ROS scavengers by locating and neutralizing radicals [Gill and Tuteja, 2010]. Bioactive properties such as free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action of flavonoids is known [Njoku *et al.*, 2011]. The antioxidant activity of the dietary phenolics considered to be superior to that of the essential vitamins and is ascribed to their high redox potential, which allows them to interrupt free radical mediated reactions by donating hydrogen from the phenolic hydroxyl groups (Beevi *et al.*, 2010).

Phenolics are secondary metabolities that behave as antioxidants due to the reactivity of the phenol moiety (hydroxyl substituent on the aromatic ring). The antioxidant activities of phenolic compounds are also attributed to their ability to chelate transition metal ions, such as those of iron and copper, which have been proposed as the catalyst for the initial formation of ROS (Knezevic *et al.*, 2011).

Ascorbic acid (vitamin C) is a vital component in human diet with the highest concentrations in animal organs like the liver, leukocytes, and anterior pituitary. It is used for its antioxidant effect [Ensafi *et al.*,2010]. Vitamin C is a major ubiquitous non-enzymatic, water soluble antioxidant (Ueta *et al.*, 2003). It acts as ROS scavenger, thus potentially protecting cells from harmful oxidative products [Fossati *et al.*, 2011]. Vitamin C functions in enzyme activation, oxidative stress reduction, and immune function. There is considerable evidence that vitamin C protects against respiratory tract infections and reduces risk for cardiovascular disease and some cancers [Schlueter and Johnston, 2011]. Tannins are group of polymeric phenolic substances. Consumption of tannin containing beverages, especially green teas and

red wines can cure or prevent a variety of illness including heart related diseases (Van-Burden and Robinson, 1981).

Swamy *et al.* (2004) tested the leaf extracts of medicinal plant, *Leptadenia reticulata* for AgNPs production and antioxidant activity studies. He observed that, 500 µg/ml of green synthesized silver nanoparticles showed maximum (64.81 %) radical scavenging activity. The silver nanoparticles were synthesized using aqueous *Piper longum* fruit extract and the aqueous *P. longum* fruit extract and the green synthesized silver nanoparticles showed powerful antioxidant properties *in vitro* antioxidant assays. Haes *et al.*, (2002).

Pourmorad *et al.* (2006) carried out a comparative study on the antioxidant potentials of some selected Iranian medicinal plant extracts. The antioxidant properties of 25 edible tropical plants were studied by Wong *et al.*,(2006). Badami and Channabasavaraj (2007) studied the *in vitro* antioxidant activities of thirteen medicinal plants collected from Western Ghats of India.

Ademiluyi and Oboh (2008) studied the antioxidant activity of methanol leaf extract of *Viscum album* by using linolenic acid peroxidation and DPPH methods. Effat *et al.*,(2008) screened thirteen medicinal plant extracts for antioxidant activity. MoniRani *et al.*,(2008) evaluated antioxidant activities of methanol extract of *Ixora coccinea* by DPPH free radical scavenging activity, reducing power and total antioxidant activity assays.

Gayatri *et al.* (2011) observed that the piperine, an alkaloid found naturally in *Piper nigrum* and *Piper cubeba*. It is widely used in various herbal cough syrups and

anti-inflammatory, antimalarial, anti-leukemia treatment. Ethanol extract of *Piper cubeba* showed high antioxidant activity.

Inbathamizh *et al.*, (2013) studied in vitro evaluation of antioxidant and anticancer potential of *Morinda pubescens* synthesized silver nanoparticles. The decolorization from purple DPPH radical to yellow DPPH molecule by the sample in a dose-dependent manner with an IC50 value of 84±0.25 µg/ml indicated the sample's high radical scavenging activity, which was closer to that of the standard whose IC50 value was found to be 80±0.69 µg/ml.

Niraimathi *et al.* (2013) investigated on biosynthesis of silver nanoparticles using *Alternanthera sessilis* (Linn.) leaf extract and determined antioxidant activities. Free radical scavenging activity of the AgNPs on DPPH radical was found to increase with increase in concentration, showing a maximum of 62% at 500 µg/ml. The standard gallic acid, however, at this concentration exhibited 80% inhibition. The IC50 value was found to be 300.6 µg/ml.

The silver nitrate extract of *Annona squamosa* and *Sapium macrocarpum* showed two times more DPPH scavenging activity than the commercial antioxidant butylated hydroxyl anisole. (Ruiz *et al.*, 2008). The silver nitrate extracts of *Melissa officinalis*, *Matricaria recuttia* and *Cymbopogan citratus* were found to possess DPPH scavenging activity. (Pereira *et al.*, (2009). Sowndharajan *et al.*, (2010) studied the antioxidant capacity and total phenolic contents present in the silver nitrate extracts of leaves, stem, and roots of *Melothria maderaspatana* were evaluated. Sathisha *et al.*, (2011) determined antioxidant potentials in silver nitrate extract of

some plants, *Curcuma longa, Coffea Arabica, Tribulus terrestris, Bacopa monnieri* and *Trigonella foenumgraceum* using various *in vitro* assays.

Iwalewa *et al.*, (2005) studied the pro and antioxidant effects of silver nitrate extracts of nine edible vegetables in southwest Nigeria using 1, 1-diphenyl-2-picrylhydrazyl free radical assay. The silver nitrate extract of *Helichrysum plicatum* had been reported to have antioxidant activity using two *in vitro* methods, namely DPPH and -carotene linoleic acid assays . (Tepe *et al.*,(2005) .

The silver nitrate extracts of *Chlorophytum borivilianum* had been shown to scavenge DPPH radical and decrease TBRAS (Thiobarbituric Acid Reactive Substances), revealing that it is a promising anti-stress agent as well as a potential antioxidant (Kenjale *et al.*, 2007).

GC-MS

The chemical composition of the essential oils from leaves and wood of *Ocotea brenesii* growing wild in Costa Rica was determined by capillary GC/FID and GC-MS. From the leaves, 64 compounds were identified, corresponding to 85.9% of the oil. and from the wood 57 compounds were identified corresponding to 69.0% of the oil (Carlos and Jose, 2005). The chemical compositions of the essential oils of *Ocimum basilicum* L. *cv.* purple and *Ocimum basilicum* L. *cv.* green cultivated in Iran were investigated by GC-MS (Seyed, 2006).

GC-MS analysis of *Jatropha curcas* leaves revealed the presence of 16 compounds. The most abundant components were 22, 23-dihydro-stigmasterol (16.14%) alpha-tocopherol (15.18%), beta amylin (7.73%) and dotriacontanol (7.02%) The content of gamma tocopherol reached 2.88% and Vitamin E reached

18.06% in the extract (Wang *et al.*, 2009). The GC-MS analysis of *Strobilanthes crispus* oil revealed the presence of 28 components. The main constituents were found to be phytol, α-cadinol, Megastigmatrienone, 2,3-dihydrobenzofuranand eugenol (Asmah *et al.*, 2006).

Nithya Narayanaswamy and Balakrishnan (2011) evaluated the antioxidant properties of 13 important medicinal plants and it showed that *Ocimum basilicum* leaf, *Alpinia calcarata* leaf, *Jatropha mulitifida* flower, *Hyptis suaveolens* leaf, *Solanum indicum* leaf and *Clitoria ternatea* leaf and flower possessed higher DPPH scavenging activity. Moussa *et al.*, (2011). The aqueous leaf extracts of 124 Egyptian plant species belonging to 56 families were investigated and compared for their antioxidant activity by DPPH scavenging assay. Safi *et al.* (2012) studied the biological activities of aqueous extract of the root of *Jatropha curcas* like antimicrobial and free radical scavenging activities. In the evaluation of DPPH free radical scavenging activity. Olabinri *et al.*, (2013) investigated *in vitro* antioxidant and nitric oxide radical scavenging capabilities of *Jatropha gossypifolia* extract.

Sermakkani M. And V. Thangapandian (2012) evaluated GC-MS analysis of *C. italica* leaves revealed the presence of seventeen compounds. The identified compounds possess many biological properties. For instance, 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- Linolenic acid (R/T 20.06) possesses anti-inflammatory, insectifuge, hypocholesterolemic, cancer preventive, nematicide, hepatoprotective, antihistaminic, antieczemic, antiacne, 5-alpha reductase inhibitor, antiandrogenic, antiarthritic and anticoronary properties. n-Hexadecanoic acid - palmitic acid (R/T 17.25) can be an antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant activities.

Fenghuan Wei *et al.*, (2015) identified thirty compounds in *Jasminum grandiflorum* by using GCMS. The major volatile components of the flower were 3,7,11,15- tetramethyl-2-hexadecen-1-o (phytol) (25.77 %), 3,7,11- trimethyldodeca - 1,6,10-trien-3-ol (12.54 %) and 3,7,11,15- tetramethyl - 1-Hexadecen-3-ol (12.42 %). The results show that phytol is the major volatile component of *Jasminum grandiflorum.*

Praveen Kumar P *et al.*, (2018) studied the identification of bioactive compounds from the Neem sap by Gas chromatography and Mass spectroscopy (GC-MS). The GC-MS analysis of the Neem sap revealed the presence of 30 volatile compounds. Among the 30 compounds, the most predominant compounds are fatty acids like Hexadecanoic acid and Pentadecanoic acid. Hence, this current attempt forms a basis for the biological characterization and importance of the compounds which could be exploited for future development of drugs.

Seventy six kinds of chemical compounds were found in methanol extract of *E.cephalotes* including aldehydes (7.9%), phenols (7.5%), fatty acids (5.8%) and furfural (5.4%) and 86 kinds of chemical compounds found in *M.anisodan* extract. Furfural, steroids, vitamin B and flavonoids are the main compounds of *M.anisodan* by S. Mohammadi *et al.*, (2019).

FTIR

A large number of medicinal plants are used as alternate medicine for diseases of man and other animal since most of them are without side effects when compared with synthetic drugs. Identification of the chemical nature of phytochemical compounds present in the medicinal plant will provide some information on the

different functional groups responsible for their medicinal properties. Iqbal Ahamed *et al*., (2006) detected major groups of compounds as the most active fraction of four plants extract by infrared spectroscopy.

Ramamoorthi and Kannan (2007) screened the bioactive group of chemicals in the dry leaf powder of *Calotropis gigantea* by FTIR analysis .Kareru *et al.* (2008) detected saponins in crude dry powder of 11 plants using FTIR spectroscopy.

Muruganantham *et al.*, (2009) carried out the FTIR spectroscopic analysis in the powder samples of leaf , stem and root of *Eclipta alba* and *Eclipta prostratea.* The FTIR analysis of aqueous methanolic leaf extracts of *Bauhinia racemosa* for phytochemical compounds was done by Gauravkumar *et al* .,(2010). Ragavendran *et al*.,(2011) detected the functional groups in various extracts of *Aerva lanata* using spectroscopic method .

Thangarajan Starlin *et al*., (2012), analyzed the ethanolic extracts of *Ichnocarpus frutescens*, by FTIR, revealed the presence of functional group components of amino acids, amides, amines, carboxylic acid, carbonyl compounds, organic hydrocarbons and halogens. Parag A. Petnekar and Bhanu Raman (2013) carried out the FTIR spectroscopic analysis of methanolic leaf extract of *Ampelocissus lantifolia* for antimicrobial compounds.

FTIR analysis for five selected green leafy vegetables(GLVs) viz., *Hibiscus cannabinus*, *H. sabdariffa*, *Basella alba*, *B. rubra* L. and *Rumex vesicarius* confirmed the presence of free alcohol, intermolecular bonded alcohol, intramolecular bonded alcohol, alkane, aromatic compounds, imine or oxime or ketone or alkene, phenol and amine stretching (Sravan Kumar and Manoj., 2015).

The functional group identification is made by FTIR analysis and the active components based on the peak value in the region of infrared radiation. The ethanolic flower extract of *Erythrina variegata* L. is passed into the FTIR spectroscopy and the functional groups of the components are separated based on the peak ratio. The results of FTIR analysis confirm the presence of functional groups such as non-bonded, O-H stretch, carboxylic group, acidic, H bonded, C-H stretch, asymmetric stretching of –CH (CH2) vibration, C=N (stretch), carbon-carbon triple bond, multiple bonding, carbonyl compound frequency, C=O stretch, C=C stretch, O-H bend, alcoholic group, C-N stretch, C-O stretch, PO3 stretch, =C-H bending and C-Cl (Priyanga *et al.*, (2017).

MATERIALS & METHODS

MATERIALS AND METHODS

Plant material

Botanical name : *Cryptostegia grandiflora (Roxb.) R.Br.*

Classification:

Division : **Tracheophyta**

Sub - Division : **Spermatophytina**

Class : **Magnoliopsida**

Order : **Gentianales**

Family : **Asclepidaceae**

Distribution :

Cryptostegia grandiflora as native to Madagascar. *Cryptostegia grandiflora* as alien, established and not invasive in the caribbean Sea, Australia, Cayman Islands, Ecuador. It is introduced and invasive in Mexico, Aruba, Cuba and Australia.

Description :

Cryptostegia grandiflora is a self supporting, Scrambling, many stemmed vine that grows to 2 meters tall with long trailing whips. A milky sap oozes from stems, leaves and seedpods when cut or broken. Leaves are dark green and glossy, 6-10cm long, 3-5cm wide and in opposite pairs. Roots have been found at a depth of 13 meters in mine shafts. Roots are seedlings are twice as long as shoots. The growth form of the vine differs depending on the surrounding conditions. They can form dense canopies of overpapping plants with long whips, form towers upto 30mts high the height of native trees and grow as freestanding shrubs in the absence of other vegetation. Flowers are large and showy, with five white to light purple petals in a funnel shape. The seedpods are rigid, 10-12cm long, 3-4cm wide and grow in pairs at the end of a short stalk. The flowers resemble those of the purple Allamanda.

Materials And Methods

Collection and processing

The leaves and stem of *Cryptostegia grandiflora* were collected from Thoothukudi, Tamil Nadu respectively. The collected samples were cut into small fragments and shade dried until the fracture is uniform and smooth. The dried plant material was granulated or powdered by using a blender, and sieved to get uniform particles by using sieve No. 60. The final uniform powder was used for the extraction of active constituents of the plant material.

Extraction (Maxon and Rooney,1972)

One gram of air- dried sample powder was taken in a 100ml flask, to which added 50ml of 1% (v/v) in methanol. The samples were shaken in a reciprocating shaker for 24h.at room temperature. The contents were centrifuged at 10,000 g for 5min.The supernatant was collected separately and used for further analysis.

Quantitative analysis of antioxidant

Total phenolic content:(Duan *et al.*,2006)

Reagents

- 50%Folin – ciocalteau reagent
- 20%sodium – carbonate
- Gallic acid – standard

Procedure

100mg of samples was homogenate with 10 ml of distilled water and filtered through a muslin cloth. 1ml of the filtrate was added to 1.5 ml of deionized water and 0.5 ml of 50% folinciocalteau reagent and the contents were mixed thoroughly. After 1min, 1 ml of 20% sodium carbonate solution was added mixed the control contained all the reagents except the sample. After 30 minutes of incubation at 37°c, the absorbance was measured at 750nm. Total phenolics were calculated as Gallic acid equivalent (GAE) per gram fresh weight.

Total flavonoid content (Zhinshen *et al.*, 1999)

Reagents

- 5% sodium nitrate (NaNo2)
- 10% Aluminium chloride (Alcl3,H2O)
- 1N sodium hydroxide (NaoH)
- Quercetin standard

Procedure

100mg of plant material was homogenized with 10ml of distilled water and filtered through a muslin cloth. 0.5 ml of the extract was added with 2.5 ml distilled water and mixed. After 6 minutes 0.15 ml NaNO, was added and again after 6min 0.3 ml of 10% $Alcl^3$ was added. After 5 minutes 1ml of 1M NaNH and 0.5 ml of water were added. Following through mixing of the solution the absorbance against blank were recorded at 510nm. Quercetin was used as standard and the results were expressed as my quercetin equivalents (QE) 1g fresh weight.

Vitamin C [Ascorbic acid] (Baker and Frank, 1968)

Reagents

- 5% of TCA
- Indophenol reagent
- 20mg of dichlorophenol indophenols was dissolved in 10ml of warm distilled water
- DT reagent 2g of 2, 4 dinitraphenyl hydrazine and 1g of thiourea were dissolved.
- 85% sulphuric acid
- L-ascorbic acid - standard

Procedure

100 mg of plant material was homogenized with 10ml of 5% Trichloro acetic acid (TCA). The homogenate was centrifuged. To 2 ml of indophenols reagent and 0.5ml of DT reagent was added and incubated at 10c for 1hour and then cooled in ice bath and 2.5 ml of 85% sulphuric acid was added and shaken well for 30 minutes (until) red colour appeared. The absorbance was measured at 540nm. 1-ascorbic acid was used as standard and the results were expressed as mg/1g/FW.

Estimation of Tannin (Julkunen-Titto, 1985)

Procedure

100 mg of sample homogenized with 10 ml of distilled water and filtrated through a muslin cloth. 1ml of aliquot of aqueous extract was mixed with 1.5ml of 4% vanillin (prepared with methanol) and 750 µl of concentrated HCL was added the

solution was shaken vigorously and left to stand at room temperature for 20 minutes in darkness the absorbance against blank was read at 500nm using UV-Visible spectrophotometer. Results were expressed as mg catechin equivalent (CE) 1g tissue.

Vitamin E (Tocopherol): Rosenberg, 1992

Procedure

The plant sample (2.5g) was homogenized in 50ml of 0.1 N sulphuric acid and allowed to stand overnight the content in the flask was shaken vigorously and filtered through what man No.1 filter paper. Aliquots of the filtrate were used for estimation.

In stoppered centrifuge tubes 3ml of extract and 3ml of water were pipette out separately. To both the tubes, 3ml of ethanol and 3ml of xylene were added, mixed well and centrifuged. Xylene (2.0ml) layer was transferred into another stoppered tube. To each tube, 2.0 ml of dipyridyl reagent was added and mixed well, the mixture (3ml) was pipette out into a cuvette and the extinction was read at 460nm. Ferric chloride solution (0.66 ml) was added to all the tubes and mixed well. The red colour developed was read exactly after 15min at 520nm. Tocopherol was used as standard.

FT-IR analysis

A little powder of plant specimen was mixed with KBrsalt, using amortar and pestle, and compressed into a thin pellet. Infra -red spectra were recorded as KBrpellets on a Thermo Scientific NicotiS5ID1 transmission, between 4000-400 cm^{-1} (Kareru *et al.*, 2008).

GC-MS Analysis:

Extract Preparation

The 50g tuber powder of *Cryptostegia grandiflora* was serially extracted with 250 ml of Methanol with the help of soxhlet apparatus. The extraction procedures were continued for 3-4 hours at 60°C -80°C[15]. These extracts were concentration under reducedpressureevaporatorandstoredinairtightvialsat4°Cfor further study.

Phytochemical analysis by GC-MS

Gas chromatography-Mass spectrometry (GC-MS) analysis of the methanolic extracts was performed by using a GC-MS (Model; QP2010series, Shimadzu, Tokyo, Japan) equipped with a VF-5ms fused silica capillary column of 30 m length, 0.25 mm dia .and 0.25µm film thickness. For GC-MS detection, anelectron ionization system with ionization energy of 70 eV was used. Heliumgas (99.99%) was used as a carrier gas at a constant flow rate of 1.51 ml/min. Injector and mass transfer line temperature was set at 200 and 240° C respectively. The oven temperature was programmed from70to220°Cat10°C/min, held is other malforl min and finally raised to 300°C at 10°C/min. 2 µl of respective diluted samples was manually injected in the splitless mode, with split ratio of 1:40 and with mass 18 scan of 50-600 amu. Total running time of GC-MS is 35min. The relative percentage of the each extract constituents was expressed as percentage with peak areanormalization.

Identification of phytochemical components

The identity of the components in the extracts was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on

the computer library and also with published literatures. NIST08s.LIB and WILEY8. LIB library sources were used for matching the identified components from the plant material.

ANTIOXIDENT ACTIVITY

Crude samples extracts were prepared by pouring 100ml of distilled water in a conical flask containing 10g of each samples separately in the ratio of 10:1 (V/W). After 24 hours, the mixture was filtrated through whatman no:1 filter paper and the filtrate was evaporated to dryness. Crude (aqueous) extracts of all samples (1mg/ml) were used for the determination of free radical scavenging activity.

Free radical scavenging assays (Hatano *et al.*, 1998)

Free radical scavenging assay was measured by 2-2 Diphenyl, 1-picryl hydrazine (DPPH) method proposed by with slight modifications. 1ml of aliquot of test sample was added to 3ml of 0.004% DPPH solution prepared in methanol. The mixture was vortexed for 1min and kept at room temperature for 30 minutes in darkness the absorbance was read at 517 nm. Allow absorbance of the reaction mixture indicated a high free radical scavenging activity. Ascorbic acid was used as standard.

DPPH scavenging activity (%)

A control –A test / A control * 100

Where, A control is the absorbance of the DPPH solution without test solution. A test is the absorbance of DPPH with the test solution. Aqueous extract was used as blank.

RESULT & DISCUSSION

RESULT AND DISCUSSION

Phytochemical analysis conducted on the plant extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities.(Sofowra.A, 1993). Analysis of the plant extracts revealed the presence of phytochemicals such as Phenol, Tannin, Flavanoid. The phenolic compounds are one of the largest and most ubiquitous group of plant metabolites. (Singh.R *et al.*,2007). They possess biological properties such as antiapoptosis, cardiovascular protection, antiaging, anticarcinogen, anti inflammation (Han.X *et al.*, 2007). Flavanoids are hydroxylated phenolic substances known to be synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against wide array of microorganisms in vitro. Various parts of *Cryptostegia grandiflora* are traditionally used as treat nervous disorders [Britto JDe and Mahesh R, 2007]. This plant species is also reported to possess various biological activities like antioxidant [De Freitas Cleverson DT*et al.*,2010], antitumour [Doskotch RW *et al.*,1972] antiviral [Vijayan K*et a*l.,2012] and control schistosomiasis [Adewunmi CO, 1984].

QUANTITATIVE ANALYSIS:

The total phenol, flavanoid, tannin, vitamin-C and vitamin-E were analysed in leaf and stem extract of *Cryptostegia grandiflora* belonging to the family Asclepidaceae.

TOTAL PHENOL:

Phenolics are the most wide spread secondary metabolites and are believed to be responsible for antioxidant activity. The total phenol contents of the leaf (3.867mg/GAE/g) were higher than that stem (2.874mg/GAE/g) in *Cryptostegia grandiflora.* Phenolic compounds are as class of antioxidant agents acts as free terminators. (Shahidi and Wanashndara, 1992). Phenolic compounds have a variety of beneficial activities. They have potential antioxidants and free radical scavenger (Meenakshi *et al.*,2012). The antimicrobial (most of phenolics) may provide a microbe - free environment with in the body.

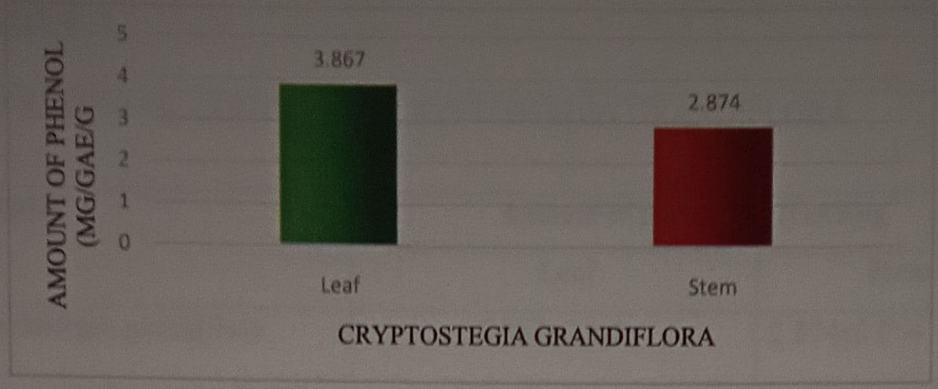


FIG:1 Total phenol content of *Cryptostegia grandiflora*

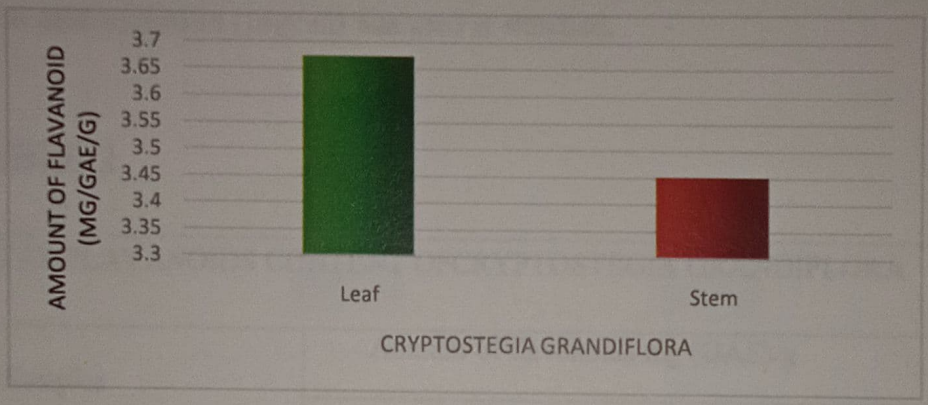


FIG:2 Total flavanoids content of *Cryptostegia grandiflora*

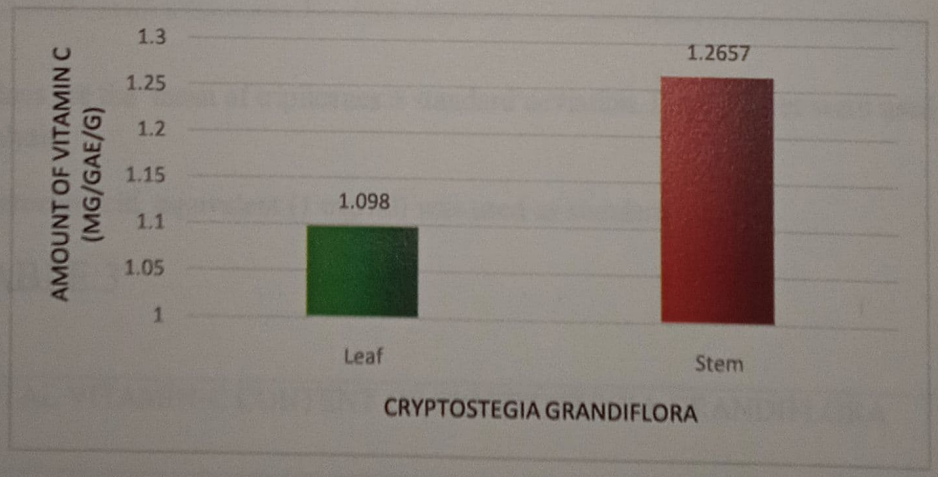


FIG:3 Total vitamin-c content of *Cryptostegia grandiflora*

TABLE:1

TOTAL PHENOL CONTENT OF CRYPTOSTEGIA GRANDIFLORA		
Samples	Amount of phenol mg (GAE)/g	
	Leaf	Stem
Cryptostegia grandiflora	3.867±0.323	2.874±0.014

Values are the mean of triplicates ± standard deviation. Dry samples were used for analysis.

Garlic acid equivalent (1mg/ml) was used as standard.

TABLE:2

TOTAL FLAVANOIDS CONTENT OFCRYPTOSTEGIA GRANDIFLORA		
Samples	Amount of flavonoids mg (GAE)/g	
	Leaf	stem
Cryptostegia grandiflora	3.675±1.009	3.453±0.451

Values are the mean of triplicanes ± standard deviation. Dry samples were used for analysis.

Quercetin acid equivalent (1 mg/ml) was used as standard

TABLE:3

TOTAL VITAMIN-C CONTENT OF CRYPTOSTEGIA GRANDIFLORA		
Samples	Amount of Vitamin-C (mg/g)	
	Leaf	stem
Cryptostegia grandiflora	1.098±0.965	1.2657±0.12

Values are the mean of triplicates ± standard deviation. Dry samples were used for analysis.Vitamin-C Equivalent (1mg/ml) was used as standard.

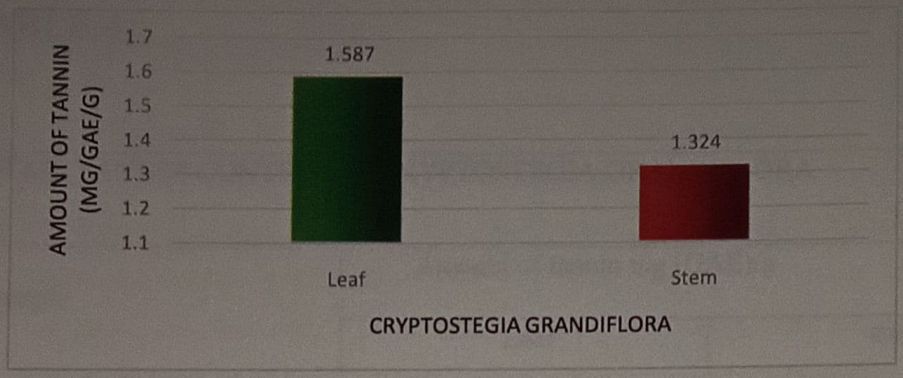


FIG:4 Total tannin content of *Cryptostegia grandiflora*

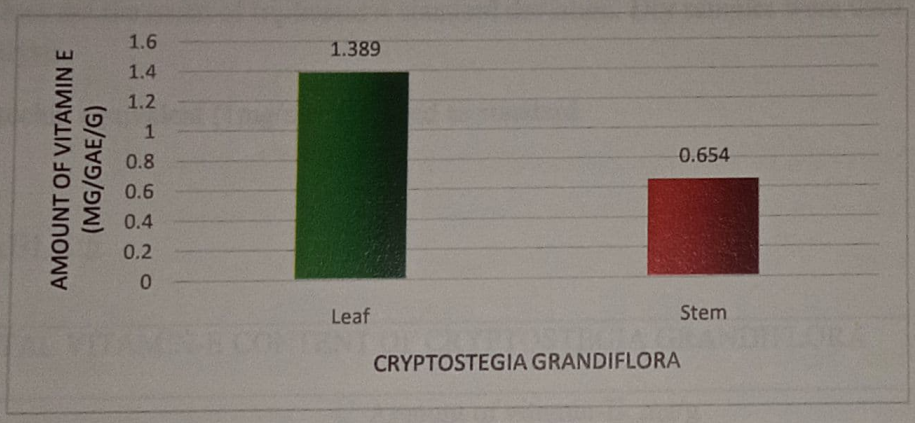


FIG:5 Total vitamin-E content of *Cryptostegia grandiflora*

TABLE:4

TOTAL TANNIN CONTENT OFCRYPTOSTEGIA GRANDIFLORA		
Sample	Amount of tannin mg (GAE)/g	
	Leaf	stem
Cryptostegia grandiflora	1.587±0.087	1.324±0.562

Values are the mean of triplicates ± standard deviation. Dry samples were used for analysis.

Catechin equivalent (1mg/ml) was used as standard.

TABLE:5

TOTAL VITAMIN-E CONTENT OF CRYPTOSTEGIA GRANDIFLORA		
Samples	Amount of vitamin-E mg/g	
	Leaf	stem
Cryptostegia grandiflora	1.389±0.004	0.654±0.032

Values are the mean of triplicates ± standard deviation. Dry samples were used for analysis.

Vitamin-E equivalent (1mg/ml) was used as standard.

TOTAL FLAVANOID:

Flavanoids are secondary metabolites and has responsible for antioxidant activity in medicinal field. The total flavanoids contents of leaf (3.675mg QE/g) were higher than that stem (3.453mg QE/g) in *Cryptostegia grandiflora.* Flavanoids are potent antioxidants and epidemic studies indicate that high flavanoids in take is correlated with decreased risk of lifestyle disease like diabetes and cardiovascular disease (Kaur *et al.*,2016). Flavanoids are potent water-soluble antioxidants and free radical which prevent oxidative cell damage and have strong anti-cancer activity (Havsteen, 2008).

TOTAL VITAMIN-C:

Cryptostegia grandiflora leaf (1.098mg/g) and stem (1.2657mg/g) contain significant amount of vitamin-C. Vitamin-C is a vital component in human diet with the highest concentrations in animal organs. Vitamin-C is a non-enzymatic, antioxidant water soluble antioxidant (Ueta *et al.*,2003). Vitamin-C functions in enzyme activation, oxidative stress reduction and immune function. It is protects against respiratory tract infection and reduces risk for cardiovascular disease and some cancer.

TOTAL TANNINS:

Cryptostegia grandiflora leaf (1.587mg CE/g) contain highest amount of tannin, and the stem of *Cryptostegia grandiflora* contain the lowest amount of tannin (1.324mg CE/g). Tannins are present primarily in the leaves of trees growing in stress conditions. They are accumulated in the vacuoles, especially these of the epidermal layer and the palised mesophyll. Tannins are useful in treating inflammation, ulcers and remarkable activity in cancer prevention and anticancer activities (Li *et al.*,2003, Akinpelu *et al.*,2009).

TOTAL VITAMIN-E:

Total vitamin-E content in *Cryptostegia grandiflora* leaf (1.389mg/g) highest and *Cryptostegia grandiflora* stem (0.654mg/g) lowest (Table-5). Vitamin-E is a fat soluble nutrient found in many foods (Jacob,1995). In the body, it acts as an

antioxidants, helping to protect cells from the damages caused by free radicals. Free radicals are compounds formed when our bodies convert the food we eat into energy (Havsteen, 1983).

FTIR:

Fourier transform infrared spectroscopy was used to analyse the functional group present in the leaf and stem of the *Cryptostegia grandiflora.*

The FTIR spectroscopy analysis of *Cryptostegia grandiflora* leaf obtained peaks at 3739.72 cm^{-1} , 3392.55 cm, 2918.1 cm^{-1}, 2848.67 cm^{-1}, 2315.38 cm^{-1}, 1809.1 cm^{-1}, 1746.42 cm^{-1}, 1643.24 cm^{-1}, 1512.09 cm^{-1}, 1455.19 cm^{-1}, 1374.19 cm^{-1}, 1317.29 cm^{-1}, 1244.97 cm^{-1}, 1159.14 cm^{-1}, 1102.24 cm^{-1}, 1074.28 cm^{-1}, 1000.99 cm^{-1}, 892.02 cm^{-1}, 835.12 cm^{-1}, 768.58 cm^{-1}, 719.4 cm^{-1}, 669.25 cm^{-1}, 598.86 cm^{-1}, 517.85 cm^{-1}. These absorption peaks are known to be associated with the stretching vibrations for N-H in Secondary amine, N-H in dilute solution one band, fairly sharp, C-H in Cyclobutane, CH_2 in Symmetric, N-H in secondary amine, CH in aromatic, C=O for membered ring stretching vibration, NH_3 in aminoacid, NO_2 in Nitro group, O-H in symmetric, NO_2 in aliphatic and aromatic nitro group, C-N in aryl tertiaryamine, C-O-C in formates acetates propionates and higher ester, C-O-C in formates acetates propionates and higher ester, C-O-C in aryl and symmetric, S=O in sulphonic acid, C-F in mono fluorinated compounds, C-H in benzene ring, C-H in Napthalene ring, C-Cl in alicyclic, N-H in secondary amine, C-Br in acyclic axial, C-Cl monochlorinated alicyclic axial, C-Br alicyclic and aromatic. Fig:6 , Table(6).

The FTIR spectroscopy analysis of *Cryptostegia grandiflora* stem obtained peaks at 3587.35 cm^{-1}, 3276.83 cm^{-1}, 2915.2 cm^{-1}, 2316.35 cm^{-1}, 1747.39 cm^{-1}, 1680.85 cm^{-1}, 1604.66 cm^{-1}, 1546.8 cm^{-1}, 1510.16 cm^{-1}, 1455.19 cm^{-1}, 1430.12 cm^{-1}, 1362.61 cm^{-1}, 1338.51 cm^{-1}, 1317.29 cm^{-1}, 1265.22 cm^{-1}, 1164.92 cm^{-1}, 1108.99 cm^{-1}, 1018.34 cm^{-1}, 892.98 cm^{-1}, 780.15 cm^{-1}, 713.61 cm^{-1}, 637.43 cm^{-1}, 579.57 cm^{-1}, 517.85 cm^{-1}. These absorption peaks are known to be associated with the stretching vibrations for O-H in polyvalent alcohols, N-H in sulphon amide, C-H in ketone stretching, N-H in facilaey amine salt, C=O in phenolic ester, C=O orthohydroxy aryl, N-H in primary amine, N-H in secondary amide, NO_2 in aliphatic and aromatic nitro group, O-H in symmetric, O-H in symmetric, NO_2 in aliphatic nitro group, SO_2

FIG:6 FTIR spectroscopy analysis of *Cryptostegia grandiflora* leaf

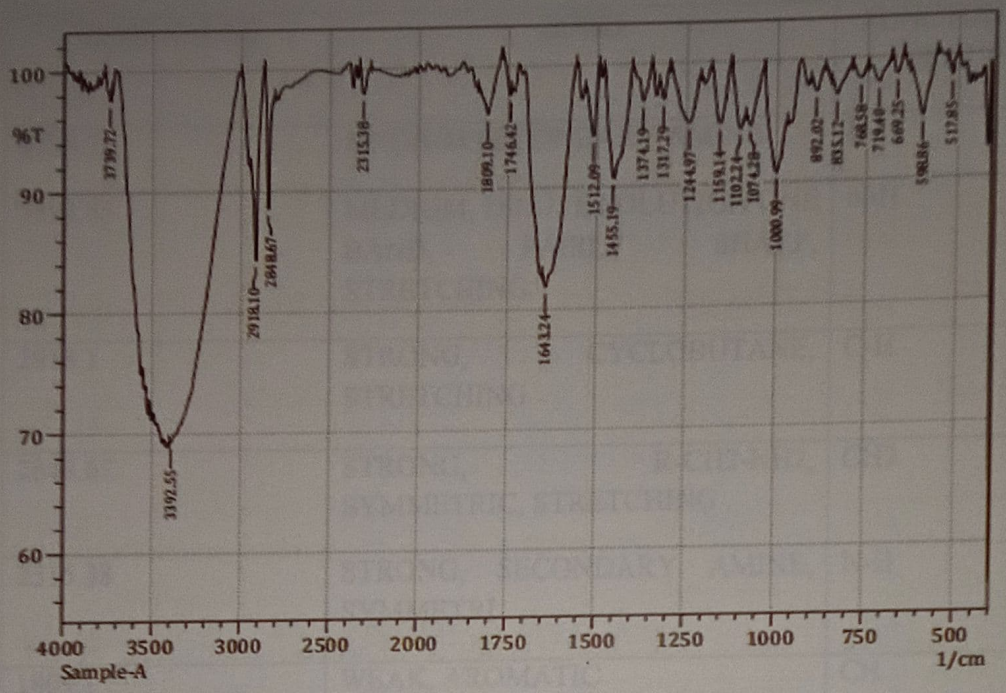


FIG:7 FTIR spectroscopy analysis of *Cryptostegia grandiflora* Stem

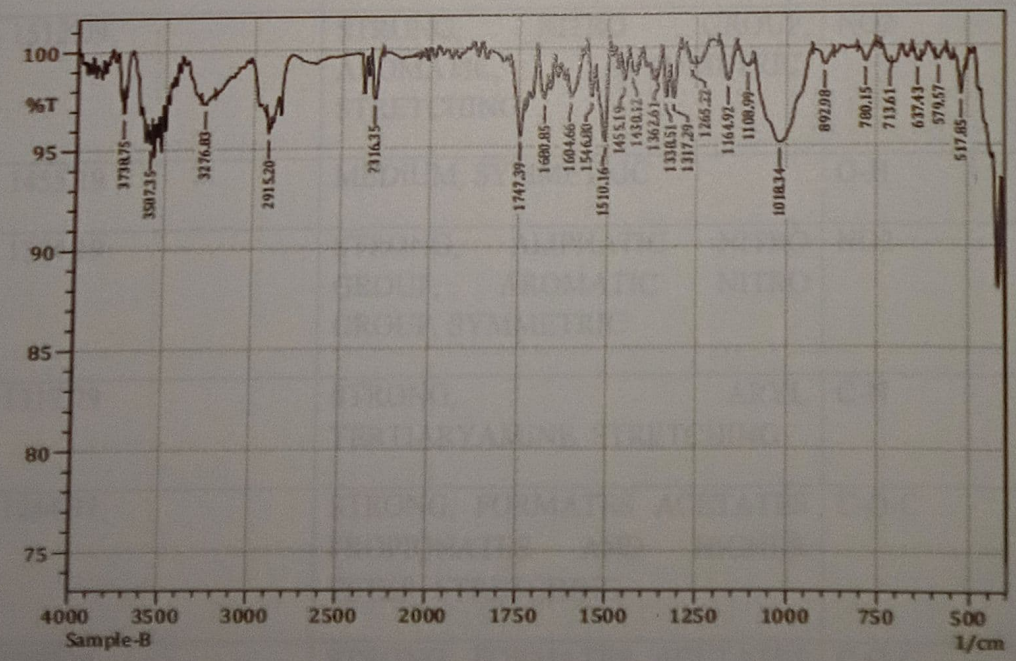


TABLE:6 FTIR spectroscopy analysis of *Cryptostegia grandiflora* leaf

PEAK VALUE	BOND	FUNCTONAL GROUP
3739.72	MEDIUM SECONDARY AMINE	N-H
3392.55	MEDIUM, DILUTE SOLUTION ONE BAND, FAIRLY SHARP, STRETCHING	N-H
2918.1	STRONG, CYCLOBUTANE, STRETCHING	C-H
2848.67	STRONG, R-CH2-NH2, SYMMETRIC, STRETCHING	CH2
2315.38	STRONG, SECONDARY AMINE, SYMMETRI	N-H
1809.1	WEAK, AROMATIC	CH
1746.42	STRONG, FOR MEMBERED RING STRETCHING VIBRATION	C=O
1643.24	WEAK, AMINO ACID, ASYMMETRIC	NH3+
1512.09	STRONG, NITRO GROUP, AROMATIC, ASYMMETRIC, STRETCHING	NO2
1455.19	MEDIUM, SYMMETRIC	O-H
1374.19	STRONG, ALIPHATIC NITRO GROUP, AROMATIC NITRO GROUP, SYMMETRIC	NO2
1317.29	STRONG, ARYL TERTIARYAMINE, STRETCHING	C-N
1244.97	STRONG, FORMATES ACETATES PROPIONATES AND HIGHER ESTER STRETCHING	C-O-C
1159.14	STRONG, FORMATES ACETATES PROPIONATES AND HIGHER	C-O-C

	ESTER, STRETCHING	
1102.24	STRONG, ARYL, SYMMETRIC	C-O-C
1074.28	STRONG, SULPHONIC ACID	S=O
1000.99	VERY STRONG, MONOFLUORINATED COMPOUNDS	C-F
892.02	MEDIUM, BENZENE RING	C-H
835.12	STRONG, MEDIUM, NAPHTHALENE RING	C-H
768.58	STRONG, MONOCHLORINATED, ALICYCLIC EQUATORIAL	C-Cl
719.4	MEDIUM, ALIPHATIC, SECONDARY AMINE	N-H
669.25	STRONG, ACYCLIC AXIAL	C-Br
598.86	STRONG, MONOCHLORINATED ALICYCLIC AXIAL	C-Cl
517.85	STRONG, ACYCLIC AND AROMATIC	C-Br

TABLE:7 FTIR spectroscopy analysis of *Cryptostegia grandiflora* stem

PEAK VALUE	BOND	FUNCTIONAL GROUP
3587.35	STRONG, MEDIUM, POLYVALENT ALCOHOLS	O-H
3276.83	STRONG, SULPHON AMIDE, STRETCHING	N-H
2915.2	WEAK, KETONE STRECH	C-H
2316.35	STRONG, STRETCHING, FECILAEY AMINE SALT	N-H
1747.39	STRONG, PHENOLIC ESTER, STRETCHING	C=O
1680.85	STRONG, ORTHO-HYDROXY ARYL, STRETCHING	C=O
1604.66	STRONG, PRIMARY AMINE	N-H
1546.8	STRONG, SECONDARY AMIDE	N-H
1510.16	STRONG, ALIPHATIC NITRO GROUP, AROMATIC NITRO GROUP, STRETCHING	NO2
1455.19	MEDIUM, SYMMETRIC	O-H
1430.12	MEDIUM, SYMMETRIC	O-H
1362.61	STRONG, ALIPHATIC NITRO GROUP	NO2
1338.51	STRONG, SULPHONAMIDE	SO2
1317.29	MEDIUM STRETCHING	C-N
1265.22	SMALL, ALDEHYDE	C-CHO

	GROUP, STRETCHING	
1164.92	MEDIUM, STRETCHING VIBRALIOR ISOPROPHYL	C-H
1108.99	VERY STRONG, POLY FLUORINATED COMPOUND	C-F
1018.34	VERY STRONG, MONOFLUORINATED COMPOUND	C-F
892.98	MEDIUM, RING STRETCHING CYCLOPENTANE	C-H
780.15	STRONG, MONOCHLORINATE, ALCYCLIC EQUATONAL	C-Cl
713.61	STRONG, POLYCHLORINATE, STRETCHING	C-Cl
637.43	STRONG, DEFORMATION, AMIDE PRESENT	OCN
579.57	STRONG, DEFORMATION, AMIDE PRESENT	OCN
517.85	WEAK, SULPHIDES,DISULPHIDES, STRETCHING	S-S

sulphonamide, C-N in stretching, C-CHO in aldehyde group, CH in vibralior isoprophyl, C-F in poly flourinated compound, C-F in mono flourinated compound, C-H in cyclopentane, C-Cl in mono chlorinated and alcyclic, C-Cl in poly chlorinated, OCN in amide present, OCN in amide present, S-S in sulphides and disulphides. Fig:7, Table (7)

From the spectral data presence of N-H, C-H, C=O, O-H, C-N, C-F, C-Cl, C-Br, C-O, S=O, S-S were identified. These bending are responsible for the presence of amine group, carboxylic acids group, Dihaloketon group, Asymmetric group, Alkyl amine group, Poly fluorinated group, Mono chlorinate, Alicyclic group, Acyclic and aromatic group, Carboxylic group, Sulphonic acid group, Sulphides group, carboxylic acid present in the medicinal plant serves as main pharmaceutical product in curing ulcer, jaundice, head ache, stomatitis, hemicranias, fever, pain in lever, treatment of edema and rheumatic joint pain, amides, amine are the main groups which are involved in protein synthesis. The study revealed that the whole plant of *Cryptostegia grandiflora* contain a considerable amount of secondary metabolites and it may considered in future to be used human disease management.

GC-MS Analysis:

The GC-MS analysis of methanolic leaf extract of *Cryptostegia grandiflora* was confirmed the presence of 30 compounds with retention time. Interpretation of mass spectrum of GC-MS was conducted using the database of NIST and WILEY libraries. Out of this 30 compounds 4 compounds are majority present in the leaf extract of *Cryptostegia grandiflora* respectively n-Hexadeconic acid(10.627%), pentadeconic acid (10.627%) Tert-Butyl (5-isopropyl-2-methylphenoxy) dimethylsilane (1.70%), Trimethylsilyl-di(timethylsiloxy)-silane (3.69%).

The 26 minor compounds such as 4-Heptafluorobutyroxytetradecane (24.16%), 1-Pyrroline, 3-ethyl(24.16%), 1H-Imidazole, 2-ethyl-4,5-dihydro-4-methyl(24.16%), Z-(13,14-Epoxy)tertradec-11-en-1-ol acetate(5.03%), 13-Octadencenal (z) (5.03%) Isopulegol (9.91%), 3,8,11-Trioxatetracyclo[4.4.1.0(2,4).0(7,9)] undecane (1.alpha.,2.beta.,4.beta.,6alpha., 7beta., 9beta) (9.91%), 3-Nonynoic acid(9.91%), Hexahydropyridine, 1-methyl-4-[4,5-dihydroxyphenyl]-(5.58%), Methyl nonyl ether (5.58%), Adipic acid, cis-non-3-

FIG:8 GC-MS chromatogram of leaf extract (Methanol)
Cryptostegia grandiflora

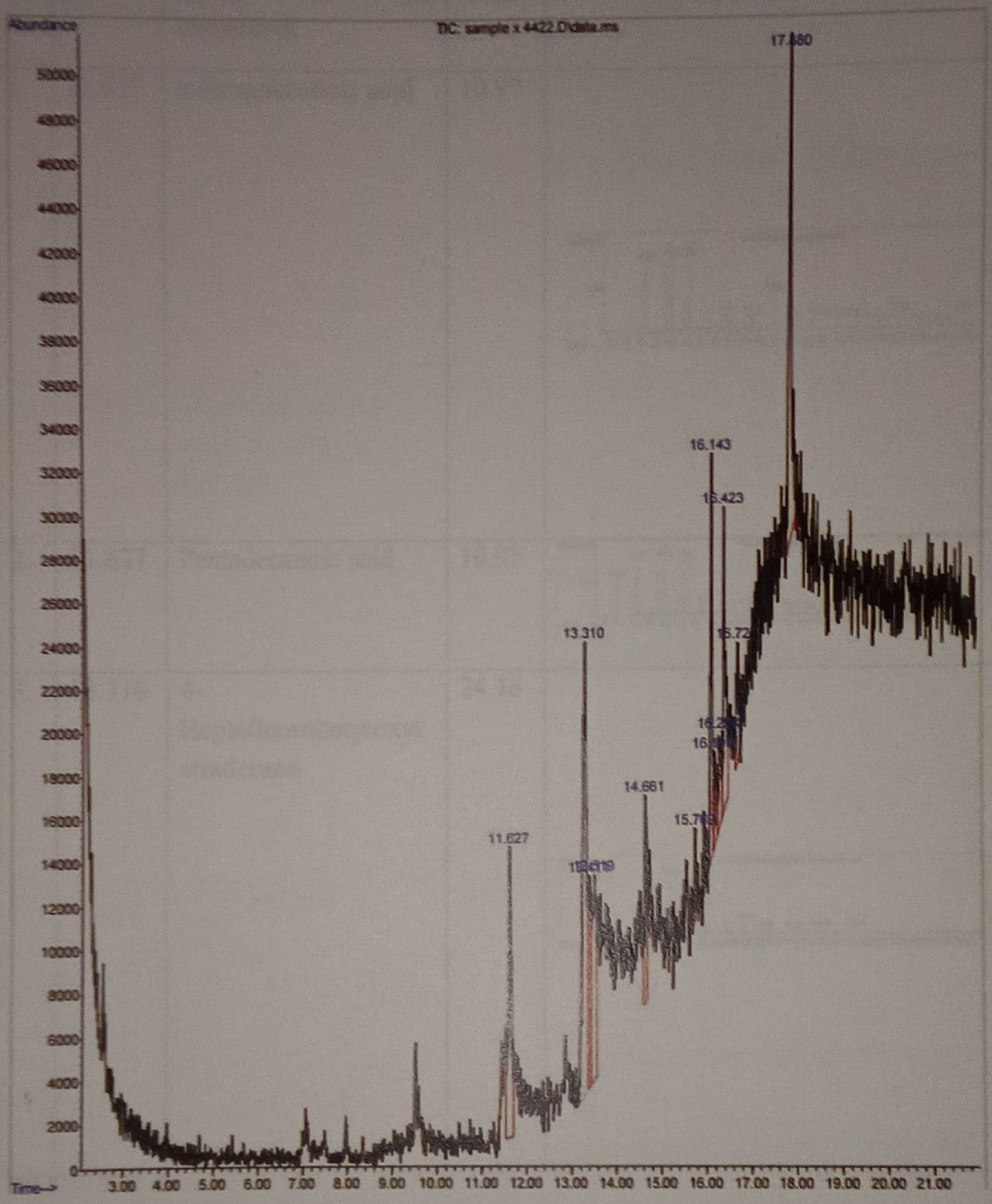
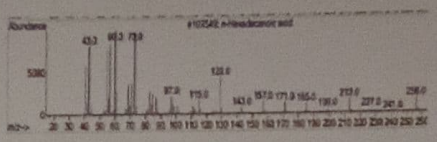
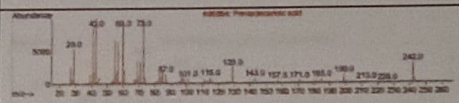
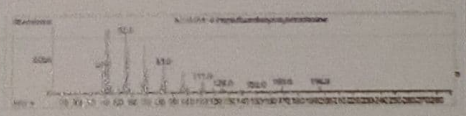
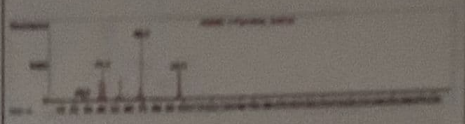
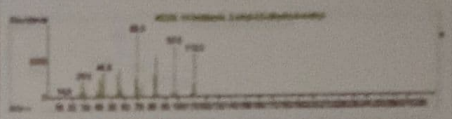
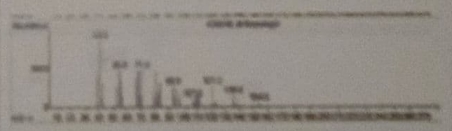
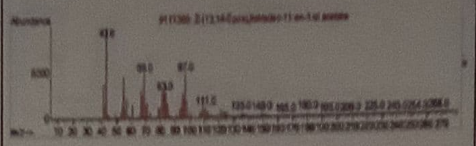
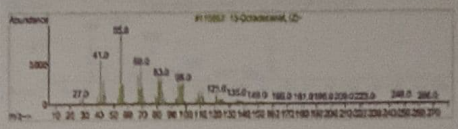
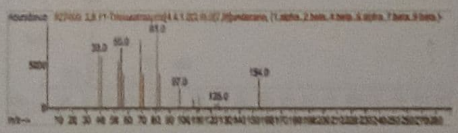
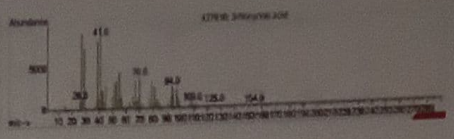
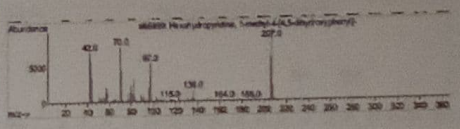
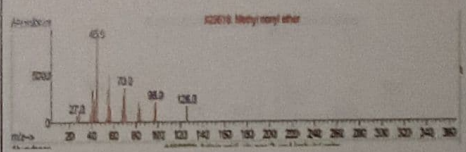


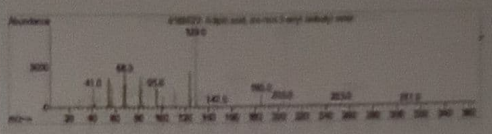
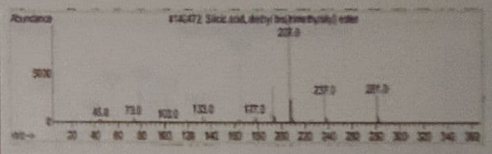
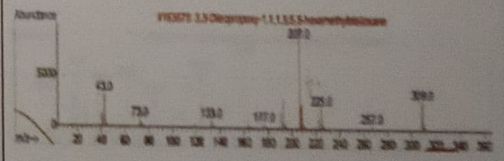
TABLE:8 *Cryptostegia grandiflora* leaf spectrum

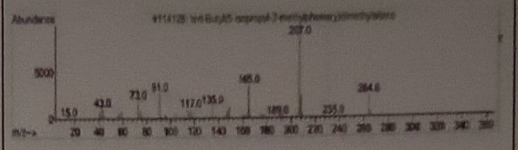
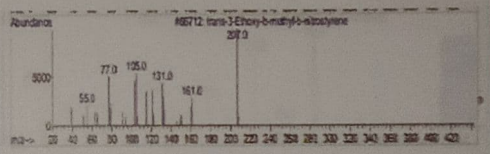
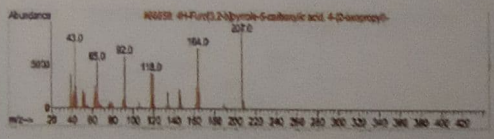
S. No	RT	Name of the compounds	Area%	Mass spectrum
1.	11.627	n-Hexadecanoic acid	10.97	
2.	11.627	Pentadecanoic acid	10.97	
3.	13.310	4-Heptafluorobutyroxytetradecane	24.16	

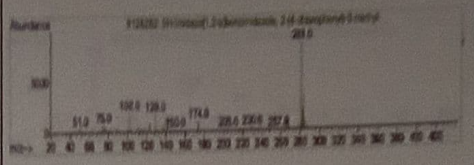
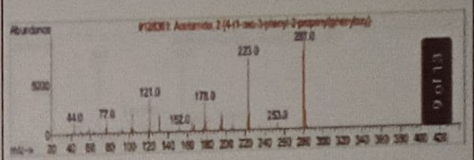
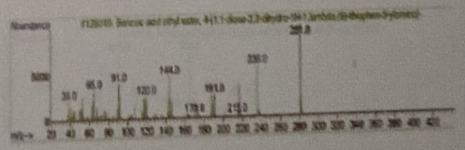
4.	13.310	1-Pyrroline, 3-ethyl-	24.16	
5.	13.310	1H-Imidazole, 2-ethyl-4,5-dihydro-4-methyl-	24.16	
6.	13.414	dl-Isopulegol	5.03	

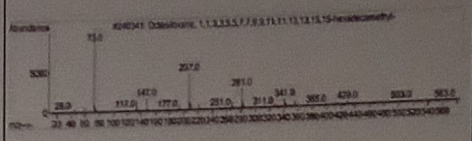
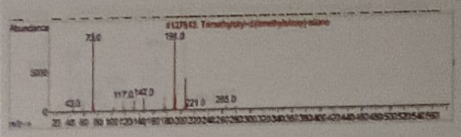
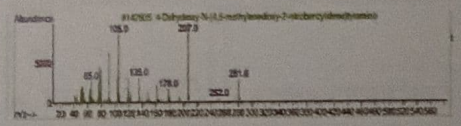
7.	13.414	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	5.03	
8.	13.414	13-Octadecenal, (Z)	5.03	
9.	13.518	3,8,11-Trioxatetracyclo[4.4.1.0(2,4).0(7,9)]undecane, (1.alpha.,2.beta.,4.beta.,6.alpha.,7.beta.,9.beta.)-	9.91	

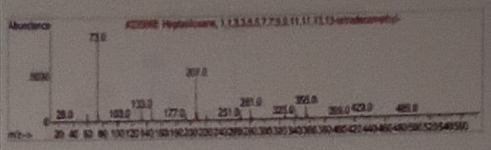
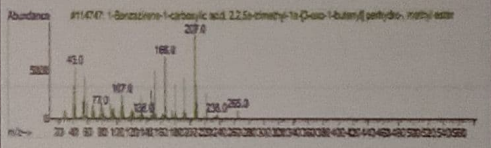
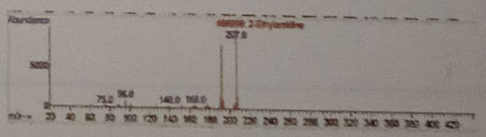
10.	13.518	3-Nonynoic acid	9.91	
11.	14.663	Hexahydropyridine, 1-methyl-4-[4,5-dihydroxyphenyl]-	5.58	
12.	14.663	Methyl nonyl ether	5.58	

13.	14.663	Adipic acid, cis-non-3-enyl isobutyl ester	5.58	
14.	15.769	Silicic acid, diethyl bis(trimethylsilyl) ester	1.70	
15.	15.769	3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyltrisiloxane	1.70	

16.	15.769	tert-Butyl(5-isopropyl-2-methylphenoxy)dimethylsilane	1.70	
17.	16.147	trans-3-Ethoxy-b-methyl-b-nitrostyrene	8.90	
18.	16.147	4H-Furo[3,2-b]pyrrole-5-carboxylic acid, 4-(2-oxopropyl)-	8.90	

19.	16.147	9H-Imidazo[1,2-a]benzimidazole, 2-(4-chlorophenyl)-9-methyl-	8.90	
20.	16.204	Acetamide, 2-[4-(1-oxo-3-phenyl-2-propenyl)phenyloxy]-	2.36	
21.	16.204	Benzoic acid ethyl ester, 4-(1,1-dioxo-2,3-dihydro-1H-1.lambda.(6)-thiophen-3-ylamino)	2.36	

22.	16.298	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- 73.0	3.69	
23.	16.298	Trimethylsilyl-di(timethylsiloxy)-silane	3.69	
24.	16.298	4-Dehydroxy-N-(4,5-methylenedioxy-2-nitrobenzylidene)tyramine	3.69	

25.	16.421	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	8.72	
26.	16.421	1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	8.72	
27.	16.724	2-Ethylacridine	2.83	

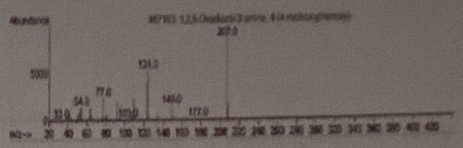
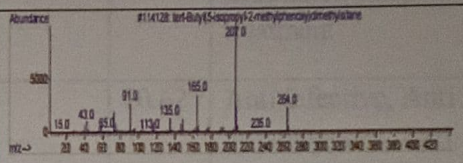
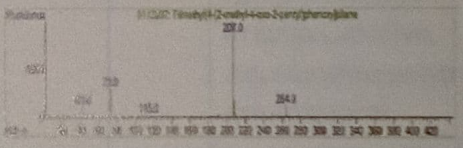
28.	16.724	1,2,5-Oxadiazol-3-amine, 4-(4-methoxyphenoxy)-	2.83	
29.	17.877	Benzene, 2-[(tert-butyldimethylsilyl)oxy]-1-isopropyl-4-methyl-	16.14	
30.	17.877	Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	16.14	

Table:9 List of chemical compounds identified from methanol leaf extract of *Cryptostegia grandiflora* through GC-MS analysis

S.NO	RT	Name of the compound	Area %	Biological Activity
1.	11.627	N-Hexadecanoic acid	10.627	Anti infective, Anti inflammatory, Anti neoplastic, Alopcia treatment
2.	11.627	Pentadecanoic acid	10.627	Anti infective, Anti inflammatory, Anti neoplastic, Alopcia treatment, Anti viral
3.	13.310	4-Heptafluorobutyroxytetradecane	24.16	Anti inflammatory, Antiacne, Antiviral, Anti infective, Antipruritic, Eye irritation inactive, Adenomatouspolyposis treatment
4.	13.310	1-Pyrroline, 3-ethyl	24.16	Anti fungal, Antibiotic, Anti diabetic, Antiviral (Influenza), Dementia treatment, Mucositis treatment, Antineoplastic
5.	13.310	1H-Imidazole, 2-ethyl-4,5-dihydro-4-methyl	24.16	Antifungal, Rhinitis tretment, Stroke treatment, Antineoplastic
6.	13.414	Z-(13,14-Epoxy)tertradec-11-en-1-ol acetate	5.03	Antipruriti, Allergic, Antiviral, Dementia treatment

7.	13.414	13-Octadencenal (z)	5.03	Anti infective, Mucositis treatment, Insecticide, Cancer assosiated disorders treatment
8.	13.518	Isopulegol	9.91	Antipruritic Allergic, Anti fungal, Antipsoriatic, Anti bacterial, Anti infective, Anti carcinogenic, Antiviral(HIV)
9.	13.518	3,8,11-Trioxatetracyclo[4.4.1.0(2,4).0(7,9)] undecane (1.alpha.,2.beta.,4.beta.,6alpha.,7beta. ,9beta)	9.91	Antineoplastic, Antiviral, Carminative, Anti mitotic, Anti seborrheic, Alopecia treatment
10.	13.518	3-Nonynoic acid	9.91	Anti protozoal, Antifungal, Anti inflammatory, Antibacterial, Antithyroid
11.	14.663	Hexahydropyridine, 1-methyl-4-[4,5-dihydroxyphenyl]-	5.58	Diabetic nephropathy treatment, Adenamatous polyposis treatment, Antihelmintic
12.	14.663	Methyl nonyl ether	5.58	Eye irritation inactive, Antienzymatic, Anti hypercholestrolemic , Skin irritation inactive
13.	14.663	Adipic acid, cis-non-3-enyl isobutyl ester	5.58	Sclerosant, Cancer associated disorders treatment,

				Inflammatory bowl disease treatment
14.	15.769	Silicic acid, diethyl bis(trimethylsilyl)ester	1.70	Antiviral, Oxidizing agent, Anti infective Carminative, Dementia treatment
15.	15.769	3,3-Diisopropoxy-1,1,1,5,5,5, hexamethyltrisiloxane	1.70	Antineoplastic, Narcolepsy treatment, Anti protozoal, Antineristalic
16.	15.769	Tert-Butyl(5-isopropyl-2-methylphenoxy) dimethylsilane	1.70	Anti inflammatory, Bonedisease treatment, Antheroclerosis treatment, Mucositis treatment
17.	16.147	Trans-3-ethoxy-b-methyl-b-nitrostyrene	8.90	Phobic disorders treatment, Antineoplastic, Capillary fragility treatment, Muscular dystrophy treatment
18.	16.147	4H-furo[3,2-b]pyrrole-5-carboxylic acid, 4-(2-oxopropyl)	8.90	Antiviral(Poxvirus), Antimyopathies, Serve acute respiratory treatment, Sickle cell anemia treatment
19.	16.147	9H-Imidazo[1,2-a]benzimidazole, 2-(4-chlorophenyl)-9methyl	8.90	Rhinitis treatment, Anti fungal, Anti carcinogenic, Stroke treatment, Anti neoplastic
20.	16.204	Acetamide, 2-[4-(1-oxo-3phenyl-2-propenyl) phenyloxy]	2.36	Anti tuberculosis, Anti diabetic, Antipyretic, Anti

				virus
21.	16.204	Benzoic acid ethyl ester, 4-(1,1-dioxo-2,3-dihydro-1H-1.lambda.(6)-thiophen-3-ylamino)-	2.36	Alopecia treatment, Antineoplastic (brain cancer), Prion disease treatment
22.	16.298	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	3.69	Antiprotozoal, Gaucher disease treatment, Fibromyalgia syndrome treatment, Intermittent claudication treatment
23.	16.298	Trimethylsilyl-di(timethylsiloxy)-silane	3.69	Anti viral, mucositis treatment, Alopcia treatment, Anti septic
24.	16.298	4-Dehydroxy-N-(4,5-methylenedioxy-2-nitrobenzylidene)tyramine	3.69	Antiseptic, Antiviral, Anti infective, Insecticide, Anti protozoal, Dry eye syndrome treatment, Antihelmintic
25.	16.421	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	8.72	Antineoplastic, Antiviral, Intermittent claudication treatment
26.	16.421	1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	8.72	Mucositis treatment, Movement disorders treatment, Uritcaria treatment, Macular degeneration treatment
27.	16.724	2-Ethylacridine	2.83	Antiseptic, Antineoplastic,Skin

				whitener, Kidney function stimulent, Narcolespy treatment, Liver fibrosis treatment
28.	16.724	1,2,5-Oxadiazol-3-amine, 4-(4-methoxyphenoxy)-	2.83	Anti neoplastic, Anti fungal, Anti inflammatory, Bowl syndrome treatment
29.	17.877	Benzene, 2-[(tert-butyldimethylsilyl)oxy]-1-isopropyl-4-methyl-	16.14	Anti infective, Antiviral, Antiulcerative, Antifungal, Antiseptic, Raynaud's phenomenon treatment, Antiprotozoal, Anti inflammatory
30.	17.877	Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	16.14	Antineoplastic, Anti fungal, Anti infective, Antiuremic, Antiviral, Keratoses actinic treatment, Insecticide,

FIG:9 GC-MS chromatogram of stem extract (Methanol)
Cryptostegia grandiflora

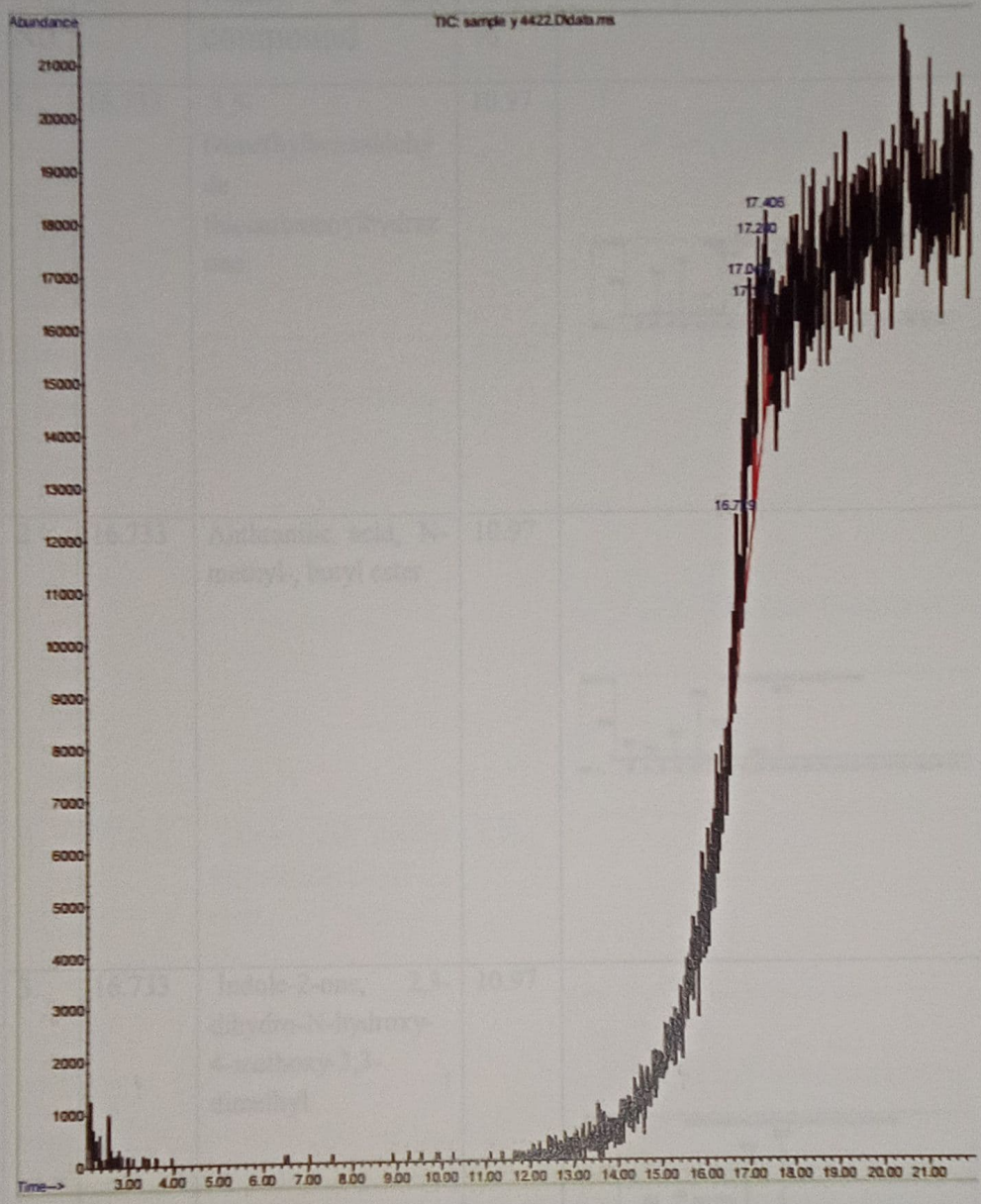
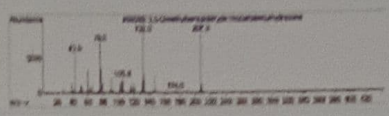
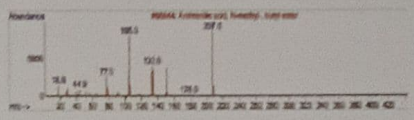
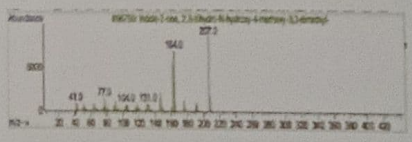
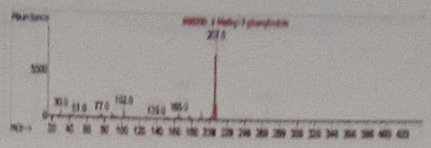
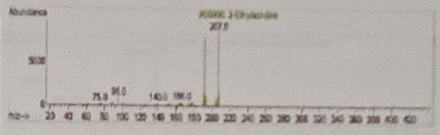
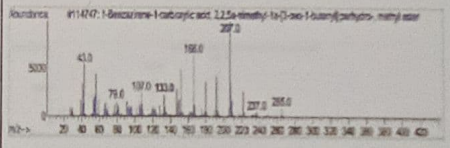


TABLE:10 *Cryptostegia grandiflora* stem spectrum

S. No	RT	Name of the compound	Area %	Mass spectrum
1.	16.733	3,5-Dimethylbenzaldehyde thiocarbamoylhydrazone	10.97	
2.	16.733	Anthranilic acid, N-methyl-, butyl ester	10.97	
3.	16.733	Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl	10.97	

7.	17.149	2-Methyl-7-phenylindole	7.06	
8.	17.149	2-Ethylacridine	7.06	
9.	17.244	1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	34.79	

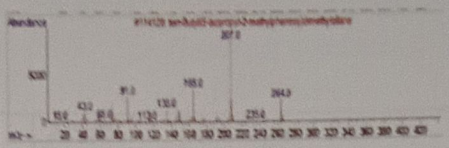
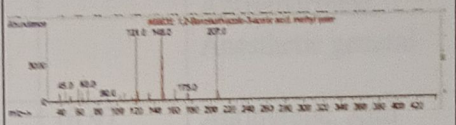
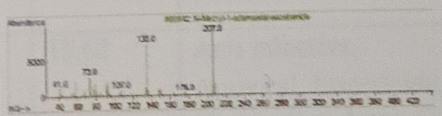
10.	17.244	tert-Butyl(5-isopropyl-2-methylphenoxy)dimethylsilane	34.79	
11.	17.404	1,2-Benzisothiazole-3-acetic acid, methyl ester	13.55	
12.	17.404	N-Methyl-1-adamantaneacetamide	13.55	

Table:10 List of chemical compounds identified from methanol stem extract of *Cryptostegia grandiflora* through GC-MS analysis

S.NO	RT	Name of the compound	Area%	Biological Activity
1.	16.733	3,5-Dimethylbenzaldehyde thiocarbamoylhydrazone	10.97	Antiseborrheic, Bone disease treatment, Phobic disorders treatment, Adenomatous polyposis treatment
2.	16.733	Anthranilic acid, N-methyl-, butyl ester	10.97	Dementia treatment, Antiseborrheic, Anesthetic general
3.	16.733	Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl-	10.97	Antipyretic, Anti neoplastic, Dementia treatment,
4.	17.036	Arsenous acid, tris(trimethylsilyl) ester	33.62	Antiviral, Anti infective, Mucositis Treatment, Carminative, Oxygen scavenger, Prostate disorders treatment
5.	17.036	1,1,1,3,5,5,5-Heptamethyltrisiloxane	33.62	Anti infective, Antiviral, Insecticide, Phobic disorders
6.	17.149	Silicic acid, diethyl bis(trimethylsilyl) ester	7.06	Antiviral, Oxidizing agent, Anti infective Carminative, Dementia treatment
7.	17.149	2-Methyl-7-phenylindole	7.06	Antiviral (Picmavirus), Antianorexic, Antiinfective, Antipyretic, Cancer associated disorders

				treatment, Anti mutagenic, Antiseptic
8.	17.149	2-Ethylacridine	7.06	Antiseptic, Prion disease treatment, Liver fibrosis treatment, Kidney function stimulent
9.	17.244	1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	34.79	Anti neoplastic Oxygen scavenger, Antiprurtic, Dementia treatment, Anti viral
10.	17.244	tert-Butyl(5-isopropyl-2-methylphenoxy)dimethylsilane	34.79	Anti inflammatory, Antheresclerosis treatment, Antiprurtic treatment, Bone disease treatment, Alopecia treatment
11.	17.404	1,2-Benzisothiazole-3-acetic acid, methyl ester	13.55	Antipyretic, Antianemic, Sepsis treatment, Dementia treatment, Antithrombotic, Diabetic neuropathy treatment
12.	17.404	N-Methyl-1-adamantaneacetamide	13.55	Anti infective, Phobic disorders, Cancer associated disease treatment

enyl isobutyl ester (5.58%), Silicic acid, diethyl bis(trimethylsilyl)ester (1.70%), 3,3-Diisopropoxy-1,1,1,5,5,5, hexamethyltrisiloxane (1.70%), Tert-Butyl(5-isopropyl-2-methylphenoxy) dimethylsilane (1.70%), Trans-3-ethoxy-b-methyl-b-nitrostyrene (8.90%), 4H-furo[3,2-b]pyrrole-5-carboxylic acid, 4-(2-oxopropyl) (8.90%), 9H-Imidazo[1,2-a]benzimidazole, 2-(4-chlorophenyl)-9methyl (8.90%), Acetamide, 2-[4-(1-oxo-3phenyl-2-propenyl) phenyloxy] (2.36%), Benzoic acid ethyl ester, 4-(1,1-dioxo-2,3-dihydro-1H-1.lambda.(6)-thiophen-3-ylamino)- (2.36%), Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (3.69%), Trimethylsilyl-di(timethylsiloxy)-silane (3.69%), 4-Dehydroxy-N-(4,5-methylenedioxy-2-nitrobenzylidene)tyramine (3.69%), Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl- (8.72%), 1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester (8.72%), 2-Ethylacridine (2.83%), 1,2,5-Oxadiazol-3-amine, 4-(4-methoxyphenoxy)- (2.83%), Benzene, 2-[(tert-butyldimethylsilyl)oxy]-1-isopropyl-4-methyl- (16.14%), Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane (16.14%) were also present from the methanolic leaf extract of *Cryptostegia grandiflora* was reported in table-8 and their GC-MS chromatogram is represented in Fig-8 Table-8&9.

The first compound identified with less retention (11.627 min) was n-hexadeconic acid, Pentadeconic acid where as Benzene, 2-[(tert-butyldimethylsilyl)oxy]-1-isopropyl-4-methyl- and Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane was the last compound which took longest retention time (17.877 min) to identify. At (17.877 min) retention time Benzene, 2-[(tert-butyldimethylsilyl)oxy]-1-isopropyl-4-methyl-, Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane was found to be high (16.14%) and the lowest percentage (1.70%) was found to be Silicic acid, diethyl bis(trimethylsilyl) ester, 3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyltrisiloxane and tert-Butyl(5-isopropyl-2-methylphenoxy)dimethylsilane.

The GC-MS analysis of methanolic stem extract of *Cryptostegia grandiflora* was confirmed the presence of 12 compounds with retention time. Interpretation of mass spectrum of GC-MS was conducted using the database of NIST and WILEY libraries. Out of this 12 compounds 2 compounds were majority present in the stem

extract of *Cryptostegia grandiflora* respectively 2-Ethylacridine(7.6%), tert-Butyl(5-isopropyl-2-methylphenoxy)dimethylsilane (34.79%).

The ten minor compounds such as 3,5-Dimethylbenzaldehyde thiocarbamoylhydrazone (10.97%), Anthranilic acid, N-methyl-, butyl ester (10.97%), Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl (10.97%), Arsenous acid, tris(trimethylsilyl) ester (33.62%), 1,1,1,3,5,5,5-Heptamethyltrisiloxane (33.62%), Silicic acid, diethyl bis(trimethylsilyl) ester (7.06%), 2-Methyl-7-phenylindole (7.06%), 1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester (34.79%), 1,2-Benzisothiazole-3-acetic acid, methyl ester (13.55%), N-Methyl-1-adamantaneacetamide (13.55%) were also reported from the ethanolic leaf extract of *Cryptostegia grandiflora* stem were reported in Table 8 and their GC-MS chromatogram is presented in Fig:9, Table-10&11.

The first compound identified with less retention (16.733 min) was 3,5-Dimethylbenzaldehyde thiocarbamoylhydrazone, Anthranilic acid, N-methyl-, butyl ester, Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl where as 1,2-Benzisothiazole-3-acetic acid, methyl ester, N-Methyl-1-adamantaneacetamide was the last compound which took longest retention time (17.404 min) to identify. At (17.404 min.) 1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester and tert-Butyl(5-isopropyl-2-methylphenoxy)dimethylsilane was found to be high (34.79%) and lowest percentage (7.06%) was found to be Silicic acid, diethyl bis(trimethylsilyl) ester, 2-Methyl-7-phenylindole, 2-Ethylacridine, the above mentioned isolated compounds from the methanol leaf and stem extract of *Cryptostegia grandiflora* have a medicinal important.

n-Hexadeconic acid in the leaf methanolic extract of *Cryptostegia grandiflora* is a main antimicrobial compound (S.Agneeswari *et al.*,2019), Hexadeconic acid have a Antibacterial, anti fungal, antioxidant, insecticide lubricant.(Anil F Bobade, 2019).

Trimethylsilyl-di(timethylsiloxy)-silane is found in leaf methanolic extract of *Cryptostegia grandiflora* is a main compound of antibacterial compound. (S.Agneeswari *et al.*, 2019).

Tert-Butyl(5-isopropyl-2-methylphenoxy)dimethylsilane is found in stem methanolic extract of *Cryptostegia grandiflora* is a main antimicrobial compound.(S.Agneeswari *et al.*, 2019).

ANTIOXIDANT ACTIVITY:

An antioxidant is a molecule capable of showing or preventing the oxidation of other molecules. In a biological system, they protect cells from the damage caused by unstable molecules known as free radicals. Antioxidants terminate the chain reactions by removing free radical intermediates, and inhabit other oxidation reactions by being oxidized themselves. They are believed to play a role in preventing the development of Chronic disease like cancer, heart disease, stroke, AD, RA and cataracts (Chakraborty *et al.*,2010).

Antioxidant chemicals found in nature inhibit or prevent oxidation of substrate leading to the formation of reactive oxygen species and reactive nitrogen species and thus protect the biological system (Hwang *et al.*,2007). Fruits and vegetables are endowed with antioxidants and consumption of these, prevent and protect from oxidative stress related diseases, inflammatory diseases viz., arthritis, autoimmune disease, carcinogenesis, neurodegenerative diseases, cardiovascular disorders etc. Several food industries use butyated hydrooxyanol, butylated hydroxy toluene and tertiary butyl hydroquinone, the common synthetic antioxidants for preventing lipid oxidation in food products while processing and storage. These synthetic antioxidants have been suspected to be carcinogenic and hence their use as food ingredients has been prohibited (Hung and Wang 2004). Natural antioxidanrs comprised non-detrimental chemical combinations are considered to be rather safer for use in food protects. Further, uncared wastes are if exploited as resource of antioxidants, will be more beneficial to human kind and protecting the environment. Flavanoids are water soluble polyphenolic molecules with antioxidant activity which has many beneficial effects on the cardiovascular system (Evans,1989). Vitamin C acts as ROS scavenger, thus potentially protecting cells from harmful oxidative products (Fossati *et al.*,). Vitamin E supplement elevates the activities of antioxidant enzymes (Kiron *et al.*,2004).

Table:12 Antioxidant activity in aqueous extract of stem and leaf of *Cryptostegia grandiflora*

S.NO	AQUEOUS EXTRACT	DPPH free radical assay (%)
		Cryptostegia grandiflora
1	Stem	56.86
2	Leaf	43.89

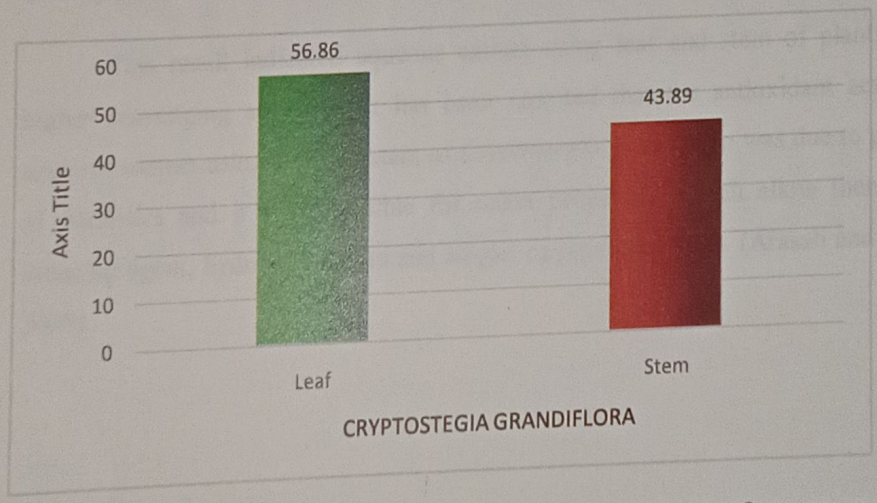


FIG:10 Antioxidant activity in aqueous extract of stem and leaf of *Cryptostegia grandiflora*

DPPH FREE RADICAL SCAVENGING ACTIVITY:

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of a specific compound or plant extracts (Wei *et al.*,2012). DPPH solution shows a strong absorption band at 517nm appearing as a deep violet colour. The absorption vanishes and the resulting decolourization is stoichiometric with respect to degree of reduction. The leaf and stem extract of *Cryptostegia grandiflora* was able to reduce stable DPPH radical to yellow colour diphenyl picrylhydrazine. The degree of reduction in absorption is the reflection of radical scavenging power of the compound.

The antioxidant activity of aqueous extract using leaf and stem of *Cryptostegia grandiflora* plants was evaluated by using DPPH scavenging essay Fig (10). Aqueous extract using *Cryptostegia grandiflora* leaf has higher scavenging activity (56.86%) followed by stem (43.89%) as shown Fig (10) and Table(12).

This result indicated aqueous extract using leaf and stem of plants shows higher scavenging activities. It has been reported that the antioxidant activity of aqueous extract using leaf and stem of *Cryptostegia grandiflora* was due to presence of phenolics and it is responsible for redox properties, which allow them to act reducing agent, hydrogen donors and singlet oxygen quenchers. (Arasali and Kadimi 2009).

SUMMARY & CONCLUSION

SUMMARY AND CONCLUSION

Cryptostegia grandiflora well known plant of family Asclepiadaceae is used as a therapeutic agent. Various part of *Cryptostegia grandiflora* are traditionally used as purgative, analgesic, wound healing remedis, antioxidant, antiviral and treatment of schistosomiasis. (K.Nagendrea Prasad 2017). The medicinal effects of plants are considered to be due to metabolites, especially secondary compounds, produced by plants. In this study, we determined flavonoid, tannin and phenol, vitamin E and vitamin C content of leaf and stem of *Cryptostegia grandiflora* using spectrophotometric methods. The result of this study showed that the leaf of *Cryptostegia grandiflora* have significant amount of phenol, flavonoid and tannin compared to the stem. The stem of *Cryptostegia grandiflora* have significant amount of vitamin E and vitamin C compared to the leaf.

The FTIR spectrum of *Cryptostegia grandiflora* showed strong IR bands characteristics of Amine (2315.38 cm^{-1}) ,Aminoacid (1643.24 cm^{-1}),Nitro group , aromatic group (1512.09 cm^{-1}), Aryl tertiaryamine (1317.29 cm^{-1}) Monofluorinated (1000.99 cm^{-1}), Monochlorinated Alicyclic group (768.58 cm^{-1}), Acyclic Axial (669.25 cm^{-1}) Polyvalent Alcohols group (3587.35 cm-1), Sulphon Amide (3276.83 cm^{-1}), Fecilaey Amine salt group (2316.35 cm^{-1}), Phenolic Ester (1747.39 cm^{-1}) Sulphonamide (1338.51 cm-1), Aldehyde group (1265.22cm^{-1}), Vibraliorisoprophyl group (1164.92cm^{-1}), Cyclopentane group (892.98cm^{-1}), Polychlorinate group (713.61cm^{-1}), Sulphides, Disulphides group(517.85cm^{-1}) Functiional group. From the spectral data , presence of NH3+,NO2, C-N, C-F, C-CL, C-Br, O-H, N-H, N-H, C=O, SO2, C-CHO ,C-H, C-H, C-CC, S-S were identified. These bonding are responsible for the presence of Amine group,Amino acid group, Nitro group, Aryl tertiaryamine group, Monofluorrinated group, Mono chlorinated Alicyclic group, Acyclic Axial group, Polyvalent Alcohols group, Sulphon amide group, Fecilaey Amine salt group, Phenolic Ester group, Sulphonamide group, Aldehyde group, Vibraliorisoprophyl group, cyclopentane group, Polychlorinate group. Sulphides, Disulphides group. The amino acid present in the medicinal plant serves as the main pharmaceutical product in Skeletal muscle function, atrophic conditions, sarcopenia, and cancer, synthesis of

hormones, immune function, cardiovascular health. Amide and amino acids are the main groups which are involved in protein synthesis.

The GC-MS analysis of amethanolic extract of *Cryptostegia grandiflora* confirmed the presence of 30 compounds with retention time. Out of these 30 compounds, 4 compounds were majority and 26 minor compounds present in the leaf extract of *Cryptostegia grandiflora*. The GC-MS analysis of methanolic stem extract of *Cryptostegia grandiflora* was confirmed in the presence of 12 compounds with retention time. The above mentioned isolated compound from the methanolic extract of *Cryptostegia grandiflora* leaf and stem has a medicinal importance.

n-Hexadeconic acid in the leaf methanolic extract of *Cryptostegia grandiflora* is a main antimicrobial compound (S.Agneeswari *et al.*,2019). Hexadeconic acid has a Antibacterial, anti fungal, antioxidant, insecticide lubricant. (Anil F Bobade, 2019). Trimethylsilyl-di(timethylsiloxy)-silane is found in leaf methanolic extract of *Cryptostegia grandiflora* is a main compound of antibacterial compound. (S.Agneeswari*et al.*, 2019). Tert-Butyl(5-isopropyl-2-methylphenoxy)dimethylsilane is found in stem methanolic extract of *Cryptostegia grandiflora* is a main antimicrobial compound. (S.Agneeswari *et al.*, 2019). PhytocompoundTert-Butyl(5-isopropyl-2-methylphenoxy) dimethylsilane at retention time of 17.244 min has an antimicrobial.

The antioxidant or free radical scavenging activity of leaf and stem extracts of this selected medicinal plant is investigated by using methods like DPPH scavenging activity. The leaf and stem extracts of *Cryptostegia grandiflora* shows maximum antioxidant activity. The findings of the present study suggest that *C.grandiflora* could be a potential source of natural antioxidants that could have great importance as a therapeutic agent in preventing or slowing oxidative stress related degenerative diseases.

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A COMPARATIVE STUDY ON PHYTOCHEMICAL ATTRIBUTES AND ANTIBACTERIAL ACTIVITY OF BANANA PEELS

A Short term Project Work Submitted to St. Mary's College (Autonomous)

Affiliated to Manonmaniam Sundaranar University in Partial Fulfillment for the Degree of

BACHELOR OF SCIENCE IN BOTANY

By

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THOOTHUKUDI - 628001

2021 -2022

CERTIFICATE

It is certified that this short term project work entitled "A COMPARATIVE STUDY ON PHYTOCHEMICAL ATTRIBUTES, ANTIBACTERIAL ACTIVITY OF BANANA PEELS " submitted to St. Mary's College (Autonomous) affiliated to **MONONMANIAM SUNDARANAR UNIVERSITY** in partial fulfilment of therequirements for the degree of Bachelor of Science in Botany, and is a record of work done in the Department of Botany, St. Mary's College (Autonomous), Thoothukudi during the year 2021 -2022 by the following students.

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It is our humble attempt to present this project "A COMPARATIVE STUDY ON PHYTOCHEMICAL ATTRIBUTES AND ANTIBACTERIAL ACTIVITY OF BANANA PEELS

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CONTENTS

INTRODUCTION

lacking, or possess relicts of male flowers. Plantains are always cooked before consumption and are higher in starch than bananas. The two groups of plantains, French and Horn, produce fewer fruit per plant than sweet bananas. The groups differ in whether the male parts of the inflorescence are present or absent.

Banana normally has a short shelf life and start deteriorating just after plucking. The most widely used part of banana is the flesh of the fruit; meanwhile, the outer skin is only used for animal feed and organic fertilizer. Plantain and unripe banana are cooked as vegetable, chips, snacks, powder etc., whereas, mature dessert banana is eaten raw. In recent times, Banana peel has been utilized for various industrial applications including bio-fuel production, bio-sorbents, pulp and paper, cosmetics, energy related activities, organic fertilizer, environmental cleanup and biotechnology related processes. The banana peel is rich in phytochemical compounds than its pulp. The antifungal, antibiotic properties of banana peel can put to be good use. The peel is used for home remedy for treating several skin problems including allergies and skin irritations. The banana peels waste is normally disposed in municipal landfills, which contributes to the existing environmental problems. However, the problem can be recovered by utilizing its high-added value compounds, including the dietary fibre fraction that has a great potential in the preparation of functional foods.

Some of the specific diseases known to be cured by banana are anemia: High in iron, bananas are belived to stimulate the production of heomoglobin in the blood and so help in cases of anemia (Amit and shailandra, 2006). Blood pressure: banana is extremely high in potassium yet low in salt, perfect food for helping to beat blood pressure (Debabandya *et al.*, 2010). Depression: This is because banana contain tryptophan, a type of protein that the body convert into serotonin known to make you relax, improve your mood and genrally make you feel happier (Grish and sathish, 2008). Morning sickness: Snacking on bananas

between meals help to keep blood sugar level up and avoids morning sickness (Amit and shailandra, 2006). Nerves: bananas are high in B vitamins that help calm the nervous system (Singh and bhat, 2003). Banana was enriching with minerals like potassium, phosphorus, magnesium and calcium. Banana peel could be good source of carbohydrates and fibers. The high fiber content also indicates that the peels could help treat constipation and improve general health and well being (Anhwange, *et al.*, 2009). Therefore the current study aimed to validate the phytochemical screening and antibacterial activities of three types of banana peel.

Scope and Objectives:

Banana is used widely because of its nutritional values. Bananas are an excellent source of vitamin B6 and contain moderate amounts of vitamin C, manganese and dietary fibre. All parts of banana plant such as flower, pulp, stem and leaves have medicinal applications. In past, there are studies that show banana plant parts, and their fruits can be used to treat the human diseases. Peels are often the waste part of various fruits. These wastes have not generally received much attention with a view to being used or recycled rather than discharged. This might be due to their unknown benefit of commercial application. Peels from about 18-33% of the whole fruit and are waste products. According to Health line banana peel rich in antioxidants, fibre and essential nutrients, helps brighten the skin reduce wrinkles. Natural products play a dominant role in the development of novel drug leads for the treatment and prevention of disease. Knowledge of the chemical constituents of plant is helpful in the discovery of therapeutic agent. However it is essential to work on locally available resource to bring out their pharmaceutical values and antimicrobial in medicine. Hence the present investigation was undertaken with the following objectives.

- To identify the phytochemicals present in the three types of banana peel (Yellow Banana, Red Banana and Green Banana)
- Elucidating the effectiveness of banana peel in controlling human pathogenic bacteria such as *Escherichia coli, Bacillus subtilis, Vibrio cholerae* , *Staphylo cocus aureus* and *Proteus vulgaris.*

The results of the study also underline the cost effective, bio friendly resources which would be tapped for development of effective drug in future.

CHAPTER III

REVIEW AND LITERATURE

Banana trees are giant perennial herbs coming from the Southeast Asia and belong to the Musa genus. From the botanical point of view, *Musa* genus can be divided into two parts: edible and wild species. It is widely believed there are more than 1,000 types of bananas in the world, which are subdivided into 50 groups. The scientific names of most cultivated bananas are *Musa acuminata*, *Musa balbisiana* and *Musa paradisiaca* for the hybrid *Musa acuminata* × *M. balbisiana*, depending on their genomic constitution.

Banana is one of the world's leading fruit crops grown in over 122 countries worldwide. Banana normally has a short shelf life and start deteriorating just after plucking. The most widely used part of banana is the flesh of the fruit; meanwhile, the outer skin is only used for animal feed and organic fertilizer.

Banana evolved in the humid tropical regions of S.E Asia with India as one of its centres of origin. Peels from about 18-33% of the whole fruit and are a waste product. According to Healthline banana peel rich in antioxidants, fibre and essential nutrients, helps brighten the skin reduce wrinkles. It even acts as a moisturiser and helps in hydrating the skin. Several studies have found banana peel to have anti-inflammatory properties, too.

Antibacterial and phytochemical analysis of Banana peel.

Ehiowemwenguan *et al* ., 2014 investigated that the *in vitro* antibacterial activity of ethanolic and aqueous extract of banana (*Musa sapientum*) peels on both gram - positive and gram - negative bacteria using agar well diffusion technique. Phytochemical result showed ethanol to be a better solvent for the extraction of the bioactive agents in banana peels which include: glycosides, alkaloids, saponins, tannins, flavonoids and volatile oil. The presence of glycosides and alkaloids in *Musa sapientum* peels may be attributed to their use by traditional

medicine practitioners in healthcare systems in the treatment of some bacterial infections such as cough, fever, cold and venereal diseases. Thus extracts from the peel can be used to control infections caused by *Salmonella typhi*, *Escherichia coli*, *Klebsiella pneumonia* and *Staphylococcus aureus*.

Infections such as bronchopneumonia, bacterial endocarditis and meningitis caused by *Micrococcus* Sp. and *Pseudomonas aeruginosa* will also find treatment with the extracts of this medicinal peel.

Banana (*Musa acuminata*) contains peels which are rich in important bioactive compounds which makes them greatly significant for therapeutic treatments. For studying phytochemical and antimicrobial properties of the peels, six varieties of bananas were chosen from Quetta. Carbohydrates, tannins, steroids, terpenoids, flavonoids, glycosides and phenols were found in the six solutions of banana peels samples through phytochemical screening. Evaluation of antibacterial activity of peel solutions through agar disc diffusion method showed inhibition zone diameters of 19 mm and 17 mm against *Escherichia* coli and Staphylococcus aureus respectively. Poisoned food technique and agar well assays were used for the antifungal activity but the BP solutions against *Aspergillus niger* were not effective. In addition to this, proximate analysis was also carried out to characterize the peel powder and the results showed 6-11% moisture, 60-75% volatile matter, 13-25% ash and 2-9% fixed carbon contents. For the analysis of elements (Iron and Copper) concentrations, Atomic Absorption Spectrophotometer was utilized which concluded that banana peels could be excellent therapeutic agent (Farrukh Bashir *et al* ., 2021)

Herbal medicines have been used to treat various infectious diseases and have been found to be effective in a number of instances. The majority of natural medicines are derived from

plant materials such as leaves, flowers, fruits and stems. These extracts may be used to develop novel antimicrobial compounds with new chemical structures and mechanisms of action that will serve as a barrier against multidrug-resistant microorganisms. A previous study discovered that the antimicrobial properties of banana peel were effective against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Salmonella enteritidis* and *Escherichia coli* (Mokbel and *Hashinaga*, 2005).

Krishna *et al.* (2013) reported that ethanol extracts of *M. paradisiaca* and *M. acuminata* have a broad spectrum of antibacterial activity against the tested microorganisms, with particularly high inhibitory potency against *P. vulgaris* and *S. paratyphi*. Phytochemical analysis revealed the presence of biologically active compounds such as glycosides, flavonoids, terpenoids and tannins. These derivatives may be used in the treatment of clinical pathogenic bacteria.

Musa acuminata peel methanolic extract exhibited variable inhibitory activity against *Escherichia coli* (ATCC 25922), S. *aureus* (ATCC 25923), *Lactobacillus casei*, *Bacillus spp.*, *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* at a 300 mg/mL concentration (Niamah *et al.*, 2014).

Mordi *et al.* (2016) reported a high antibacterial potential of oil from methanolic peel extract (30 mg/mL) against several bacterial species. This finding is likely due to the presence of 2-methyl-5-(1-methylethyl) phenol in peel, which is a potent antimicrobial compound. The presence of tannins has also demonstrated some antimicrobial activities against the three tested microorganisms (*E. coli*, *S. aureus* and *P. aeruginosa*) (Aboul-Enin *et al.*, 2016).

Banana is used widely because of its nutritional values. In past, there are studies that show banana plant parts, and their fruits can be used to treat the human diseases. Banana peel is a part of banana fruit that also has the antibacterial activity against microorganisms but has not

been studied extensively. Since, there are no studies that relate the antibacterial activity of banana peel against periodontal pathogens.

Suraj primal kapadia pushpa *et al*., 2015 studied the antimicrobial activity of banana peel extract on *Porphyromonas gingivalis* (*P. gingivalis*) and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*). Detection of antimicrobial activity of banana peel (*Musa paradisiaca* L.) on *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*:

Nishant Kumar., *et al*. (2021) have done the A Comprehensive review on phytochemical, nutritional, and therapeutic importance of *Musa* acuminata (*Musa acuminata*) Acetone : water; phenolics, anthocyanins, dopamine, catecholamines , Methanol; Glycoside, tannin, saponins, flavonoids, alkaloids, steroids, terpenoids, triterpenes, phenols, palmitic, oleic and linoleic acid and their methyl esters, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one,5-(hydroxymethyl) 2furancarboxyaldehyde, methyl hexadeconoate, methyl-9, 12-octadienoate, methyl1-9-octadeconoate, 9,12-ocatadecanoic acid, 13-octadecanoic acid, octadecanoic acid, 2-methyl-5-(1-methyl ethyl) phenol, pentadecanoic acid, cis-9- hexadecenal, cis-9-hexadecenoic acid, benzoic acid, pyrogallol, sesamin, epi-sesamin , Hexane; Glycosides, tannins, saponins, flavonoids, carbohydrates , Ethanol; Saponins, carotenoids, phenolics, flavonoids, tannins

Velumani (2016) investigated that phytochemical screening and antioxiant activity of Banana peel (*Musa paradaisica*) The phytochemical analysis of aqueous and organic solvent extracts of banana peel (*Musa paradaisica*) reveal the presence of alkaloids, flavonoids, carbohydrates, proteins, tannins, terpenoid, saponins, Glycosides and Anthroquiones. The Phytochemicals alkaloids, flavonoid and tannins, have a very good antioxidant property while saponins and terpenoids have antimicrobial activity.

Farrukh Bashir *et al.*, 2021evaluated the phytochemistry and antimicrobial activities of different varieties of Banana (*Musa acuminata*). Banana (*Musa acuminata*) contains peels which are rich in important bioactive compounds which makes them greatly significant for therapeutic treatments'. For studying phytochemical properties of the peels, six varieties of bananas were chosen from Quetta. Carbohydrates, tannins, steroids, terpenoids, flavonoids, glycosides and phenol were found in the six solutions of banana peels samples through phytochemical screening.

Carbohydrates have been isolated from *M. sapientum* (*Anhwange*, 2008). Catecholamines such as norepinephrine, serotonin, dopamine (Waalkes *et al.*, 1958; Vettorazz, 1974), tryptophan, indole compounds (Shanmugavelu and Rangaswami, 1962), pectin have been found in the pulp. Several flavonoids and related compounds (Leucocyanidin, quercetin and its 3-O-galactoside, 3-O-glucoside, and3-O-rhamnosyl glucoside) were isolated from the unripe pulp of plantain (Lewis *et al.*, 1999; Lewis and Shaw, 2001; Ragasa *et al.*, 2007). Serotonin, nor-epinephrine, tryptophan, indole compounds, tannin, starch, iron, crystallisable and non-crystallisable sugars, vitamin C, B-vitamins, albuminoids, fats, mineral salts have been found in the fruit pulp of *M.paradisica* and M.*sapientum* (Ghani,2003)

Acyl steryl glycosides such as sitoindoside-I, sitoindoside-II, sitoindoside-III, sitoindoside-IV and steryl glycosides such as sitosterol gentiobioside, sitosterol *myo*-inosityl-β-D-glucoside have been isolated from fruits of *M. paradisiacal* (Ghoshal, 1985).

Jang *et al.* (2002) isolated a bicyclicdiarylheptanoid,*rel*-(3*S*,4a*R*,10b*R*)-8-hydroxy-3-(4-hydroxyphenyl)-9-methoxy-4a,5,6,10b-tetrahydro-3*H*-naphtho[2,1-*b*]pyran, and1,2-dihydro-1,2,3-trihydroxy-9-(4-methoxyphenyl)phenalene, hydroxyanigorufone, 2-(4-hydroxyphenyl)naphthalic anhydride, 1,7-bis(4-hydroxyphenyl)hepta-4(*E*),6(*E*)-dien-3-one.

Ragasa *et al.* (2007) reported the isolation of several triterpenes such as cyclomusalenol, cyclomusalenone, 24-methylenecycloartanol, stigmast-7-methylenecycloartanol, stigmast-7-

en-3-ol, lanosterol and amyrin. An antihypertensive principle,7, 8-dihydroxy-3-methylisochroman-4-one, was isolated from thefruit peel of *M. sapientum* (Qian *et al.*, 2007).

Cycloartane triterpenes such as 3-epicycloeucalenol, 3-epicyclomusalenol, 24-methylenepollinastanone, 28-norcyclomusalenone, 24-oxo-29-norcycloartanone have been isolated from the fruit peel of *M. sapientum* (Akihisa *et al.*, 1998).

Cellulose, hemicelluloses, arginine, aspartic acid, glutamic acid, leucine, valine, phenylalanine and threonine have been isolated from pulp and peel of *M. paradisiaca* (Ketiku, 1973; Emaga *et al.*, 2007).

Among fruits, banana is one of the widely consumed fruit in the world. Once the fruit is eaten the peel is discarded. But Banana peels are as useful as the banana. They are known to contain antifungal, antibiotic and enzymatic properties. It is rich in vitamins and fiber content. In the present study phytochemical and antifungal activity of ripe banana peels are studied. The Scalp fungi were isolated on Sabouraud's Dextrose Agar and observed for growth. The grown fungus were identified by morphological characteristics & Lactophenol staining and the antifungal activity of the extract of banana peel powder and ash prepared using distilled water were evaluated for the isolated fungi. The fungi isolated were *Aspergillus niger, Aspergillus flavus* and *Penicillium* sps. The phytochemical analysis showed that all the three varieties of banana peel in powder and ash showed the presence of carbohydrates, phenols, terpenoids and saponins were present in powdered form of all the three banana peel varieties. In the study, it was found that the extract of dried Kadali banana peel powder and ash exhibited antifungal activity against *Aspergillus niger* (Bharathi Prakash *et al.*, 2017)

Said Behiry *et al* ., 2019 evaluated *Melia azedarach* wood samples with the methanolic extract of *Musa paradisiaca* L. peels for their antibacterial and antifungal activities against *Agrobacterium tumefaciens*, *Dickeya solani*, *Erwinia amylovora*, *Pseudomonas cichorii*, *Serratia pylmuthica*, *Fusarium culmorum*, and *Rhizoctonia solani*. The strongest

antibacterial activity was only found against *A. tumefaciens* (inhibition zone 90 mm), while the other bacterial strains showed resistance to wood that was treated with the extract. Potential antifungal activity against *F. culmorum* and *R. solani* was observed; the mycelial growth inhibition percentages reached 68.88% and 94.07%, respectively, in wood samples that were treated with the 3% methanolic extract of *M. paradisiaca* peel. HPLC analysis demonstrated the presence of seven phenolic compounds and three flavonoid compounds, as their peaks were matched with the standard compounds in a HPLC analysis. The major constituents of phenolic and flavonoid compounds in mg/100 g dry extract (DE) were ellagic acid (16.19), gallic acid (7.73), rutin (973.08), myricetin (11.52), and naringenin (8.47). The results demonstrated the potential effects of banana peel extract as a natural compound that can protect wood from molds while in use.

Giri *et al* ., 2016 evaluated the feasibility of *Musa acuminata* (banana) peels as a feed additive, effects of banana peel flour (BPF) on the growth and immune functions of Labeo rohita. Diets containing five different concentrations of BPF (0% [basal diet], 1%[B1], 3%[B3], 5%[B5], and 7%[B7]) were fed to the fish (averageweight:15.3g) for 60days.The final weight gain and specificgrowth rate were higher ($P < 0.05$) in the B5 group. These results suggest that dietary BPF at 5% could promote growth performanceand strengthen immunity in *L. rohita*

Sri Atun *et al*., 2010 isolated some compounds from methanol extracts of peel of banana (*Musa paradisiaca* Linn.) which had activities as antioxidant, and determined this structure. Method of this study was extracted powdered peel of banana with methanol at room temperature. Extract was concentrated in vaccuo and then successively was partitioned with n-hexane, chloroform, etyl acetate, and buthanol. Antioxidant test from each fractions was measured by hydroxyl radical scavenger test with Fenton reaction method. The result of this study showed activity each fractions as hydroxyl radical scavenger activity of chloroform, ethyl acetate, and buthanol fraction were IC50 693.15; 2347.40; and 1071.14 mg/mL respectively.

The isolation of secondary metabolite compounds from chloroform fraction obtained two isolate compounds. Identification by spectroscopy IR, MS, 1H and 13C NMR one and two dimension showed that the compounds are 5,6,7,4'-tetrahidroxy-3,4-flavan-diol and a new compound cyclohexenon derivative (2-cyclohexene-1-on-2,4,4-trimethyl-3-O-2'-hydroxypropyl ether).

Banana is an important crop worldwide; plantation generates tonnes of residues after each harvest season and during processing to obtain banana pulps. The depositions may include leaves, pseudostem, stalk and inflorescence, but 35%–50% of the total mass fruit represents the banana peel (Gomes *et al.*, 2020). Banana peels are typically dumped into the environment without any treatment. In some cases, banana peel may be used as organic fertiliser and animal feed because of its low tannins and high fibre content (Pereira and Maraschin, 2014). Approximately 36 million tons of banana peel is produced every year, and their current endpoint is associated with adverse environmental impact and economic losses (Gomes *et al.*, 2020). Every day, several tonnes of banana peel wastes is generated in fruit markets and home garbage, causing an unpleasant odour because of anaerobic digestion of the biomass, which produces gases that disturb the natural balance of air. Although ripe bananas are consumed unprocessed, significant quantities of bananas are industrially processed into banana flour, chips and other processed goods, resulting in vast banana peel waste. Previously, banana peels were disposed of in a landfill by food manufacturing industries. The agriculture industry would benefit financially from the conversion of banana peel into a valuable product.

Several epidemiological studies indicate that dietary antioxidants reduce the risk of diseases such as diabetes, cancer and CVD, which are typically associated with oxidative stress (Nisha and Mini, 2014). Dietary antioxidants may play a role in preventing and complementing the treatment of these diseases by scavenging free radicals and minimising

oxidative stress. They can also protect food from oxidation; thus, dietary antioxidants are a potential alternative to synthetic antioxidants that are strictly regulated for use because of their possible health risks (Agourram *et al*., 2013). Their use can be expanded to the food industry because of the low cost and large quantities of plant biowastes produced, where they can be used as antioxidants to invent new functional foods. Phenolics are important secondary metabolites, which are present in banana peels at high levels relative to other fruits. Numerous phenolic compounds, such as gallic acid, catechin, epicatechin, tannins and anthocyanins, are found in banana peel (Sidhu and Zafar, 2018). In addition, gallocatechin in banana peel is five times higher than that in pulp, implying that peel is a high source of antioxidant compounds (Someya *et al*., 2002).

Anti Cancer activity:

Phytochemical constituents found in fruit and vegetable extracts is more effective at preventing cancer than their individual components. In addition, edible phytochemicals provide a readily available, suitable and attainable basis for cancer control and management. In a study by Dahham *et al*. (2015), the banana peel extract prepared from hexane solvent exhibited the highest toxicity towards HCT-116 (colorectal carcinoma cell line from humans) with 64.02% cell inhibition of cell proliferation. In a separate study, Durgadevi *et al*. (2019) demonstrated that the aqueous methanol extract of Nendran banana peel had a significant cytotoxic activity against MCF-7 breast cell lines. Vijayakumar *et al*. (2017) demonstrated that banana peel crude extract could also be used to synthesise gold nanoparticles that inhibited the biofilm formation of Gram-positive bacteria *Enterococcus faecalis*, which were cytotoxic to human lung cancer cells.

Flavonoids could inhibit the ROS-scavenging enzyme activities, induce apoptosis, arrest cell cycle and subsequently suppress tumor production . The proposed mechanism of action of flavonoids as an anticancer agent. In another study by Phacharapiyangkul *et al*. (2019),

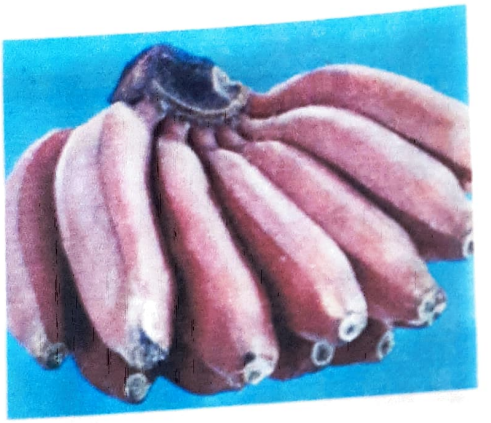
the ferulic acid that is highly identified in sucrier banana peel potentially act as anti-melanogenesis by regulating the growth factor of vascular endothethelial expression, initiating nitric oxide synthase, and acting as a suppressor gene of tumor.

CHAPTER- IV

MATERIALS AND METHODS

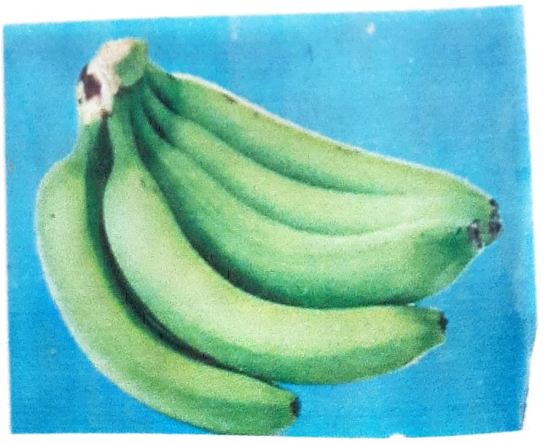
Class : Monocotyledonae
Order : Zingiberales
Fmily : Musaceae
Genus : Musa

(a) Red Banana



(b) Yellow Banana

(c) Green Banana



Sample Collection and preparation:

Fresh banana such as red banana, green banana and yellow banana were obtained from Thoothukudi market during December 2021. After collecting the banana peels were removed, they were carefully washed with running tap water to remove any dust or foreign materials. The fresh peels were taken and cut into smaller bits and allowed to air dry. The dried pieces ground into powder with a mechanical blender and sieved with a mesh of size 0.50 mm. The powdered samples obtained were thereafter stored in clean bottles at room temperature (28±2°C) until needed for use

Preparation of banana peel extracts:

10 grams powdered sample was sequentially extracted with 200 ml of benzene, acetone, ethanol, methanol and aqueous solution using in soxhlet apparatus. The prepared extracts were tested for phytochemical screening and anti bacterial activities.

Phytochemical qualitative analysis:

The phytochemical tests were done for analyzing different chemical groups present in the extracts. These were done to find out the presence of bioactive chemical constituents such as alkaloid, flavonoids, tannis, glycosides, steroids, saponins and resins. Detection of achieve phytochemical constituents was carried out for all the extracts using the standard procedures

Test for Glycosides:

To 1ml of the extract was added 2ml of acetic acid and then cooled in an ice bath at 4°C. To this mixture 1ml of concentrated tetraoxosulphate (vi) acid (H_2SO_4) was added dropwise.

The formation of an oil layer on top of solution indicated the presence of glycosides (Odebiyi and Sofowora, 1978).

Test for Alkaloides:

To 3ml of the extract was added 1ml of 1% HCL. This resulting mixture was then treated with few drops of Meyer's reagent. The appearance of a creamy white precipitate confirmed the presence of alkaloids (Ogukwe *et al.*, 2004).

Test for Saponins:

Five drops of olive oil was added to 2ml of the plant extract and the mixture shaken vigorously. The formation of a stable emulsion indicated the presence of saponins (Trease and Evans, 1996)

Test for Tannins:

Two drops of 5% $FeCl_3$ was added to 1ml of the plant extract. The appearance of a dirty-green precipitate indicated the presence of tannins (Trease and Evans, 1996)

Test for Flavonoids:

To 1ml of the extract was added 3 drops of ammonia solution (NH3+) followed by 0.5ml of concentrated HCl. The resultant pale brown colouration of the entire mixture indicated the presence of flavonoids (Odebiyi and Sofowora, 1978).

Test for Steroids:

To 1ml of the plant extract was added 1ml of concentrated tetraoxosulphate (vi) acid (H2SO4). A red colouration confirmed the presence of steroids (Trease and Evans, 1996).

Test for Resins:

To 5ml of the extract was added 5ml of copper acetate solution. The mixture was shaken vigorously and allowed to separate. The appearance of a reddish-brown precipitate indicated the presence of resins (Elmahmood and Doughari, 2008).

Antibacterial activity - Disc diffusion Assay

Antibacterial activity of each plant extract was analysed using human pathogens., Gram positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli*, *Proteus vulgaris* and *Vibrio cholerae* obtained from the Department of Microbiology; St. Mary's College (Autonomous), Thoothukudi. Each bacterial pathogen was subcultured in agar medium and maintained. What man No. 1 sterile filter paper discs (5mm) were impregnated with 2.5 mg/ ml and dried aseptically at room temperature. The spread plates were prepared by proper concentration of inocula. Each sample loaded disc was placed in the seeded agar plate. After 24-48 hours of ±37°C incubation, the diameter of the inhibition zone was measured. For positive control, streptomycin disc (100 µg/ ml) was used, whereas for negative control, respective solvents were loaded on sterile discs.

RESULT AND DISCUSSION

Result and Discussion

Phytochemical Analysis

The present study carried out on the Banana peels waste materials reveals the presence of medicinal and active compounds. The phytochemical active compounds of materials were qualitatively analysed from the five types of extracts such as methanol, ethanol, acetone, benzene and aqueous extract and the result were presented in table 1, 2 and 3 based on the color change indicate positive and negative result. In this screening process we were identified different types phytochemicals which has shown their activity in different solution.

The present study carried out in three types of banana (yellow banana, red banana and green banana) revealed the presence of alkaloids, flavonoids, tannins, saponins, steroids, glycosides, saponins and resins.alkaloids are nitrogen containing naturally occurring componds found to have antimicrobial properties due to their ability to intercolate with DNA of the microorganisms (*kasolo et al., 2010)*

Alkaloids was observed in the yellow banana of all extracts and it was completely absent in red banana. Alkaloids are used as basic medicinal agents because of their analgeric, antiplasmidic and antibacterial properties (*stray, 1998)*

Flavonoids was present in all extracts of red banana .it was absent in acetone and benzene extract in green banana.In yellow banana it was present in ethanol,acetone and methonal and it was completely absent in benzene and aqueous extract.flavoind was useful in reduce body heat anf remarkable activity in cancer activity (Veerachai,2011)flavoind compounds are the sub group of polyphenols.polyphenols ,a secondary plant metabolies are most abundant antioxidants in human diet.These compound are designed with an aromatic ring carring one or

Table 1: Preliminary phytochemical analysis of red banana peels

(+ : present; - : absent)

S.NO	Phytochemical constituent	Phytochemical analysis of *red banana* extract				
		Ethanol	Acetone	Methanol	Benzene	Aqueous
1	Alkaloids	–	–	–	–	–
2	Flavonoids	++	++	+++	–	+++
3	Tannins	+	–	+	–	–
4	Glycosides	+++	++	+++	+	+
5	Steriods	+	+	–	–	–
6	Saponins	–	–	–	–	–
7	Resins	–	–	–	–	–

Table 2 :Preliminary phytochemical analysis of green banana

(+ :present ; - : absent)

	Phytochemical analysis of *green banana* extract					
S.NO	Phytochemical constituent	Ethanol	Acetone	Methanal	Benzene	Aqueous
1	Alkaloids	++	–	+	+	++
2	Flavonoids	++	–	++	–	+++
3	Tannins	+	+	+	–	–
4	Glycosides	++	+++	+++	+	++
5	Steriods	–	+	+	–	+
6	Saponins	+	+	+	–	+
7	Resins	–	–	–	–	–

Table 3:Preliminary phytochemical analysis of yellow banana

(+ :present ; - : absent)

	Phytochemical analysis of *yellow banana* extract					
S.NO	Phytochemical constituent	Ethanol	Acetone	Methnal	Benzene	Aqueous
1	Alkaloids	++	++	+++	+	+
2	Flavonoids	++	+++	+++	–	–
3	Tannins	+	–	+	+	–
4	Glycosides	++	+++	+++	+	–
5	Steriods	+	+	–	–	–
6	Saponins	+	+	++	+	–
7	Resins	–	–	–	–	–

more hydroxyl moieties .two main group of polyphenols termed flavoinds and nonflavoinds. flavones, flavones, dihydroflavonols, flavonls, flavanols, anthocyanidins, isoflavones, and proanthocynidins. Flavonoids are known to be synthesized by plants in response to microbial attack.their activity is probably due to their ability to react with extracellular and soluable protiens and to complex with bacterial cell walls leading to deathnof the bacteria(Idris *et al* .,2009)

Tannins reduce the risk of coronary heart disease (ahani et al .,2003).Tannins was highly present in ethanol and methanol extracts of yellow banana,green banana and red banana.tannins are also reported to have various physiological effects like anti irritant , antisecaretolytic, antiphlogistic, antimicrobial and antiparasitic effects.(Westendrap, 2006).

Glycosides was present in all extract of red banana and green banana. In yellow banana, it was absent in aqueous extract. Glycoside are used in the treatment of heart diseases.

Banana peel extract also contains active componds of steroids. Steroids was present in acetone extract of red, green and yellow banana. It was absent in methanol,benzene and aqueous extract of red banana. steroids are a group of antioxidant that has antifungal effects and commonly used as raw meterials for the biosynthesis of corticosteroids drugs. (prihatman, 2001)

Saponins was absent in all extract of red banana, green banana, saponin was absend in benzene extract. saponin was present in methanol, ethanol and acetone extract of yellow and green banana. Saponin decrese blood lipids, lower cancer risks and lower blood glucose response.

Resins was absent in all extracts of red, yellow and green banana. And it was absent in all extract of yellow and green banana. Resins are chemical and solvent resistance. The

phytochemicals alkaloids, flavonoid and tannins have a very good antioxidant property while saponins and terpenoids have antimicrobial activity.

Antibacterial activity

The antibacterial activity of three banana peel extracts (Yellow, Green and Red banana) in five different solvents (Ethanol, Methanol, Acetone, Benzene and Aqueous extracts) were measured against five human pathogenic bacteria (*Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Vibrio cholerae* and *Proteus vulgaris*) and the results were presented in table (4,5 and 6).The inhibition zone against these species ranged in size from 7 to 20mm in diameter. All of the extracts examined prevented the growth of all of the pathogens in the sample. The ethanolic peel extract of red banana had the highest activity against *E. coli* (19 mm) our results agreed with the reports of Singh *et al.* (2013).The benzene extract of red banana had lowest activity against *Bacillus subtilis* (7mm)

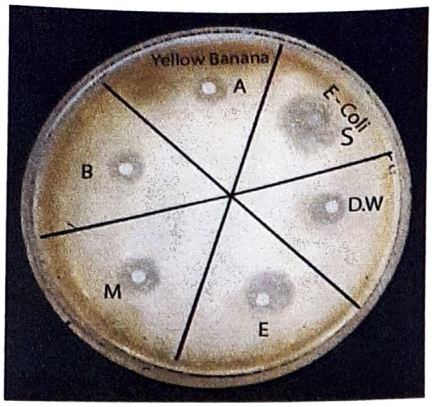
The highest zone of clearance was obtained from ethanol extract of green banana (18 mm) against *Staphylococcus aureus*. This was followed by acetone and aqueous extract against *E.coli* and *Bacillus subtilis* with a diameter of 17 mm. The lowest zone of inhibition was noted in benzene peel extract (7 mm) against *Bacillus subtilis*

In ethanolic extract of yellow banana peel showed a maximum zone of inhibition of 15 mm against *Vibrio cholerae* .The minimum zone of inhibition was noted in acetone and aqueous extract against *E. coli* , *Vibrio cholerae* and *Bacillus subtilis*. Sumathy *et al.*, 2011 studied the anti properties of Yellow banana fruit peel and found that it is effective against gram positive and gram negative bacteria.

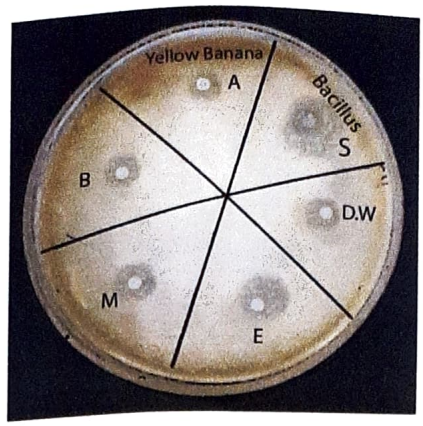
Flavonoids, tannins, alkaloids, glycosides and terpenoids were found to be present in the peels of genus *Musa*. These phytochemicals have been reported to exert multiple biological and pharamacological effects (antibacterial, antihypertensive, antidiabetic and anti-inflammatory activities). The presence of these bioactive substances in banana peels therefore suggests that the peels possess valuable medicinal potential yet to be explored. As the bioactive

Plate 3

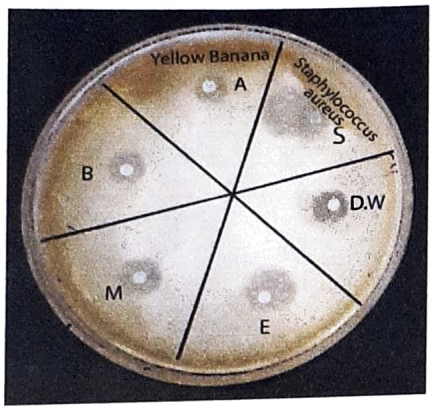
In vitro Antibacterial activity of Yellow banana peel extract against human pathogens



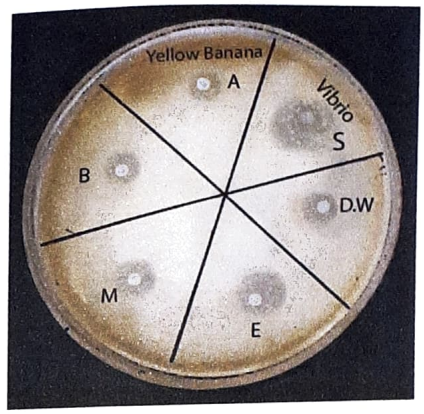
E . Coli



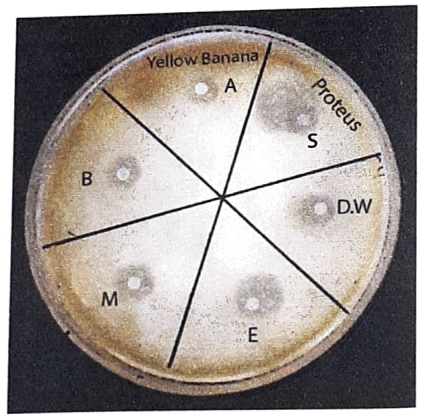
Bacillus subtilis



Staphylococcus aureus



Vibrio cholerae



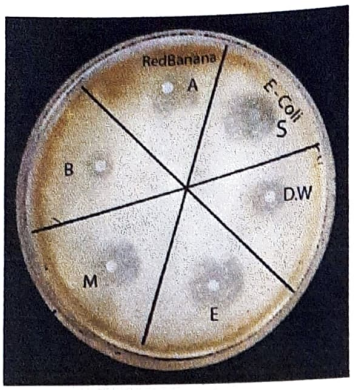
Proteus vulgaris

Antibacterial activity is revealed as clear zone around the disc and is represented as zone of inhibition.

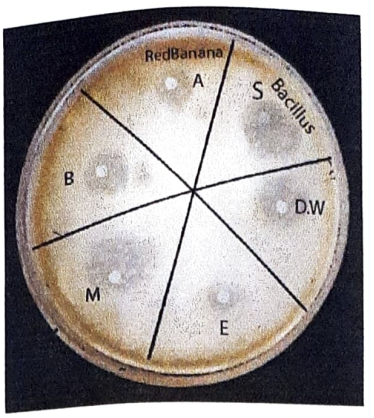
B- Benzene, M- Methonal, E- Ethanol, A-Acetone, D-Distilled water S-Streptomycin Banana peel extract (2.5 mg/ml effect concentration)

Plate 4

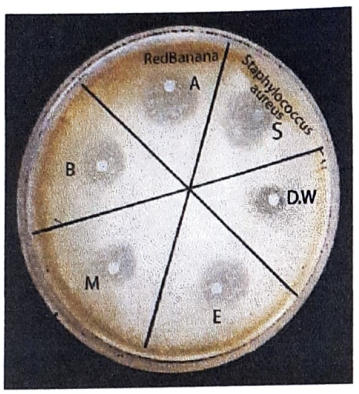
In vitro Antibacterial activity of Red banana peel extract against human pathogens



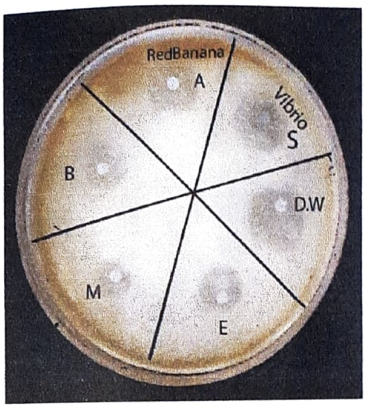
E . Coli



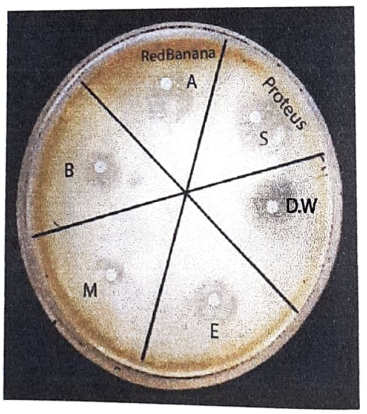
Bacillus subtilis



Staphylococcus aureus



Vibrio cholerae



Proteus vulgaris

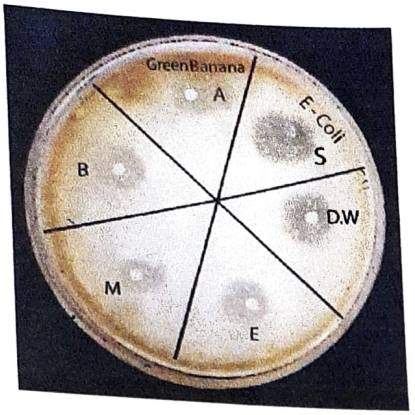
Antibacterial activity is revealed as clear zone around the disc and is represented as zone of inhibition.

B- Benzene, M- Methonal, E- Ethanol, A-Acetone, D-Distilled water

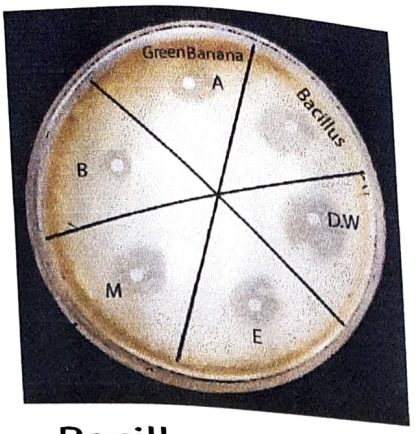
S-Streptomycin Orange peel extract (2.5 mg/ml effect concentration)

Plate 2

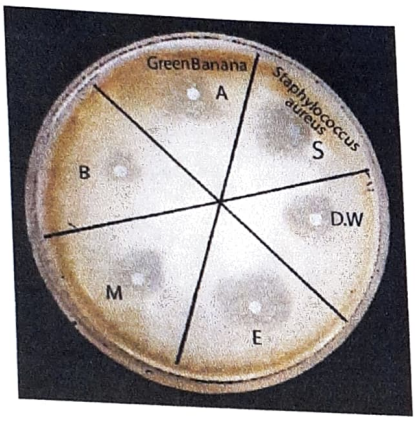
In vitro Antibacterial activity of Green banana peel extract against human pathogens



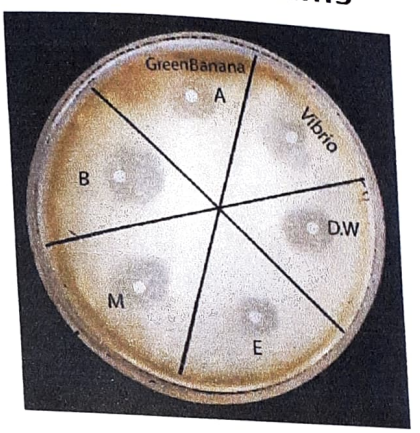
E . Coli



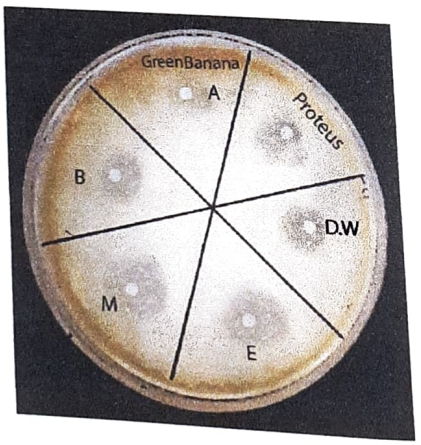
Bacillus subtilis



Staphylococcus aureus



Vibrio cholerae



Proteus vulgaris

Antibacterial activity is revealed as clear zone around the disc and is represented as zone of inhibition.

B- Benzene, M- Methonal, E- Ethanol, A-Acetone, D-Distilled water S-Streptomycin Banana peel extract (2.5 mg/ml effect concentration)

Table : 5

Antibacterial Activity of Yellow Banana peel extracted with different solvent against human pathogens

S.No	Organism	Zone of inhibition (mm)					
		Acetone	Ethanol	Methonal	Aqueous extract	Benzene	Streptomycin
1	*E-Coli*	10	16	14	11	11	9
2	*B.subtilis*	9	16	15	12	12	10
3	*S.aureus*	13	17	15	15	13	11
4	*V. cholerae*	10	16	14	13	12	10
5	*P.vulgaris*	11	17	16	14	13	10

Values are the mean of 3 replicats ± SD

Control = streptomycin (100µg /m)

Banana peel extract = 2.5mg/ml (effect concentration)

NS = No sensitivity

Table: 6

Antibacterial Activity of Red Banana peel extracted with different solvent against human pathogens

S.No	Organism	Zone of inhibition (mm)					
		Acetone	Ethanol	Methonal	Aqueous extract	Benzene	Streptomycin
1	*E-Coli*	15	19	18	11	10	12
2	*B.subtilis*	12	10	18	17	13	14
3	*S.aureus*	18	16	15	11	15	12
4	*v.cholerae*	16	14	12	17	13	10
5	*P.vulgaris*	16	15	9	14	14	18

Values are the mean of 3 replicats ± SD

Control = streptomycin (100µg /m)

Banana peel extract = 2.5mg/ml (effect concentration)

NS = No sensitivity

Table: 4

Antibacterial Activity of Green Banana peel extracted with different solvent against human pathogens

S.No	Organism	Zone of inhibition (mm)					
		Acetone	Ethanol	Methonal	Aqueous extract	Benzene	Streptomycin
1	*E-Coli*	17	11	10	15	14	9
2	*B.subtilis*	11	14	15	18	8	10
3	*S.aureus*	15	18	13	12	8	10
4	*V.cholerae*	15	10	17	16	16	8
5	*P.vulgaris*	11	17	18	15	13	10

Values are the mean of 3 replicats ± SD

Control = streptomycin (100µg /m)

Banana peel extract = 2.5mg/ml (effect concentration)

NS = No sensitivity

compounds contained in plants are majorly responsible for their medicinal properties (Ighodaroro *et al.*, 2009).

The consumption of banana is good because of its nutritional value. It is used in anemia (Roy and saraf, 2006), depression (Girish and Satish , 2008), heart burn (Mokbel and Hashinaga , 2005) , Strokes (Roy and Saraf , 2006) , Stress (Girish and Satish , 2008) etc.,

Banana peel which is an outer shell of banana also have been studied for the treatment of mosquito bites (Odebyl & Sofowora , 1978), gastrointestinal disorders (Pannang petch,2001), warts (Roy and Saraf, 2006) and nipple fissures caused by *Staphylococues aureus* (Novak *et al*, 2003).

All extracts of red, yellow and green banana peels could be considered as a good antibacterial against both gram positive and negative bacteria to replace the synthetic medicines in treatment of diseases caused by these bacteria.

SUMMARY AND CONCLUSION

Summary and Conclusion

Banana is one of the world's leading fruit crops grown in over 122 countries worldwide. Banana normally has a short shelf life and start deteriorating just after plucking. The most widely used part of banana is the flesh of the fruit; meanwhile, the outer skin is only used for animal feed and organic fertilizer.

Banana evolved in the humid tropical regions of S.E Asia with India as one of its centers of origin. Peels from about 18-33% of the whole fruit and are waste products. According to Health line banana peel rich in antioxidants, fibre and essential nutrients, helps brighten the skin reduce wrinkles. It even acts as a moisturizer and helps in hydrating the skin. Several studies have found banana peel to have anti-inflammatory properties, too.

Banana contains peels which are rich in important bioactive compounds which makes them greatly significant for therapeutic treatments. For studying phytochemical and antimicrobial properties of the peels, three varieties of bananas were chosen from Thoothukudi.Carbohydrates, tannins, steroids, alkaloids, flavonoids, glycosides and Resin were found in thefive solutions of banana peels samples through phytochemical screening. Evaluation of antibacterial activity of peel solutions through agar disc diffusion method. The inhibition zone against these species ranged in size from 7 to 20mm indiameter. All of the extracts examined prevented the growth of all of the pathogens in the sample. The ethanolic peel extract of red banana had the highest activity against *E. coli* (19 mm) our results agreed with the reports of Singh *et al.* (2013).The benzene extract of red banana had lowest activity against *Bacillus subtilis* (7mm)

The highest zone of clearance was obtained from ethanol extract of green banana (18 mm) against *Staphylococcus aureus*. This was followed by acetone and aqueous extract against *E.coli* and *Bacillus subtilis* with a diameter of 17 mm. The lowest zone of inhibition was noted in benzene peel extract (7 mm) against *Bacillus subtilis*

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against *Vibrio cholerae* .The minimum zone of inhibition was noted in acetone and aqueous extract against *E. coli* , *Vibrio cholerae* and *Bacillus subtilis.* Sumathy *et al.*, 2011 studied the anti properties of Yellow banana fruit peel and found that it is effective against gram positive and gram negative bacteria.

Flavonoids, tannins, alkaloids, glycosides and terpenoids were found to be present in the peels of genus *Musa.* These phytochemicals have been reported to exert multiple biological and pharamacological effects (antibacterial, antihypertensive, antidiabetic and anti-inflammatory activities). We conclude that banana peels are high in phytochemicals that are utilized to treat illness in humans.

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STUDY ON ISOLATION AND IDENTIFICATION OF MICRO ALGAE FROM MULLAKADU MARINE ECOSYSTEM

A short term project work submitted to St. Mary's College (Autonomous) affiliated to MANONMANIAM SUNDARANAR UNIVERSITY in partial fulfilment of the requirements for the Degree of Bachelor of Science in Botany

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2021-2022

CERTIFICATE

It is certified that this short term project work entitled **"ISOLATION AND IDENTIFICATION OF MICRO ALGAE FROM MULLAKADU MARINE ECOSYSTEM".** Submitted to **St. Mary's College (Autonomous)** affiliated to **MANONMANIAM SUNDARANAR UNIVERSITY** in partial fulfilment of the requirements for the degree of Bachelor of Science in Botany, and is a record of work done in the Department of Botany, St. Mary's College (Autonomous), Thoothukudi during the year 2020-2021 by the following students.

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CONTENTS

INTRODUCTION

Introduction:

Micro algae is a single celled microscopic, green organism in the marine, brackish and fresh water aquatic systems. Its electro chemical characters play on very important role in the industry. Nowadays micro algae have received the attention on renewable energy resource. Because micro algae in the source of an building blocks of polyunsaturated fatty acids, amino acids antioxidant and vitamins. It plays vital role in aquatic ecosystem because microalgae is responsible for 40% of global photosynthesis. Microalgae has a good economic value in production of nutrition food and biofuels. Some blue green algae has bioactive molecules on horticulture and agronomy. Micro algae plays as a bioremediation organism in the waste water treatment management. Microalgae is used to manufacture the essential products in more efficient way. Cyanobacteria (blue green algae) has received the significant attention in bio refinery industries in recent years by the variety of pigments such as chlorophyll, carotenoids, carbohydrates and lipids etc. There advances in application of microalgae is due to the vast biodiversity of micro algal species.

Tmutanda *et al.*, (2017) says that the biochemical diversity in the micro algae result for industrial and biotechnological applications. He also found that the microalgae are the natural biofactories that are gaining rapidly in tropical prominence in long term versatile and sustainable applications as well as food and food for bioenergy and biochemical production of global climate change. Ratanapuram *et al.* (2018) says that the outlook to the future development, future market enlargement of cultivating microalgae for commercial production. Hence the role of micro algal diversity in ecosystem is increasingly recognized. Microalgae are the large group of aquatic organisms that are extremely diverse and heterogeneous form evolutionary and ecological viewpoints. Among the vast diversity of algae only a few hundred have so far been investigated. Thus, the isolation and characterization from the unique aquatic environments is a continuing effort for screening of strains, screening of microalgae needs to be carried out is Local habitats, because it is

expected to have a competitive advantage Ramachandran *et al*., (2011) .aquatic environments as they have fluctuating provide a higher chance of isolating large lipid accumulating microalgae (Nuveen *et al*., 2004, Duong *et al*., 2012). Based on the above considerations, an attempt was made to study the marine water micro algal distribution in Mullakadu Coast, Thoothukudi District, Tamilnadu.

SCOPE
&
OBJECTIVES

Scope and Objectives

Microalgae are one of the most important components of marine ecosystems, providing the fuel required to sustain higher trophic levels. Being at the base of aquatic food webs, these communities are generally the first to respond to anthropogenic disturbances particularly opportunistic species and can often be used effectively as indicators of ecosystem health. The purpose of this the present study unravels the micro algal diversity and dynamics of a tropical estuarine ecosystem (Mullakadu, Thoothukudi of Indian south east coast) and applies tools like isolation of useful species to utilize in aquaculture as well as to conserve the native strains.

The objective of the this studies are

- Collection, isolation and preservation of microalgae from the selected site
- Identification of the isolated microalgae
- Find out the abundance of distribution of micro algae

LITERATURE REVIEW

Literature review:

Aquatic microalgae are photosynthetic microorganisms that live with a variety of other species and meet different ecological requirements (Richmond A, 2004). They represent one of the first forms of life on earth, and they have been found in oceans for more than 3 billion years since terrestrial environmental components were installed (Falkowski P.G, 2004 and Sathasivam R, *et al*, 2017). Fifty thousand microalgae species with diverse groups like Cyanobacteria, prokaryotic and eukaryotic microalgae have been discovered in oceans and freshwater lakes, ponds, and rivers around the world, however, only thirty thousand of them have been analysed (Richmond A,2004). Thanks to their biological property, microalgae can be used as a new source of compounds in several biotechnological applications, including waste water treatment (Crags R.J, *et al*, 1996), biodiesel production (Demarrias A. *et al,* 2011), and as supplements for human and animal dietary (Ensign C, 2014 and Solara P, 2006).A large amount of funding has recently been invested to select the best species of microalgae with high bioactive metabolites (Yuan J *et al*, 1985 and 2005). Microalgae represent several sources of bioactive compounds, such as polyphenols, carotenoids, polysaccharides, omega-3, fatty acids, and polyunsaturated fatty acids (PUFA) (De Jesus Repose M.F., 2013, Pang J, *et al*, 2011, Haier A., *et al*, 2012 and Go iris K., *et al* 2012). The lipid concentrations in microalgae are between 20% and 70%, and the fatty acid composition in algal cells is highly dependent on genetic and phenotypic agents, including environmental and culture conditions (Nazi G., *et al* 2013). Large scale lipid production will command the use of competitive species that are easy to grow and adapt to local environmental conditions. Isolating strains of microalgae with rapid growth, high intrinsic lipid content, and high biomass productivities is a primary necessity (Hannon M, *et al* 2010, Elliott L.G, *et al*, 2012, Abdul-Aziz A.E.M., *et al* 2014 and Poblete-Castro I., *et al* 2012). The quantity of total lipids in the form of glycolipid, phospholipid, and neutral lipid is varied considerably among and within groups of microalgae (Ensign C., *et al*, 2014). Many prior studies have identified the percent of omega-3 fatty acids between 30 and 40% of their total fatty acids in several species of microalgae like *Nannochloropsis* sp. (EPA),

Schizochytrium limacinum (DHA), and *Phaeodactylum tricornutum* (Poblete-Castro I .,*et al* 2012). As a consequence, microalgae have great potential in the human diet as supplements for the treatment of physiological aberrations, prevention management, and used as synthetic dietary supplements to providing sustainable natural resources (Betel K., *et al* 2016).

The interest toward biomass as alternative sources of energy and raw material is on the rise due to concerns about depleting petroleum reserves and greenhouse gas problem. Biomass is considered as a renewable energy resource with net zero carbon emissions due to the fact that atmospheric CO_2 is fixed through photosynthesis. (Richmond A. 2004 and Falkowski P.G.2004) Increasing demand for biofuels has exposed a great need for the discovery of more productive, nonfood sources of biomass that can be converted to biodiesel and other transportation fuels. The "Aquatic Species Program," which was supported by the US Department of Energy during 1978-1996, has shown the merits of microalgae as a renewable and sustainable source for biodiesel production.(Sathasivam R., *et al*,2017) Algae are generally defined as all photosynthetic eukaryotes (with the exception of land plants),(Crags R.J. ,*et al*,1996) although prokaryotic cyanobacteria, also known as blue-green algae, are sometimes included in this broad circumscription.(Demarrias A., *et al*, 2011) Unicellular microalgae are the fastest growing, photosynthesizing organisms and can complete an entire growing cycle every few days if adequate amounts of sunlight, water, carbon dioxide, and nutrients are available.(Ensign C., *et al* ,2014 and Solara P., *et al*, 2006) .Some aquatic microalgae species have high oil contents (up to 60% of their biomass dry weight) and the potential to become a significantly more productive source of biodiesel when compared with terrestrial crops such as soybeans. (Yuan J., *et al* ,2011, De Jesus Repose M.F., *et al* ,2013, Pang J., *et al* , 2011 and Haier A., *et al* ,2012) Algae are non-food resources that are amenable for cultivation on nonproductive land using saline water and wastewater. Temperature, light intensity, amount and type of nutrients, amount of CO_2, and pH are the key factors influencing algal growth. Algae represent a very diverse and heterogeneous complex of organisms belonging to many different phyla, and characterized by very different physiological attributes. A direct consequence of this

great diversity is that different species of algae have very different growth requirements. Therefore, location is a key determining factor for the selection of microalgae strains that can be used to produce biomass. (Go iris K., *et al*, 2012) Of the many algal strains available for the investigation of growth rates and biofuel production potential, the ideal strain will likely be different for each location, particularly if outdoor cultivation is utilized. The environmental conditions of a specific area can greatly influence micro algal populations and their growth dynamics. Therefore, the most logical approach is to screen for highly productive strains with maximum lipid contents at selected sites, and optimize the growth conditions for large-scale cultivation. Tropical or subtropical regions with an abundant availability of solar radiation throughout the year, and saline water (either from the sea or as groundwater), are generally the best for mass cultivation of algae. (Nazi G., *et al*, 2013 and Hannon M., *et al* 2010) In the United States, the South western states are characterized by these conditions, which is the reason they are considered the most suitable regions for growing algae. (Elliott L.G., *et al*, 2012) Bioenergy and microalgae. The rapid growing population of the world continuously increases the global demand for fuel energy. The intensive use of fossil fuels worldwide leads to its depletion and will bring them close to the point of exhaustion due to unsustainable and nonrenewable nature.

Thus, biofuels are now a growing opportunity throughout the world as alternative to fossil fuels. Some developed countries are already producing biofuels at the commercial level. Biofuels such as biodiesel and bioethanol are proving to be excellent alternative fuels and can be produced from several resources of biomass, such as food crops, crop wastes or fruits, woody parts of plants, garbage, and algae (Yuan ., *et al* ,2011 and De Jesus Repose M.E., *et al,*2013). The advantageous features of biofuels produced from biomass are renewability and a significantly smaller contribution to environmental pollution and global warming. The emission of greenhouse gases mainly CO_2 from burning of fossil fuels are the main cause of global warming. Fossil fuels are responsible for 29 gig tons /year release of CO_2 with a total of 35.3 billion tons CO_2 till now (Pang J., *et al,* 2011). Biofuels including algal fuels have oxygen

levels of 10–45% and very low levels of sulphur emission while petroleum-based fuels have no oxygen levels with high sulphur emission. Biofuels are non-polluting, locally available, accessible, sustainable and reliable fuel obtained from renewable sources. Microalgae algae-based fuels are ecofriendly, nontoxic and with strong potential of fixing global CO_2. It has been reported that 1 kg of algal biomass is can fix 1.83 kg of CO_2 furthermore some species use SO x and NO x as nutrient flow along with CO_2 (Haier A., *et al,* 2012). CO_2 constitutes 50% of dry weight of algal biomass. The selection and development of biomass is a crucial, cost-limiting phase in biofuels generation for adjusting and optimizing energy structure and cost. Selection of biomass for biofuels production is also directly related to greenhouse gas emissions, environmental and economic sustainability (Go iris K., *et al*, 2012). The current focus is on microalgae as a feedstock for bioenergy production as the most promising raw material to compel ate and balance the ever-increasing demands for biofuels, food, feed and valuable chemicals production (De Jesus Repose M.F., *et al*, 2013 and Pang J., *et al*, 2011). Many countries in Asia, Europe, and America have started industrialization of bioenergy from microalgae biomass. Microalgae are rapidly growing photosynthetic organisms having potential of transforming 9–10% of solar energy (average sunlight irradiance) into biomass with a theoretical yield of about 77 g/biomass/m^2/day which is about 280 ton/ha/year (Nazi G., *et al* ,2013 and Hannon M., *et al* ,2010). At lager scale cultivation this yield is lower both in outdoor and indoor culture system. In Photo bioreactors the actual yield is lower due to loss of absorbed active radiation (Elliott L.G., *et al*, 2012, Abdul-Aziz A.E.M., *et al*, 2014 and Poblete-Castro I., *et al*, 2012), Proper shaking and mixing of the culture in the bioreactor is necessary for uniform distribution of light energy to avail the same strength to all the cells to convert maximum light energy to biomass. In several aspects, microalgae feedstock is competent and preferable to produce biofuels for examples microalgae don not require cultivable land and fresh water for cultivation, they are not edible therefore no effect on human and animal's food chain, can be grown to serial folds irrespective to seasonal conditions, mitigation of atmospheric CO_2 and treatment of waste water (Poblete-Castro I., *et al*, 2012 and Betel K., *et al*, 2012). Absence of

lignocellulose materials in microalgae cell wall facilitate the pretreatment process and reduce overall cost of production. Microalgae can feed on industrial wastes and the processing energy is less than the energy produced by the algae (Ismail N.M., *et al*, 2015, Singh A, *et al*, 2011 and Zamora Castro JE, *et al*, 2008). Second generation biofuels involve terrestrial plants, especially food crops as feedstock's, a highly controversial issue, since biofuels production from food crops can only occur at the expense of their use as food and feed. Additionally, crop foods require arable land and large amounts of water, which makes their (Gavrilescu M, 2005 and Hannon M, *et al,* 2010). The algal fuels technology is still incipient, and much improvement is required to make it commercially attractive to both, investors and consumers. Most of the microalgae species are favorable for biodiesel production due to high lipids contents 50–70% and may reach to 80% such as in case of the microalga *B. Brunei* which accumulate up to 80% of oil in it biomass (Christi Y. 2007, Powell EE, 2009 and Mata TM, 2010). Microalgae are capable of producing algal oil 58,700 L/hack which can produce 121,104 L/hack biodiesels (Gundy TS, 2013).The infeasibility of algal biodiesel is due to the associated high operational, maintenance, harvesting and conversion cost (Patel V, *et al*, 2008). Bioethanol is one of the major and clean biofuel used as transportation fuel. Bioethanol has many advantages over fossil fuels, such as (i) high octane number in bioethanol prevents knocking of cylinders in engines (ii) due to the presence of higher oxygen contents, bioethanol burning produces much less greenhouse-effect gasses (iii) Bioethanol is the only biofuel that can be used directly in the current automotive industry without any modifications (iv) Bioethanol can be mixed with oil (Balata M, *et al*, 2008, Balart M, 2011 and Sanchez OJ 2008). Global production of biofuels has increased from 4.8 to 16.0 billion of gallons from 2000 to 2007 (Jegannathan KR, *et al*, 2009). Currently, the USA and Brazil are the world leaders in the production of bioethanol. Their contribution is approximately 75–80% of the world total bioethanol production (Walker GM. 2011 and Jegannathan KR, *et al* ,2009).The USA have 187 commercial bioethanol plants, which mainly produce bioethanol from corn grain (balata M, 2008). In 2013, Brazil produced 37 billion l of bioethanol using sugar cane as the main feedstock. The European Union (EU),

uses wheat and sugar beet as the preferred feedstock for bioethanol production and produces 2.0 billion gallons annually (balata M, *et al*, 2008 and Walker GM, 2011). Biofuels from renewable and sustainable feedstock are the future permissive energy sources in place of fossil liquid fuels. Today, bioethanol is the most common biofuel, mainly produced from sugars of corn and sugarcane, but the technology is now shifting towards algal carbohydrates as potential raw materials for bioethanol production (Botha's RJ 2005, Basso LC, 2009 and Goldenberg J. 2009). Global bioethanol production has vigorously increased from 1 billion to 39 billion l within a few years and will reach 100 billion l soon (Lich FO 2006). Microalgae possess high contents of different carbohydrates, such as glycogen, starch, agar and cellulose, etc. which can be easily converted to fermentable sugars for bioethanol production (Ueda R, *et al*, 1996). Figure. 2 present's carbohydrate-rich microalgae species suitable for algal bioethanol production. Extraction of the stored carbohydrates from algae cells needs lists of the cells. This may be accomplished in different ways, such as enzymatic, acidic or solvent extraction. However, ethanol yields depend on the method followed for the extraction. The extracted sugars need pretreatment to break the polymer molecules to monomeric forms that can be subjected to microbial fermentation to yield bioethanol (Haran R, *et al*, 2010).Bioethanol production from microalgae is an excellent effort in the development of sustainable biofuels, still there are some challenges regarding large-scale production and commercialization of this clean biofuel. The main areas in the development of algal bioethanol technology, which should be optimized for the commercialization of bioethanol are, selection of the algal biomass, pretreatment and an efficient fermentation process. Fermentative ethanol yield greatly depends on the potential of the fermenting microorganisms used. The fermentation process should be carried out in an aseptic environment to avoid contamination, which greatly reduces final yields (Horn SJ, 2000). The potential of microalgae to become a competitive feedstock for bioethanol production will require a continued effort to overcome current limitations regarding (i) culturing algae and producing carbohydrate- rich biomass (ii) dewatering and harvesting (iii) pretreatment of biomass (iv) ensuring maximum yielding fermentation. Development of a cost-

effective algal system can be achieved by improvement and optimization of each of the above mentioned. Both biomass and carbohydrate productivity of algal cells need to be increased for economical and feasible production of bioethanol . Some carbohydrates rich microalgae like *Chlamydomonas reinhardtii* and *Chlorella vulgaris* are considered to be potential for techno-economic analysis (TEA) of bioethanol production (Pulls O, 2014). TEA of commercial bioethanol production from microalgae estimate suitability of the plant with respect to total investment, total cost ant total net profit (Malian TI, *et al*, 2011)

Increasing the feasibility of microalgae for higher bioethanol yields through fermentative production, requires careful consideration of several parameters. The key factor for economic feasibility of microalgae biofuels are maximizing the algal biomass with reduction of operational and maintenance cost (Singh J, 2010). It has been stated that the economic viability of algal fuels need at least more 10 years research and development to achieve a stable position (Gundy Tahini S, 2013). US Renewable Fuels Standard (RFS) estimated to obtain 36 billion gallons of microalgae-based fuels by the year 2022 (Kari, *et al*, 2017).

Microalgae based fuels are estimated to be economical for crude petroleum sell higher than 100 USD per barrel (Yusuf C, 2011). Although the algal biofuels are not yet economically feasible, there are many companies in USA, Europe and other regions of the word that are producing algal fuels at commercial scale (Gundy TS, 2013). According to the TEA model biodiesel from algal biomass below \$5/gallon gasoline equivalent and bioethanol at the **cost** \$2.95/gal are economically viable (Davis R, *et al* 2014). Some studies suggested the feasible and economically viable price of algae competitive to other biofuels is \$1/L (Park H, 2016). Several companies, e.g. Algernon, Sapphire Energy and Seam biotic etc. are involved in commercial scale production of bioethanol with output 1 billion gallons/year costing at 85 cents/l (Duvall MN 2009)

Algae culturing:

Large-scale microalgae cultivation will decisively contribute to the development of a sustainable industry for biomass production as well as generating cost-effective high- value products. Many species of microalgae show potential for large-scale cultivation, but there is insufficient information to run commercial trials. A huge amount of microalgae biomass is required to compete with other feed stocks for sustainable production of bioethanol. Successful microalgae culturing technologies will need to create larger amounts of biomass, which will make the use of food stuffs for bioethanol production comparatively less attractive.

Microalgae can be cultured by different methods and under different conditions. They need light as an energy source to convert the absorbed water and CO_2 into biomass through photosynthesis (Oz Kurt I. 2009). Photosynthetic products accumulate in various forms, such as cell components or storage materials, and vary from 20 to 50% of total biomass (Christi Y. 2007). Algae also need nitrogen and phosphorus as major nutrients, which account for 10–20% of algae biomass (Baseman J. 1996). Other requirements for growth are the macronutrients Na, Mg, CA, and K; micronutrients, such as Mo, MN, B, Co, Fe, and Zn; and other trace elements.

Wastewater is a good source of the required nutrients for microalgae cultivation. Thus, application of organic effluents from the food and agriculture industries can nourish microalgae.

During growth, the algae cells pass through different phases (e.g., lag, exponential, stationary, death). Different species of microalgae may vary in their need for growth media. However, the major requirements are the same for almost all species and include essential nutrients, an organic or inorganic carbon source, nitrogen, phosphorus, and iron (Grobbelaar JU. 2004). One of the important parameters in algae culturing is the type of bioreactor used. This should be designed according to the species and the purpose of culture. On a large scale, algae can be cultured in open ponds (high-rate ponds). Open culture systems are comparatively inexpensive, but they become easily contaminated. Other bioreactors have continuous or batch culture facilities (Adel WH 1996 and Crags RJ 2000). Some species of algae grow very well in heterotrophic culture (Morales-Sánchez D, *et al*, 2016). For commercial cultivation it is feasible

to grow microalgae in waste water treatment plant to get dual advantages of water treatment and biomass production. See water is also a good alternative for commercial cultivation of microalgae. Using see water instead of fresh water for microalgae culturing will reduce the cost of production. Marin water is a good media for microalgae culture avoiding nutrients cost and enhancing productivity of lipids and other useful products in microalgae biomass (Park H, *et al*, 2018). Most recently ocean cultivation system has attracted attention for commercial scale production of algae due the advantages like mixing of the culture by ocean waves, utilizing dissolve nutrients large area availability, etc. which result in the reduction of culturing and maintenance cost (Park H, 2018 and Novoveska L, 2016 and Kim ZH, 2016). To make the algae biotechnology sustainable, feasible and economically viable it is necessary to develop successful culturing technologies for targeted production of biomass. For a feasible algal culture, the biomass output should be > 30 g/m^2-day (AL acid E, 2014). Approximately 40,000 different species of microalgae have been reported (Fuentes-Grunewald C, *et al* 2012). Microalgae culture depends on the species and is affected by factors such as nutrient availability (N, P, K, etc.), temperature, pH, salinity, inorganic carbon, oxygen, light intensity, and CO_2 (Mata TM, *et al,* 2010). Other important factors that determine the success of culture include stirring and mixing, width and depth of the bioreactor, harvest frequency, and dilution rate. Following are the important parameters of culture which has great influence and impact on the overall yield of biomass and bio products in microalgae.

Light:

Light intensity is one of the major limiting factors in microalgae cultivation. Light duration and intensity directly affect photosynthesis of microalgae and has influence on the biochemical composition of microalgae and biomass yield (Krzemińska I, *et al,* 2014). In modelling of the outdoor or indoor algal culture system, growth rate and biomass productivity are predicted as a function of light (Hussmann MH, *et al* 2013). Light intensities vary inside the culture and reduce in culture depth this should be taking in consideration for modeling of the

bioreactor or open pond system. Algae species vary in terms of their light requirements for maximum growth and biomass accumulation.

At very low and very high light intensities, microalgae cannot grow efficiently (Martins AA, 2010 and Alibi AO, 2009 Sforza E, 2012 Zhang MC, 2012). At the compensation point, where photosynthetic CO_2 uptake exactly matches respiratory CO_2 release, net growth is zero. Higher light intensities will increase photosynthetic rate to some maximum point, after which it levels off until the photosynthetic rate is balanced by photorespiration and photo inhibition. Thus, optimal light intensity needs to be determined experimentally in each case to maximize CO_2 assimilation with a minimum rate of photorespiration and as little photo inhibition as possible (Thirumaran G.*et al*, 2012). A specific duration of light/dark periods is required for algal photosynthesis. Light is required for synthesis of ATP and NADPH, which drive the dark reactions of photosynthesis that produce carbon skeletons (Cheerily B ,*et al*,2012). There is a direct relationship between microalgae growth and light intensity and duration, up to the saturation point. (Khomeini Z, *et al*, 2011) experimentally proved the differences in the growth rate and biomass yield by growing the same algae strain under different light intensities and for various durations. The growth rate and biomass productivity decreased with decreasing light duration (Jacob-Lope, *et al,* 2009). Most studies have shown that 16 h light/8 h dark is most suitable for algae growth. Appropriate light intensity and duration is necessary in bioreactors for microalgae to avoid photo-oxidation and growth inhibition (Carvalho AP.*et al* 2020). Appropriate penetration and uniform distribution of light is also needed to avoid photo inhibition, also called the self-shading effect, in which algae at lower layers are shaded from the light by upper layers. LED lights are a good choice for this purpose, although fluorescent tubes can also be used (Wu H. 2016). Mata et al. [Caetano NS.*et al* 2010] reported that an aerated culture of microalgae under 12,000 lx intensities for 12 h of daylight produced a higher biomass yield, whereas biomass decreased when the light intensity was reduced. Khan et al. (Khan MI, *et al*, 2016) reported that *microcytic aeruginosa* give maximum biomass and carbohydrates productivity at red LED light of about 5000 lx. Dairy *et*

al. most recently reported the maximum growth rate and lipid production by *Chlorella vulgaris* at light intensities of 5000–7000 lx (Dairy S, *et al*, 2017). The optimum level of light intensities for most of the microalgae species are about 200–400 3M photons/m^2/s (Schulman's RM, *et al* 2015). Photo inhibition can be prevented by increasing the light intensity or by thoroughly mixing the culture continuously. Hence, light directly affects the final yield of biomass and synthesis and accumulation of carbohydrates in the algal cells. Katya et al. (Schulman's RM, *et al* 2005) experimentally proved that a light intensity of 100 µmoll/m^2/s is optimum for some microalga species.

Temperature:

Temperature is another important factor in the growth of microalgae and directly influences the biochemical processes, including photosynthesis, in the algal cell factory. Each species has its own optimal growth temperature. Increasing temperature to the optimum range exponentially increases algal growth, but an increase or decrease in the temperature beyond the optimal point retards or even stops algae growth and activity (Bechtel Q, *et al* 2017). The optimum temperature range for most algal species is 20–30°C (Singh SP, 2015) although thermophile algae such as *Analysis nodules* and *Chaetoceros* can endure temperatures up to 40 °C and algae growing in hot spring near temperature 80 °C (Covarrubias Y, 2016). Growing Micro algae cultures at non-optimal temperatures will result in high biomass losses, particularly in outdoor culture systems (Alibi AO, 2009 and Zhang CW, 2006 and Lee CG, 2015). Temperature is important factor for large scale cultivation especially in open pond culture and need careful monitoring as the algae experience significant temperature change over time (Bechtel Q, *et al*, 2010).

Low temperatures affect photosynthesis by reducing carbon assimilation activity, whereas too-high temperatures reduce photosynthesis by inactivating the photosynthetic proteins and disturbing the balance of energy in the cell. Higher temperature also reduces cell size and respiration. The decline in photosynthesis results in a decreased growth rate (Atkinson D, 2003). The key effect of temperature on photosynthesis is due to a decline in the activity of

ribulose-1, 5-bisphosphate (Rubicon), an enzyme with dual functions. It can act as an oxidase or as a carboxylase, depending on the relative amounts of O_2 and CO_2 present in the chloroplasts. CO_2 fixation activity of Rubicon enzyme increases with rising temperature up to a certain level and then declines (Saluki ME, 2004). Hence, temperature is a limiting factor for algal growth rate and biomass production through its influence on the affinity of rib lose for CO_2.

Temperature can also be used as a stress treatment to induce the production of valuable metabolites (Moller AP *et al*, 2000 and Convert A, *et al*, 2009) found that a culture of Chlorella vulgaris produced more carbohydrates and lipids if grown at 25 °C than at 30 °C. Katya *et al.* (Katya Y, 2005) found that temperatures between 27 and 31 °C were optimum for several microalgae species.

Nutrients:

Different microalgae species may vary in their nutritional needs; however, the basic requirements are same for all species. Nitrogen, phosphorus, and carbon form the backbone of microalgae ($CH_{1.7}$ $O_{0.4}$ $N_{0.15}$ $P_{0.0094}$) (Juneau A, 2013) and are classified as macronutrients required for algal growth. Some marine microalgae species also require silicon as a macro nutrient. Microalgae absorb O_2 and H_2 from water. The quantities of macronutrients such as nitrogen and phosphorus may vary for different species of microalgae. It has been /reported the growth of *chlorella* declined when the concentrations of nitrogen and phosphors reduced from 31.5 and 10.5 mg/l respectively (Aslant S, 2006). Quantities of the available nitrogen in the culture directly alter cell growth. Nitrogen limitation in the microalgae culture, can reduce growth and biomass productivity although they increase production of carbohydrates and lipids. 0.5 g/l nitrogen has been proved to be optimum concentration for *Chlorella vulgaris* at which it produces 3.43 g/l biomass (Dairy S, 2017). the micronutrients Mo, K, Co, Fe, Mg, MN, B, and Zn are only required in trace amounts but have a strong impact on microalgae growth, as they influence many enzymatic activities in algal cells (Zhang CW 2006) Usually, inorganic nitrogen and phosphorus are absorbed as nitrates and phosphates. Urea is also a suitable source and a cost-effective alternative to other inorganic nitrogen sources. Carbon can be added to the

algae culture in organic forms, such as glycerol or acetates, or as CO_2. However, for large-scale cultivation of microalgae, environmental CO_2 must be used as a carbon source, which is not only low cost but adds the benefit of CO_2 mitigation. P, N, and C are the primary inorganic nutrients that are essential for microalga growth (Bold HC, 1978). Nutrient deficiency greatly affects the microalgae growth rate and results in low biomass (Chosen-Goldberg I, 2006, Somerfield M, 2008, Mohan SV. 2012 and Tanaka M, 2012). The nutrient supply strongly affects the synthesis and accumulation of carbohydrates and lipids in microalgae (Devi MP 2012). For commercial production of microalgae biomass, the culture must be grown rapidly; thus, providing the proper nutrients is very important to speed algal growth. Some strongly limiting substances can be used as growth enhancers for microalgae. In addition, certain bacteria can enhance the growth rates of microalgae by supplying important nutrients. These bacteria degrade nutrients into forms that can be readily assimilated by microalgae, such as ammonia or nitrate (Keota T. 2011).

Mixing:

Mixing and aerating provide uniform distribution of nutrients, air, and CO_2 in microalgae culture. They also enable the penetration and uniform distribution of light inside the culture and prevent the biomass from settling and causing aggregation (Tang MSY *et al*, 2017). If all the other requirements are met but there is no mixing, biomass productivity will be lowered significantly. Thus, microalgae cultures must be continuously mixed to keep all cells in suspension with free access to light. A proper mixing system in a photo-bioreactor not only enables nutrient dissolution and light penetration into the culture but also provides for efficient gaseous exchange (Daquan MK 2011) pH and salinity.

The pH of the culture media is another important factor affecting microalgae growth. Microalgae species have different pH requirements. Most grow well in the pH range from 6 to 8.76 Different sources of growth media have different pH values. Most algae species are pH sensitive and few can endure a range of pH as broad as that tolerated by *C. vulgar* (Dragonet G, *et al*, 2010). *C. vulgaris* can grow in broad range of pH however the maximum

growth rate and biomass productivities are reported at pH 9-10 (Tanaka M, *et al*, 2012). Increasing the pH will increase the salinity of the culture media, which is very harmful for algae cells (Ceballos RM, 2013).

Mycotrophic cultivation:

In autotrophic cultivation, microorganisms rely on light energy to generate energy, whereas in heterotrophic cultivation, organic carbon sources are used for metabolism. Mycotrophic conditions combine autotrophic and heterotrophic models so that the cultured microorganisms have both a supply of inorganic carbon to be fixed via photosynthesis and some organic carbon source, such as glucose, glycerol, and acetate (Dragonet G, et al, 2010). The microorganisms in mycotrophic cultures grow faster and can synthesize compounds through both autotrophic and heterotrophic pathways. Moreover, they have a reduced cost of light energy (compared with autotrophic culture) and organic compounds (compared with heterotrophic culture) (Creon Garcia MC, *et al*, 2005)

Modifications and improvement of the algal strains:

The future feasibility of bioethanol as an alternative to fossil fuels will largely depend on its economic advantages. At present, microalgae biomass production does not permit commercial production of bioethanol (Pianos PT 2009). The low carbohydrate content in algal biomass is a strong limitation. The amount of algal carbohydrate can be induced to reach a higher level than normal, either by controlling environment conditions or by introducing genetic modifications. Growing algal cells under certain stress conditions can alter certain biochemical pathways, leading to enhanced synthesis of carbohydrates (Chen CY, 2012 and Seat M, 2011). These stress conditions may be limitation of nutrients, such as nitrogen and phosphorus; change in light intensity, salinity of the growth media, or pH; or application of UV radiation. Regarding genetic modification, metabolic pathways inside the algal cell can be modified to increase the production of carbohydrates, lipids, and other important compounds of interest. It is important

to select microalgae strains that allow easy, multidimensional modification of biochemical pathways. This novel approach uses new, powerful, rapidly evolving genetic engineering tools to identify and selectively modify the right genes. Hence, efforts are increasing to develop carbohydrate-rich microalgae strains with the help of engineering approaches. Rapid development in genetics has made a number of transformation methods available, and successful trials encourage the use of genetic tools for a variety of purposes. Although the technology has not made satisfactory progress in the field of algal bioethanol yet, expectations for the near future are high.

Pretreatment of algal biomass:

Pretreatment of algal biomass involves the degradation or disruption of biomass to convert, accumulate, and process the carbohydrates and lipids it contains (Fig. 3). On a Large scale, biomass pretreatment is a bottleneck and potentially costly step in biofuels production. Many different methods have been described for the pretreatment of algal biomass, but there is still no optimal, highly productive method. Researchers need to develop feasible and economical methods of biomass pretreatment for bioethanol production, optimized for different feed stocks.

Carotenoids:

Carotenoids are important bio compounds having strong role in food, feed cosmetics and biopharma (Henriquez V, 2016). Have Algae synthesize different types of pigments that possess important biological activities and thus are of great commercial interest. Among the most important are the phycobiliproteins, phycocyanin, phycoerythrin, β-carotene, lutein and astaxanthin (Zhang D, 2016). Phycobili protein pigments are used in microscopy as fluorescent agents (Perez-Garcia O, 2011) Phycocyanin and other pigments from red algae have antioxidant and anti- inflammatory effects; thus, they are used in food and cosmetic products (Kumar D, *et al*,2014 and Datla P. 2011)The micro algal *Dunaliella saline* produces the carotenoid pigment β-carotene in quantities that represent about 10–14% of its dry mass

(Sathasivam R, 2013) β-carotene has a crucial role in vision and the immune system, due to its relation to vitamin A. Another important carotenoid pigment is astaxanthin, which is sold for 2500 US$/kg in the market. The microalga *Haematococcus Pluvial is* produce 4–5% astaxanthin per dry biomass (Sathasivam R, 2017). Dried biomass of *Haematococcus pluvial is* has been commercialized as astaxanthin rich Source. Because of their strong antioxidant activity, carotenoids are therapeutic in oxidative stress–related diseases and their complications, such as diabetes, aging, cancer, obesity, and stroke (Repose MFJ, *et al*, 2001, Chidambaram-Murthy KN *et al*, 2005 and Lin J, *et al*, 2016) β-carotene and astaxanthin also have strong effects on the enzymatic antioxidant defines system by preventing oxidative stress through scavenging of free radicals. β-Carotene protects membrane lipids from peroxidation, which is linked with many severe and lethal diseases, such as cancer, cardiovascular disease, Parkinson's disease, and atherosclerosis (Lobo V, 2010, Uttar B, 2009 and Pham-Hay LA 2008) The cist and trans forms of β-carotene are different isomers that confer the anticancer effect (Deming-Adams B, 2002).Many other compounds besides carotenoids have strong antioxidant activities, such as phenolic compounds and vitamins.

Sterols:

Sterols produced by plants are called phytosterols. Microalgae have a good contribution in the production of phytosterols and are considered potent and promising sources for the large-scale production. Some microalgae species have high levels of sterols. Micro algal sterols have some beneficial health effects like hypo-cholesterolemia, anticancer, anti-inflammatory and neurological diseases like Parkinson disease (Deva raj S, 2004 and Kim HJ, 2008) Phytosterols used in pharmaceutical formulation for health benefits and nutraceuticals as food ingredients (Fernandez P, 2007 and Wrigley CT 2015). Some microalgae species, such as those in the *Pavlov* and *Thalassiosira* genera, are rich in sterols (Lou X, 2015 and Volkmann JK 1996).The micro algae *Chaetoceros* has been reported to produce 27.7 mg sterols per gram of dry weight (Santosh S, 2016) 40 different sterols have been identified in 100 different species of diatoms. These sterols are differed in chemical structures and some genera,

e.g. *Amphora* produce distinctive types of sterols (Volkmann JK 2016). Recently the microalgae *annochloropsis* sp, *Pavlov Luther*, *Tetrasellimis* sp have been screened to produce sterols with a net yield of 0.4–2.6%/dry weight) (Ahmed F, 2015). *Euglena graceless* produce a mixture of sterols, 0.68–3.24 mg/g of dry biomass (Zhang Y, 2011). The major types of sterols reported in *Glaucocystophyte* are sitosterol, camp sterol and stigma sterol, (Leland JD 2011). 24-Ethylcholesterol are mostly produced by cyanobacteria (Volkmann JK 2003). Din flagellates mostly produce 4α-methyl sterols and 24 propylidenecholesterol is mostly produced by *Pelagophyceae* (Thomson PG 2004, and Ginner JL, 2009). Synthesis of sterols is also influenced by a number of factors in microalgae affecting the final yield.

Proteins and enzymes:

Some proteins, peptides, and amino acids have strong therapeutic effects on health or are necessary for cells and tissues to perform their normal activities. If the human body is unable to synthesize them, they must be obtained from an external source, usually food. Many species of microalgae produce higher quantities of various essential amino acids and proteins, which can be utilized in food and to protect against several diseases. Some species of microalgae can produce as much proteins as other rich sources of proteins, e.g. egg, meat and milk etc. (Gouveia L, *et al* 2008). Microalgae proteins have comparatively high nutritionals value. Microalgae produce 2.5–7.5 tons / Ha / year of proteins (Bleakly Stephen, 2017) the green micro algae *Chlorella* is a rich source of different types of proteins, which have been marketed. Another protein-rich microalga is *Arthrospira*. Proteins from microalgae or plants cam reduce cholesterol levels by activating cholecystokinin. They also have important enzymatic effects (See DF, *et al* 2008). *Lyngbya majuscule* produces microcline-A, an immunosuppressive agent (Arya V, 2001). *Nastic* produce the protein Cyanovirin which have been reported for its antiviral activities against HIV an influenza virus (Zapped H, 2008). *Anabaena* and *Porphyridium* produce the enzyme SOD (superoxide dismutase), which protects against oxidative damages, while *Isochrysis galbanum* produces the vital enzyme carbonic anhydrase,

which plays a crucial role in converting CO2 into carbonic acid and bicarbonate. *M. aeruginosa* produces a variety of amino acids, including praline, serine, glycine, and valise.

Polyunsaturated fatty acid:

Polyunsaturated fatty acids are important in tissue integrity and have beneficial health effects. Omega-3 and omega-6 fatty acids in particular are crucially important for humans, but the human body cannot produce these fatty acids. Thus, intake from external sources such as foods or cosmetics is essential. DHA, linoleic acid, eicosapentaenoic acid, arachidonic acid, and gamma-linoleic acid have been shown to suppress cholesterol levels, delay aging, guard membrane integrity, and prevent cardiovascular diseases (Annenberg G, 2017 and Hu FB, 2002). Many microalgae species (e.g. *Porphyridium) centum, Arthrospira platens is, Donatella, I. galbanum*) have been explored for their ability to synthesize these valuable fatty acids. *Pavlov Luther* produces polyunsaturated fatty acids in large quantities, (Guides ACA ,2010) while *A. platens is* produces and accumulates stigma sterol, sitosterol and γ-linoleic acid (Santosh S, *et al* 2016).Eicosapentaenoic acid (EPA) and docosahexaenoic acid DHA are the medicinally important Omega-3 Polyunsaturated crucial in inflammatory diseases, heart problems, arthritis, asthma, and headache etc. [401]. EPA, DHA are produced by serial microalgae species sustainable and promising source and the only alternative to fish oil. Which are limited and unable to fulfill the required demands of EPA and DHA (Sorbet B, 2008 Armenta RE, 2013). Some efforts have been made recently to enhance the production of EPA and BHA by altering the metabolic pathways via genetic manipulation (Alarmed-Vega TC, 2014). Most recently *Phaeodactylum tricornutum* attracted attentions as a potential source of EPA and DHA production (Hamilton M, 2014 and Daisha RB, 2013 Keller M, 2014). The diatom *P. tricornutum* has been genetically sequenced and modified for enhance production of Omega-3 polyunsaturated fatty acids like EPA and DHA etc.

It has been reported that the genetically engineered strain of *P. tricornutum* produce a maximum yield of 36.5 and 23.6% of DHA and EPA per total fatty acids of biomass indicating its feasibility for production of EPA and DHA at commercial scale (Chatom MS ,*et al*

2015) However, commercial scale production of these significant and useful products such as EPA and DHA from microalgae need to overcome several hurdles and challenges which are responsible for its low product yields (Hamilton ML., 2016). Up scaling required optimization in several areas, e.g. screening and selections of strains, culture development, and products induction and extraction technologies. The algae growth and productions of Omega-3 Polyunsaturated fatty acids is greatly influence by carbon sources and light strength (Gardner RD, *et al*, 2012, Muss F, *et al*, 2013 and Hussein Afresh A, 2009).

Vitamins:

Microalgae are also rich sources of different vitamins. *Has lea ostrearia* is a rich source of vitamin E (tocopherols). *P. centum* produces high quantities of vitamins E and C, as well as β-carotene (vitamin A) (Toussaint JP, *et al* 2013). The microalga *D. saline* readily produces vitamins .A and E, pyridoxine, nicotinic acid thiamine, riboflavin, and biotin (Hussein Afresh A, 2009).

Toxic metabolites:

Most microalgae species, especially cyanobacteria, produce a variety of toxic substances, generally called cyan toxins. The most common examples are the micro cyst INS produced by the blooming *Microcytic* species. *M. aeruginosa* is the dominant microalga of the bloom-secreting microsystems, which have been reported to be lethal for animals and humans. The bloom is also responsible for shellfish poisoning, due to the presence of toxins. Different types of microsystems have been identified. The microsystems are hepatocyclic peptides with a C20 amino acid chain, which determines the degree of toxicity (Jungle AD, 2006). Among all the microsystems produced by *M. aeruginosa*, microsystem LR is the most toxic and causes the death of animals and humans upon oral contact. Studies have shown that cyanobacteria toxins can treat tumours (Ahmed WA et al, 2017). Cyan toxins are of great interest as environmental hazards and due to the chemistry of their toxicology they are broadly classified on the basis of (1) effects on vertebrates, divided into neurotoxins, hepatotoxins, and dermatotoxins; and (2)

chemical structure as cyclic peptides, alkaloids, or lipopolysaccharides (Ferrao-Filho AS, 2011). These toxic substances also have important antibacterial and antifungal activities (Burma AM, *et al*, 2001 and Volk R-B, 2006). Other microalgae species have been explored for the production of toxic products. The diatom *Nietzsche pungent* produces demonic acid, which causes poisoning of shellfish (MOs Lizzy, 2001). *Gambier discus toxics* produces gambieric acids, which have antifungal activities (Donia M, 2003). Karatungiols are active antimycotic agents produced by *Amphidinium*, which also have antiprotozoal activities (Washed K*ept al*, 2006).

Biological activities of natural products from microalgae: Antioxidant:

Antioxidants are very important substances used by the human body to protect itself from the hazardous effects of free radicals. ROS (reactive oxygen species) and NOS (nitrogen reactive species) attack biomolecules like DNA, proteins, and membrane lipids, leading to many severe diseases including cancer, coronary arteries disease, obesity, diabetes, ischemic stroke, and Alzheimer disease (Ngo DN, 2006) Free radicals cause lipid peroxidation both in food lipids and biological membranes. Peroxidation results in various diseases and complications. In food materials, lipid peroxidation reduces shelf life and nutritional value. Antioxidants can prevent oxidative damage to cells and tissues by scavenging free radicals. The human body has its own enzymatic antioxidant system that prevents oxidative stress and protects the body from the hazardous effects of free radicals. However, when free radicals overcome the body's natural antioxidants, oxidative stress occurs, which is one of the major causes of various dangerous and life-threatening diseases. In such cases, the uptake of external antioxidants is crucially important. Many natural antioxidant compounds have been reported. Compounds such as flavonoids, carotenoids, and vitamins like ascorbic acid and tocopherols have strong antioxidant activity.

In the pharmaceutical and food industries, several synthetic or natural antioxidants have been used to prevent oxidation and peroxidation processes (Park PJ 2005). As synthetic antioxidants have been shown to have side effects, natural antioxidants are sought after (Pena-Ramos E, 2001). Recently, the exploration of natural antioxidants for outrace Utica's and pharmaceuticals industries has increased. Researchers are looking for antioxidants from natural sources, such as medicinal plants. Because of their huge potential for producing biologically active natural products, microalgae are one of the richest and most economical sources of natural compounds with strong antioxidants effects (Cornish M, 2010). The antioxidant potential of these substances has been determined by various methods, including ABTS, DPPH radicals scavenging assay, ferric reducing potential, and metals chelating essays. Structural features such as a phenyl chain, a porphyria ring, and conjugated double bonds are responsible for the antioxidant qualities (Le Tutor B, *et a*l, 1998). Chlorophyll a and its metabolites produced by microalgae species are reported to have antioxidant activities (Cho M, 2011) as do most of the pigment metabolites of microalgae. The pigment fucoxanthin and its derivatives, such as auroxanthi . Fucoxanthin is reported to have higher antioxidant effects than β-Carotene in tests of rat liver and plasma (Ravi Kumar S, and Sangeetha R, 2009). The chemical structure of fucoxanthin shows two hydroxyl groups in a ring structure, which are considered the active moiety for free radical scavenging (Kang He *al*, 2010). Phycobiliproteins (e.g. C-phycocyanin, R a-phycoeryth (Sear S, 2008) Phycoerythrobili(Mahindra N, *et al*,2007)n, produced by some species of microalgae, has been shown to possess antioxidant activity (Abut Y, *et al*, 2010) Hence, the widespread existence of natural products with strong antioxidant activity increases the economical and nutritional potential of microalgae for the food, pharmaceutical, and nutraceutical industries.

Anti-antigenic, cytotoxic, and anticancer activities:

Angiogenesis is the physiological process of developing new blood vessels from preexisting blood vessels. Angiogenesis proceeds rapidly during uterus development, embryogenesis, and wound healing. Angiogenesis breaks cell-to-cell contact and degrades the endothelium and extracellular matrix. The process involves the proliferation and migration of endothelial cells and formation of capillary tubes (Carmelite P. 2003) although angiogenesis is a normal process, it may become pathological under certain conditions, such as cancer, atherosclerosis, arthritis, diabetic retinopathy, and ischemic stroke (Armstrong AW, *et al*, 2011) Pathologic angiogenesis promotes tumours and helps them to grow Therefore,pangiogenesis is considered the cause of tumor growth and expansion in cancer. Several activators and inhibitors are involved in the regulation of angiogenesis. The major antigenic factors and proteins are VEGF, PDGF, angiopoetin-1 angiopoietin-2, platelet- derived growth factor, interlukin-8, interlukin-8, beg, and angiotensin II (Jerri Roskoski, 2007 and Emanuel C, *et al*, 2002)

Many reports indicate the potential of natural products, including those produced by microalgae, to treat cancer and tumors by inhibiting angiogenesis. Fucoxanthin, found in many species of microalgae, significantly inhibits human blood cell proliferation and tube formation of HUVECs (human umbilical vein endothelial cells). Fucoxanthin and fucoxanthinol have been shown to inhibit the angiogenesis process in the aortic ring of rate by suppressing the growth of micro vessels (Sugawara T, *et al*, 2006). Some species of algae produce siphonaxanthin, which also possess antiangiogenic activity (Gamesman P, *et al*, 2010)Fucoxanthin also has therapeutic effects for diabetes and induced the synthesis of arachidonic acid and DHA content in mouse livers (Tusked T, *et al*, 2009)It inhibits skin melanogenesis by negative regulation of the transcriptional factors involved (Sugawara T, *et al*,2006)Moreover, fucoxanthin has been shown to protect .DNA from photo oxidation (Gamesman P, et al ,2010). Aerucyclamide is used in pharmaceutical products as an anti-plasmodia agent isolated from the blooming of *M.aeruginosa* (Scar one L, *et al*, 2012).

Microalgae, particularly blue-green algae, are now being considered potential sources of active ingredients that can be utilized in the treatment of cancer. Many studies have shown the anti-cancer activity of these active products in the lab (Russo P, 2013). The mode of action and the mechanism of the activities may differ. Some of the microalgae-derived anti-cancer agents have been shown to induce apoptosis in tumorous cells by destroying the chromatin network, leading to cell death (Martins RF *et al*, 2008). Cyanobacteria produce various metabolites by the ribosomal or non-ribosomal pathway. Most of these compounds are either peptides or alkaloids (Silone K, 2010). Peptides, including but not limited to those from cyanobacteria, tend to be toxic substances possessing strong cytotoxic activity. Cytotoxicity of these compounds is crucial in inducing apoptosis, which leads to cell death (Zhang JY 2002).

Many species of cyanobacteria (blue - green algae) produce apoptosis-inducing compounds. Apoptotic cells can be identified by their specific morphology, as they typically have a large cytoplasm and compressed organelles, with alterations of the chromatin materials. Extracts of *Synechocystis* sp. and *Synechococcus* sp. have been shown to drive HL-60 cells into apoptotic conditions. After treatment with extracts, cells express apoptotic markers, such as fragmentation of nucleus, shrinkage of cells, and release of apoptotic substances (Martins RF *et al*, 2008). Similarly, *Lyngbya* sp. produce the glycoside biselyngbyaside, which can drive the mature osteoclasts into apoptotic conditions (Yonezawa T, *et al*, 2012). Extracts of Anabaena sp. have induced apoptosis in a leukemia myeloid cell line (Foetal L, *et al,* 2010). Some species of *Nastic produce* cryptophytic, which is several hundred to a thousand times more active on cancer cells, such as human colorectal cancer, more effective than vinblastine or taxon (Singh RK, *et al*, 2011). The Oscillatory Bryana extract was active against human breast cancer (Nair S, 2013). *Microcytic* sp. in particular have great potential in the fields of toxicology and pharmacology, due to their production of bioactive metabolites and toxic substances with anti-cancer activity. The isolation of these compounds and determination of their biotechnological and toxicological applications continues to be studied by various researchers (Welker M, 2006).

Anti obesity activity of algal products:

Obesity is the over accumulation of adipose tissues (fats) in the body (Kong CS, 2000). Obesity is considered a multifactorial metabolic disorder linked to many complications anddiseases, such as cancer, cardiovascular disease, diabetes mellitus, and aging (Copeland PG ,2000). The over growth of adipose tissue occurs because of abiogenesis, so obesity can be controlled by guarding the cells against abiogenesis (Peiris TH, *et al*, 2008). Research hasrevealed various anti-hyperlidimic and fat-lowering agents from natural sources, such as medicinal plants. Microalgae are now being studied as potential sources of these products. ROSand NOS have also been reported to be involved in the progression of obesity. Thus, antioxidants can be used to control free radical–induced accumulation of fats. Fucoxanthin andfucoxanthinol can inhibit the differentiation of 3T3-L1 cells to adipocytes. Fucoxanthin and fucoxanthinol inhibit adipocyte differentiation by down-regulating peroxisome proliferator–activated receptor (Hayat M, *et al*, 2006) Okada *et al.* (Makai M, *et al*, 2008) reported thatneoxanthin and fucoxanthin inhibited the accumulation of fats and stated that allelic and hydroxyl groups must be present to differentiate adipocytes. These compounds are reportedlycrucial for lowering fat in high-fat mouse feeds (Maeda H, *et al*,2007) In obese mice, fucoxanthin suppresses adiposity by activating the mitochondrial protein UCP1 (Uncoupling protein1) in abdomen WA T (Miyashita K. 2014) Fucoxanthin significantlyreduced body fat in obese individuals in a clinical trial by Abide et al. (Ramazanov Z, 2010)*Cylindrotheca closterium* and *Phaeodactylum tricornutum* are the two microalgae species that produce fucoxan thin, (Hosokawa M e*t al*, 2012) which shows potential as an anti-cancer, anti- oxidant, anti-obesity, anti-diabetic, and anti-inflammatory agent (Hosokawa M, *et al*,2007).

Antimicrobial activities of microalgae metabolites:

Bacteria and fungi are the major causes of severe diseases in plants and animals, including humans. They reduce crop yields and are the major causes of food spoilage. The widespread use of various antibiotics over the past few decades has given rise to increased

resistance of microbes to antibiotics, making it necessary to search for new antimicrobial agents. Synthetic antibiotics have not achieved a satisfactory level of disease control due to side effects, high cost, and risk of generating severe resistant pathogenic strains. Therefore, researchers are searching for new natural antibiotics with a broad action spectrum from natural sources like plants and microorganisms. Natural products have comparatively fewer or no side effects. Microalgae show a wide range of bioactive natural products that are effective, either in crude or purified form, as antioxidant, anti-cancer, and anti-microbial agents. The first reported antibacterial products in microalgae were in the green microalga *Chlorella*, which significantly inhibits the growth of both Gram-positive and Gram-negative bacteria (Koyama T, *et al*, 2006). Some microalgae also produce compounds that have antifungal activity (Furner FH, 2006). Kodiak acid and ciguatoxin are effective antifungal agents produced by *Prorocentrum Lima* and *G. toxics*, respectively. Antimitotic activities have been reported for karatungiols, a group of compounds synthesized by the din flagellate *Amphidinium* (Washita K, *et al*, 2005). *Chaetoceros lauder* produces lipid metabolites that have been found to inhibit the growth of serial bacteria strains. *M. aeruginosa* is a rich source of several toxic metabolites that possess strong cytotoxic and antimicrobial effects. The crude extract of *M. aeruginosa* displays high antifungal and antibacterial activity (Khalid MN, *et al*, 2010). *Dunaliella salina* also produces compounds that are active against several bacterial and fungus strains (e.g. *Staphylococcus aurous, Escherichia coli, Candida albinos, Pseudomonas aeruginosa, Aspergillums Niger*) (Mendoza JA *et al*, 2008). The extract of *D. saline* significantly inhibits the growth of *Klebsiella pneumonia*. Substances synthesized by *Dunaliella primolecta* also showed antibacterial activity against *S. aurous* and against other bacterial strains (Pane G, *et al*, 2015).

Characteristics of micro algae:

Started that several species of micro algae are known to be rich in colored high-valuable components that, although remarkable, are poorly explored as natural sources of pigments for cosmetics.(Gianpiero Potaro, *et al*, 2019) Pigments associated to

photosynthetic activity include chlorophyll, astaxanthin , xanthophyll and phycobiliproteins, many of which have shown high potential as cosmetic activities due to their antioxidant, immune-enhancing and anti-inflammatory properties. These microalgae pigments are being exploited for different applications in a variety of industries, from food dyes to health products. (Fernando Pagels , *et al,* 2020)The increased demand for natural pigments by consumers and consequently by industry has encouraged the creation of new patents and stimulated the market for these compounds, which has "bloomed "in recent years. Microalgae pigments are commonly utilized as health supplements. Light–harvesting pigments. Such as chlorophyll and phycobilins, and photo protective carotenoids are some of the most common micro algal pigments (Ramaraj sathasivam, 2019). Microalgae are one of the important component in food chains of aquatic ecosystems and have been used for human consumption as food and as medicines. Micro algae are known to be rich uncolored high-valuable components that although remarkable are poorly explored as natural sources of pigments for cosmetics. Pigments associated to photosynthetic activity include chlorophyll, astaxanthin, xanthophyll and phycobiliproteins, many of which have shown high potential as cosmetic activities due to their antioxidant, immune-enhancing and anti-inflammatory properties. (Daniel Salvatore, 2020)These microalgae pigments are being exploited for different applications in a variety of industries, from food dyes to health products. The increased demand for natural pigments by consumers and consequently by industry has encouraged the creation of new patents and stimulated the market for these compounds, which has "bloomed" in recent years. Micro algae pigments are commonly utilized as health supplements. Light -harvesting pigments. Such as chlorophyll andphycobilins, and photo protective carotenoids are some of the most common micro algal pigments .Microalgae are one of the important component in food chains of aquatic ecosystems and have been used for human consumption as food and as medicines (Helana Melo Amaro, 2020). The study aimed at evaluating the effect of pulsed electric fields pre-treatment on the extractability of pigments from microalgae Nannochloropsisoceanica by supercritical CO2 extraction.(Gianpiero

Pateros *et al.*, 2019) Microalgae are considered promising functional food ingredients due to their balanced composition, containing multiple nutritional and health-beneficial components.(lore Greisen *et al.*, 2019)Photoautotrophic microalgae are actually rich in structural biopolymers such as proteins, storage polysaccharide, and cell wall related polysaccharide, and their presence might possibly alter the rheological properties of the enriched food product. An interesting source of Carbohydrates, Proteins and minerals. Different polymer interactions result in distinct technologic features. Phaeodactylumtricornutum extracts may be tuned for different food formulations. Bioactive peptides from of Schizochytriumlimacinum were produced. The microalgae proteins showed good digestibility. Antioxidant activities of hydrolysate fraction were evaluated. Schizochytrium limacinum proteins can be used in production of functional foods. (Pedro Ferreria Santos, 2021).

Biochemical composition of micro algae:

Microalgae has high quantity of protein and also has essential amino acids exceeding 0.90;and 13.49% of fatty acids are present in microalgae and they are highly unsaturated and also has organic carbon and some dehydrogenase also present in it .The factors which affecting the microbial growth and composition such as temperature , intensity of light , available of nutrition and its metabolism .The carotenoids and lipids, they negatively affected by light intensity .The study involved the effects of temperature composition (20-36°) and nitrogen concentration (37.1-78.8 mgL-1 N-NO3) and also light intensity on biochemical composition of *pseudoneochloris marina* has biomass productivity. The highest amount of carotenes and xanthophyll were 1.25+or- 0.01mg g-1 and 2.productivitmg g-1 at 20°C and 28°C.Therefore the major carotenoids are all trans-lutein(36.0 + or- 2.9%) and also all trans beta carotene (13.2 +or- 1.2%) of total carotenoids. Nitrogen act as a critical element and has a high biological value macro molecules such as chlorophylls and DNA play a fundamental role in cultivation of micro algae. Therefore the changes in the concentration leads to affect the lipid and protein content and also carbohydrate synthesis in the microalgae (Panchal *et*

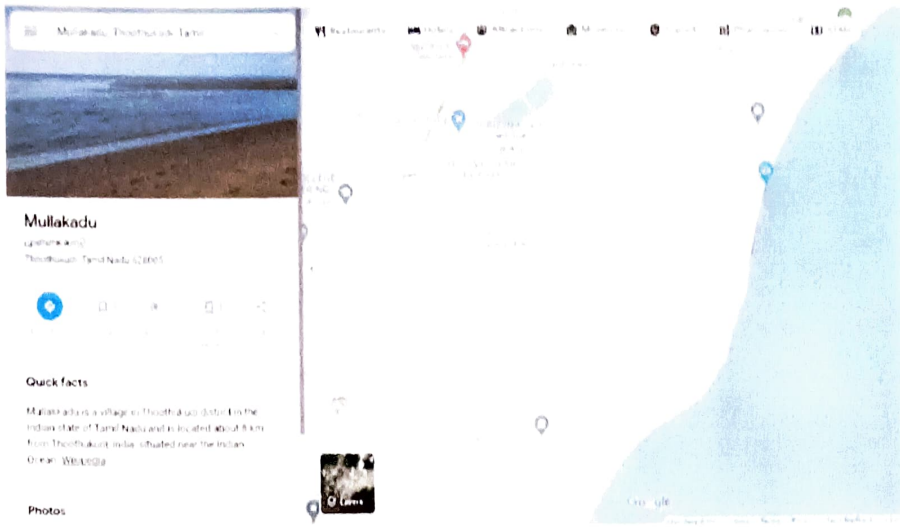
al., 2014).Many researchers ,have showed that nitrogen in the starvation conditions, it can affect all the biochemical composition, and also it affects the rate of photo concentration in the microalgae (Solovchenko et al., in 2013); (Fantail *et al.*, in 2014) In *Isochrysisgalbana*is the important micro algae species used in biodiesel production (Bouffant *et al.*, in 2018).The dries biomass of*Isochrysisgalbana*has the high lipid content(20-30%) on dry weight used for industries, to the production of biodiesel (Roncarati *et al.*, in 2004) Microalgae has the resource to increasing the demand of proteins and amino acids. Microalgae species are having their resistant on cell wall and suitable for depuration process to recover the nutrient content against in adverse conditions. To obtain protein and amino acids from microalgae resistant fresh biomass, avoid the pretreatments and drying .It includes two process namely,

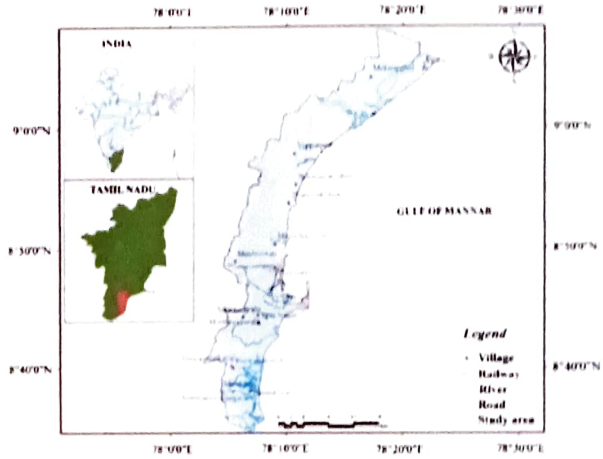
- Sequential reactions
- Extraction methods

Theextractionof proteins byenzymaticreaction by the enzyme protease the evaluation by using Microalgae consortium consists of *Nannochloropsis* species (predominant species).As result, the total protein of microalgae was calculated by using the calculation by the total nitrogen. The nitrogen can be determined by combustion Dumas method (loco CHNS 932; Lecco Corporation, USA) Amino acids can be analyzed by HPLC -High performance liquid chromatography. Ammonia can be determined by also the ion exchange chromatography. The biomass of the extract can be determined blowy method. The soluble proteinaeous material was determined separately among free amino acids and peptides Microalgae contain essential amino acids and nutrients to determine the growth, survival and resistance to disease (Gouveia *et al.*, in 2008).It can accumulate the greatest amount of omega 3 Poly unsaturated fatty acids (PUFA) (Patel *et al.*, in 2007). It has the important fatty acids for the gametogenesis process (Ehteshami *et al.*, in 2011).Now-a-days ,the algal production can be sold for animal as an feed (spoliator *et al.*, in 2006), the varieties of species can be used for commercial aquaculture industries (Enright *et al.*, in 1986).Sometimes ,the microalgae are

known as only described as the ability of algae to store or accumulate the oil. For example *chlorella*spand *Nannochloropsis* sp , the number and composition or position of the double bonds un the carbon chain may vary depending on the microalgae species and its cultural able Conditions. Under optimal conditions them, promote the conversation of fatty acid to glycerol-bases membrane lipids and synthesized of triacylglycerol's (neutral lipids). Microalgae contain Poly unsaturated fatty acids such as arachidonic acid and eicosapentaenoic acid. Politic acid is one of the most predominant fatty acid in the microalgae species. Some cyanobacteria can synthesize monounsaturated and saturated fatty acids. Some microalgae has high in omega 3 fatty acids particularly in *Schizochytrium.* Scarf *et al*., 2020 noted that the effect of photoperiod in the biochemical profile of some species such as *Chlorella vulgaris* and *Scendesmusobliquus*sit has longer photoperiods and reduce the production of alpha – linoleic acid and can induce the linoleic acid. On the other side linoleic acids content was increases the light intensity.

Study Area





Mullakadu is a Village in Thoothukudi Block in Tuticorin District of Tamil Nadu State, India. It is located 10 KM towards South from District headquarters Thoothukudi. 9 KM from Thoothukudi Rural. 625 km from State capital Chennai. Mullakadu, Gulf of Mannar located along the southeast coast of India and declared as a marine national park with high ecological diversity faces threat due to anthropogenic interventions as a result of industrialization, sand and coral mining, etc. A large number of coastal habitations were present adjacent to the beach along the study area. Coastal erosion, slope and relative sea level rise are the major factors affecting the coastal vulnerability in Thoothukudi (Sandeep *et al.*, 2019).

METERIALS AND METHODS

Materials and methods

Collection:

Collection of micro algae have done during early morning hours at around 6 am in marine water by during low tides of mullakadu coast, Thoothukudi district, Tamilnadu. Planktonic algae have collected by using a plankton net and also collected by using bottles. The bottom living algae have collected with the help of long glass tube.

Attached algae have collected along with the substrate, if the substrate is small and placed in a container without any difficulties. The algae have scraped off from the surface of the substrate and transferred to another container while collecting the samples, the following details have been recorded viz., date, place, name of the water body , nature of the substrata from which samples were collected .

Preservation of plankton:

The collected marinevwater samples (1L), the microalgae were fixed by adding 10 ml of lugol's iodine solution. To 1L of sample, add 10ml of lugol's iodine solution, left un disturbed for 24 hours then decant water. Collected settled plankton in small bottles and preserve for further studies.

Strain Identification

Purified marine microalgae strains isolated from the collected water samples were identified using their morphological features. The different purified colonies were examined using a light microscope. The identification of the algal strains was made using a field guide ("phytoplankton of the Indian seas – an aspects of marine botany")

RESULT AND DISCUSSION

Result and discussion:

Micro algae communities have a vital role in sustaining the fertility of aquatic and terrestrial habitats. This understanding the actual biodiversity of these communities is a taxa of almost importance. Micro algal diversity assemblage serve as a biological indicators of water body's environment. The study recorded 15 genera of microalgae micro algal species belonging to (Xanthophyceae, chlorophyceae, zygnematophyceae, trebouxiophyceace, chlorophyceae, fragilariophyceae, chlorodendrophyceae, zygnematophyceae,) classes.

They are colonial or single celled organism were absorbed in our study. These algae identified by using manual " phytoplankton of the Indian seas an aspects of marine botany" by R. Santana , N. Ramanathan, K. Venkataramanujam and ,G. Jegatheeran. They were categorized into divisions based on various characteristics such as morphological features and pigmentation.

The microscopic study of the purified microalgae resulted in the identification of several taxa (Table 1). The identified species represent 8 classes (Chlorophyceae, Zygnematophyceae, Treand *Trebouxiophyceae)* (Table 2). *Chlorococcum humicola* were the most abundant among the isolated species (50%), followed by *Phytophthora oedogonium* and *Chlorella vulgaris* (10%) while hormidium subtile represent only 8% (Figure 1).

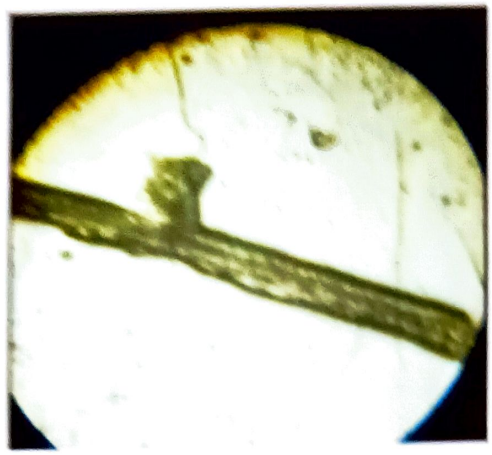
The diversity of marine microalgae is vast and represents an intact resource (Singh, J., and Saxena, R. 2015). The microalgae which were easily identified by their morphology and pigment were identified using microscope in our study area. While some coccoid forms were generally difficult to distinguish based on microscopy. So some algae we were not included in this study. In this regard, species identification using LSU rDNA sequencing was a powerful technique. So further study is needed. Microalgae are unique to ecological sites of isolation. It was interesting to observe that studies on algal diversity in various countries showed different predominant genera.

In aquatic food chain, microalgae play a vital role and they are valuable in aquaculture and have been used as live natural feeds for all bivalve mollusks and for larval or juvenile

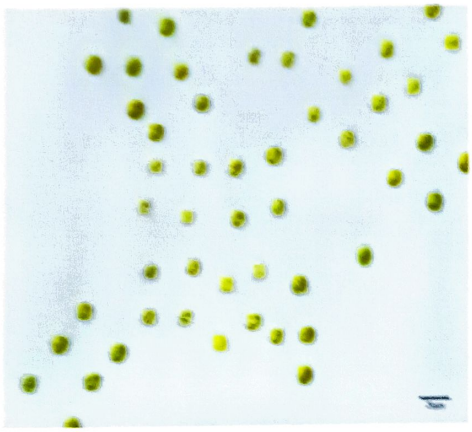
crustaceans and finfish, as well as for raising the zooplanktons required for feeding of juvenile (Ju, Z. Y et al., 2009) Diatoms and green algae are largely used in aquaculture especially for larval shrimp and in fish hatcheries (Roy, S. S., and Pal, R. 2014). The most diatoms and green algae used in aquaculture are *Navicula sp.*, *Chaetoceros sp.*, *Nitzschia sp.*, *Chlorella sp.* and *Dunaliella sp.* (Shah, M. R 2017 and Gallardo, W. G., and Been 2003) Hence, the interest of the microalgae that we have isolated in this work. Moreover, microalgae are one of the most promising sources of biomass for biofuel production

In our study area is dominated by *Aphanochaete repens, Chlamydomonas reinhardtii, Chlorella vulgaris, Chlorocloster spinigera, Chlorococcum humicola, Chlorococcum longissima, Cosmocladium saxonicum, Hormidium subtile, Phytophthora oedogonium, Spirogyra sp., Spharellopis fluviatilis, Tabellaria fenestrate, Tetraselmis chui, Tetraspora sp., Vaucheria sp.* The abundant species isolated from our study area is *Chlorococcum humicola* which has bioactive phytochemicals for antimicrobial activity (Bhagavathy S *et al.*, 2011). Besides *Chlorococcum humicola*, another group of microalgae dominated the isolates is *Phytophthora sp.* The isolated microalgae are a diverse group of prokaryotic photosynthetic microorganisms that can grow rapidly due to their simple structures and growth requirements as well as efficient use of light, CO_2 and other inorganic nutrients (Parmar *et al.*, 2011).

Plate I: Photomicrographs of dominant microalgae found at the Mullikadu marine water

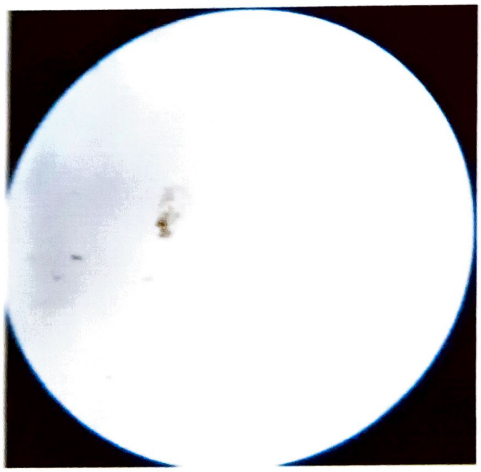


Vaucheria sp

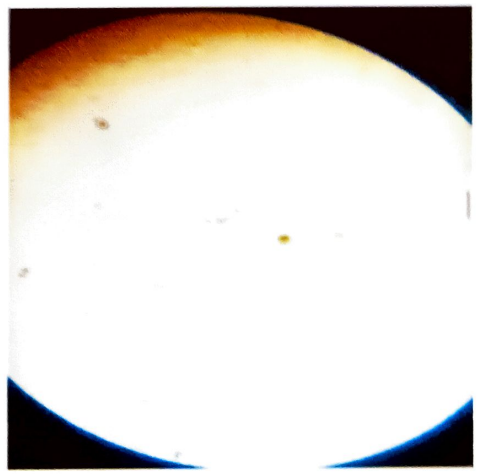


Tetraspora sp

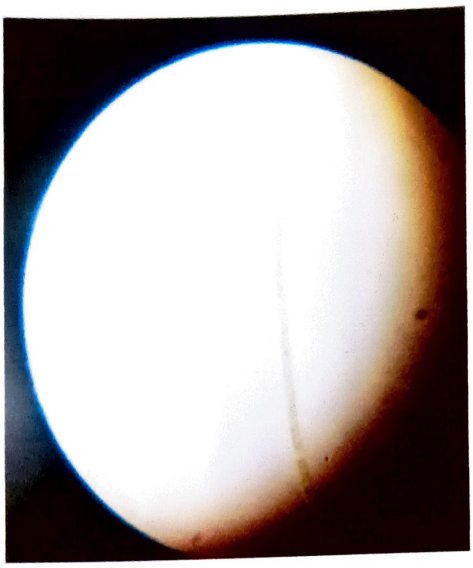
Spirogyra sp



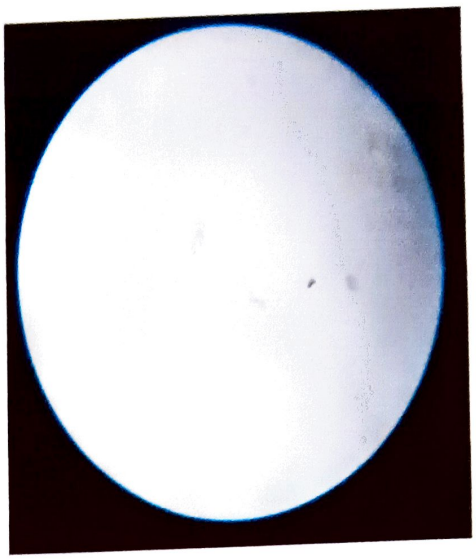
Spharellopis fluviatilis



Chlorococcum humicola

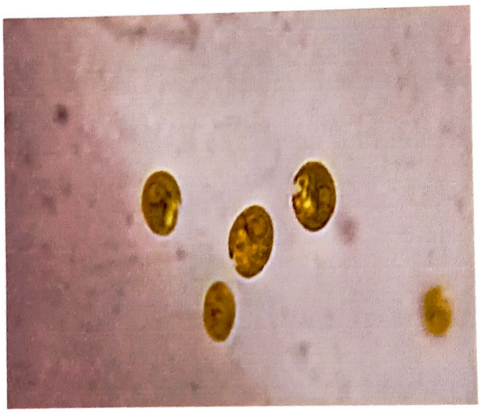


Hormidium subtile

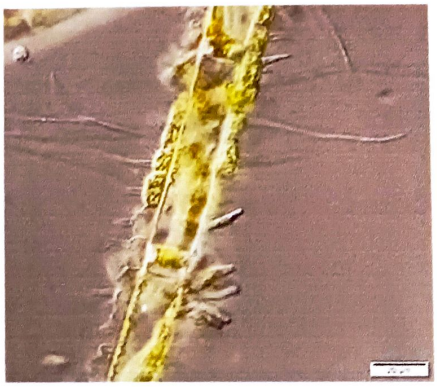


Chlorocloster spinigera

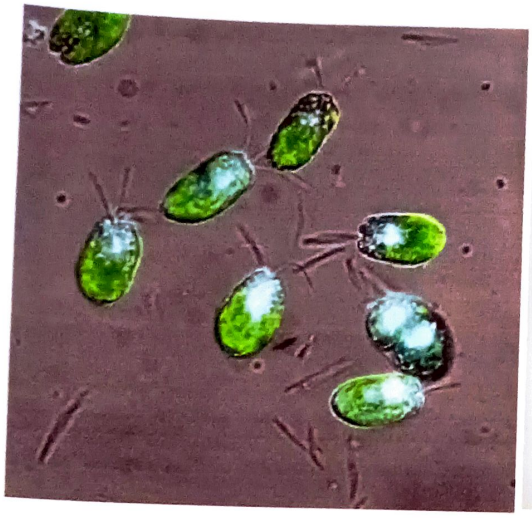
Tabellaria fenestrata



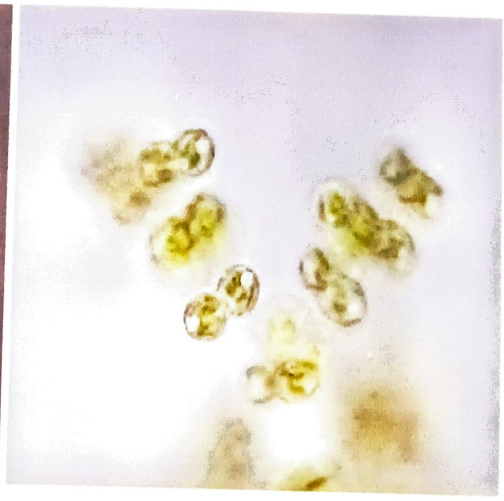
Chlamydomonas reinhardti



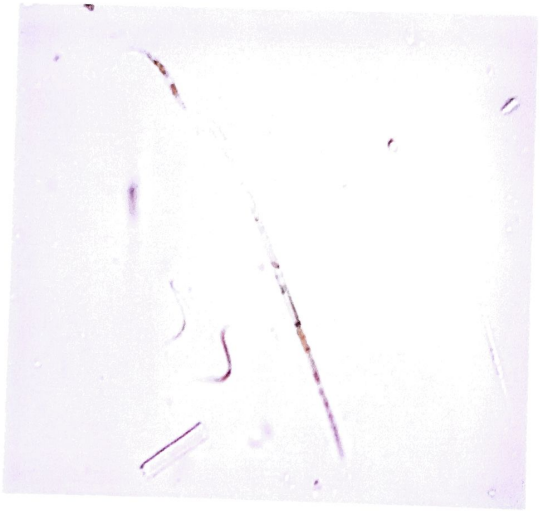
Aphanochaete repens



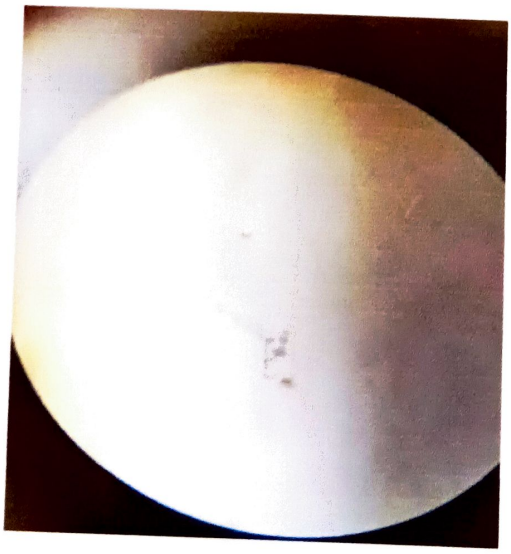
Tetraselmis chui



Cosmocladium saxonicum



Closteriopsis longissima



Phytophthora oedogonium

Table 1: List of microalgae identified in the Mullakadu marine water

S. No.	Identified Microalgae	Abundance (%)
1.	*Aphanochaete repens*	1 %
2.	*Chlamydomonas reinhardtii*	5 %
3.	*Chlorella vulgaris*	10%
4.	*Chlorocloster spinigera*	2 %
5.	*Chlorococcum humicola*	50 %
6.	*Chlorococcum longissima*	7 %
7.	*Cosmocladium saxonicum*	1 %
8.	*Hormidium subtile*	8 %
9.	*Phytophthora oedogonium*	10 %
10.	*Spirogyra sp.*	3%
11.	*Spharellopis fluviatilis*	2%
12.	*Tabellaria fenestrata*	1 %
13.	*Tetraselmis chui*	5 %
14.	*Tetraspora sp*	7 %
15.	*Vaucheria sp*	5 %

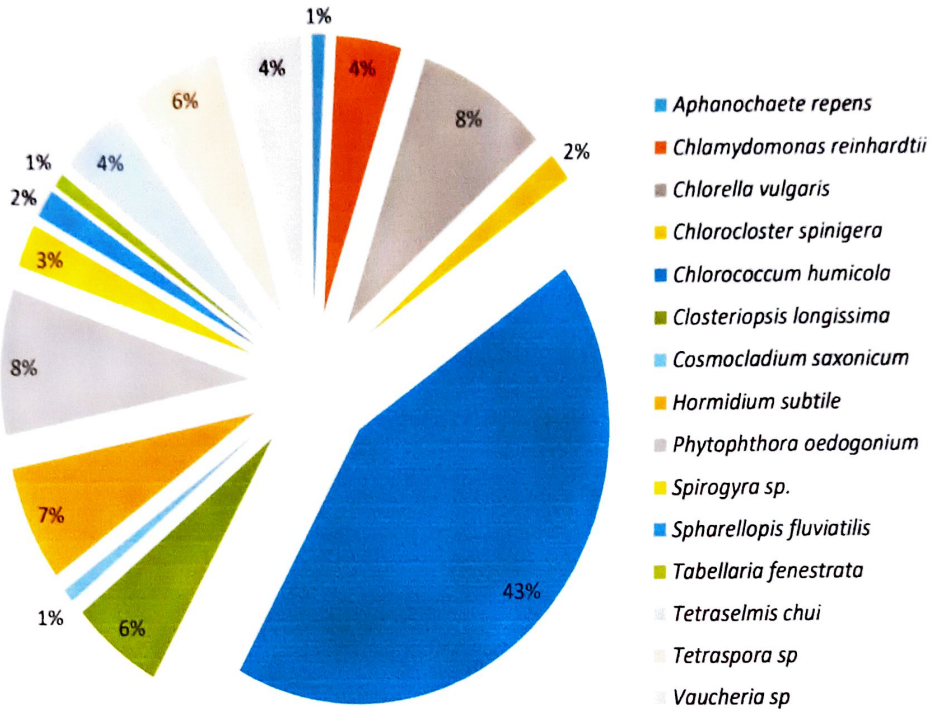


Fig. 1. Abundance of microalgal distribution in one drop of Mullakadu marine water

Table :2 Taxonomical data of micro algae isolated from the mullakadu marine water

S.No	Identified Micro algae	Class
1	*Aphanochaete*	*Chlorophyceae*
2	*Chlamydomonas reinhardtii*	*Chlorophyceae*
3	*Chlorella vulgaris*	*Trebouxiophyceae*
4	*Chlorocloster spinigera*	*Zygnematophyceae*
5	*Chlorococcum humicola*	*Chlorophyceae*
6	*Chlorococcum* longissima	*Chlorophyceae*
7	*Cosmocladium saxonicum*	*Zygnematophyceae*
8	*Hormidium subtile*	*Trebouxiophyceae*
9	*Phytophthora oedogonium*	*Chlorophyceae*
10	*Spirogyra sp.*	*Zygnematophyceae*
11	*Spharellopis fluviatilis*	*Chlorophyceae*
12	*Tabellaria fenestrate*	*Fragilariophyceae*
13	*Tetraselmis chui*	*Chlorodendrophyceae*
14	*Tetraspora sp*	*Chlorophyceae*
15	*Vaucheria sp*	*Xanthophyceae*

SUMMARY

Summary

Microalgae are referred to as green gold because of their commercial value. They are cultivable throughout the year, have a low land demand and are a rich source of organic compounds. The three major uses of algae are biofuels (biochar, bioethanol, oil, biohydrogen), direct use (food and supplements for humans and animals), bioproducts (fatty acids, antioxidants, coloring agents, vitamins, anticancer and antimicrobial drugs. Micro algal diversity assemblage serve as a biological indicators of water body's environment. The study recorded 15 genera of microalgae micro algal species belonging to (Xanthophyceae, chlorophyceae, zygnematophyceae, trebouxiophyceace, chlorophyceae, fragilariophyceae, chlorodendrophyceae, zygnematophyceae,) classes.

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CONCLUSION

Conclusion

Interest in microalgae research has increased last decades in various fields and the microalgae sector is very dynamic throughout the world. In India, the exploitation of marine microalgae for their bioactive substances is still limited, even if the potential remains important due to its favorable climatic conditions and to its specific geographical position. In this work, fourteen marine microalgae were purified, identified and classified using morphological features. The isolates obtained belong to important groups of microalgae (diatoms, green algae). These groups include species that are highly exploited on an industrial scale for the production of different molecules of interest or used as food or feed. This study is only an initiation of research on marine micro algae.

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POLLEN VIABILITY AND POLLEN GERMINATION TESTS IN SELECTED PLANTS OF THOOTHUKUDI DISTRICT

A short term project work submitted to

ST.MARY`S COLLEGE (Autonomous), THOOTHUKUDI

Affiliated to

MANONMANIAM SUNDARANAR UNIVERSITY

in partial fulfilment of the requirement for the degree of

BACHELOR OF SCIENCE IN BOTANY

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CERTIFICATE

This is to certify that this project work entitled **"POLLEN VIABILITY AND POLLEN GERMINATION TESTS IN SELECTED PLANTS OF THOOTHUKUDI DISTRICT"** is submitted to **St. Mary's college (Autonomous), Thoothukudi** affiliated to **MANONMANIAM SUNDARANAR UNIVERSITY** in partial fulfilment of the award of the degree of **Bachelor of science in Botany**, and is a record of work done in the Department of Botany, St. Mary's College (Autonomous), Thoothukudi during the year 2021 – 2022 by the following students.

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INTRODUCTION

Palynology The term pollen was first introduced by the great Swedish botanist Linnaeus and in his words; they are microspores of seed plants. Pollen refers to a highly reduced male gametophyte, and it is the most vital unit of angiosperm flower, both with regard to form and function, and represents an essential genetic bridge between one generation to the next (Erdtman, 1952). The potentialities of pollen and spores as a morphological entity in plants have become increasingly understood, since the time Hooke developed a prototype microscope in 1665. The first comprehensive attempt on the study of pollen morphology is that of Wodehouse (1935), whose book "Pollen Grains" has documented the fundamental features of the angiosperm pollen morphology that marked the beginning of a meaningful study of these microscopic grains. The monumental work of Erdtman (1952) entitled "Pollen morphology and Plant Taxonomy: Angiosperms" has laid a strong foundation for pollen studies, and it has demonstrated the importance and implication of pollen morphology in angiosperm taxonomy.

1.1.1. Palynology as an interdisciplinary science

Due to the advent of advanced technologies in the field of palynology, the science has widened its scope of interest, with the result that various aspects of palynological studies have been delimited under two main divisions, namely, basic palynology and applied palynology (Erdtman, 1969). Basic palynology covers pollen and spore morphology and the theoretical aspects of applied palynology. Apart from palynotaxonomy, applied palynology is comprised of aeropalynology (study of frequency and distribution of pollen in air), palaeopalynology (study of fossil pollen grains and spores), melittopalynology (study of pollen and spores in honey), pharmacopalynology (drugs based on pollen or pollen extracts), latropalynology (medical

aspects of pollen and spores in connection with hay fever etc.), copropalynology (study of pollen and spores in excrements), and forensic palynology (study of pollen and spores in connection with criminology). Palynological studies are also involved in agriculture, horticulture, plant breeding and plant physiology. Now, the scope of palynology is immense and the science is very vast and is presently recognized all over the world as an important offshoot of botanical sciences.

1.1.2. Palynology in relation to taxonomy and phylogeny

Pollen grains are provided with an extremely hard outer wall (exine) and an inner soft wall (intine) surrounding the cytoplasms including nuclei and organelles. The exine is extremely hard and resistant, which can be treated by strong acids or bases without being destroyed. Chemically the exine is composed of sporopollenin, a substance formed through polymerization of hydrocarbons, carotenoides and carotenoid esters and has an ornamentation which is characteristic for the species, genus or family.

The morphological characteristics of pollen grains and spores are contained in the exine and are important criteria in considerations of the taxonomy and inter-relationships of plants especially at lower levels. Pollen morphology is considered to be unique in as much as no other single discipline can obtain as great an amount of information from so little material within a short period (Walker and Doyle, 1975). The pollen morphological features especially exine architecture and apertures (number, position, character) are relatively conservative, provide the best diagnostic features which make them useful in taxonomic as well as phylogenetic discussions (Nilsson, 1992). The principal pollen characters which are phylogenetically useful at different taxonomic levels include the aperture type, pollen wall sculpture, symmetry, shape and size, of which the aperture characteristics are considered to be of paramount importance because

of their evolutionarily conservative nature, which in most cases, is an excellent means of taxonomic elucidation, especially at the generic and lower levels.

1.4. Endemism

Endemism encompasses taxonomic units of any rank or taxa which occur in a biogeographical area usually isolated by geographical, ecological or temporal barriers. Such species are known as endemic species and are very significant, throw light on the biogeography of the area, centre's of speciation, areas of extinction, vicariance and adaptive evolution of the flora in that area (Ahmedullah and Nair, 1987). Depending upon the nature, endemics may be palaeoendemics, neoendemics or holoendemics. Palaeoendemics are ancient endemics, which represent remnants of older floras usually occurring in geologically older land masses.

Pollen, which is a carrier of male gametes, includes three domains that are different in their chemical composition, morphological structure and their physiological and biological significance. The three domains of pollen grain include exine, intine and nucleus. The complex exine structures of pollen are storage sites for carbohydrates, glycoproteins, lipids, terpenoids and phenolics. The pollen nucleus is rich in chromatin material and viable pollen stains pink to deep red with acetocarmine, while sterile pollen does not take any stain and thus remains almost white and transparent. A viable or fertile pollen is one which, after landing on stigma of the same plant or other plants of the same variety or species, under normal conditions would start growing a pollen tube and finally discharge its male gametes in the embryo sac effecting fertilization. According to Rigamoto and Tyagi (2005), pollen fertility, which can be determined using pollen viability teats in-vitro is very important in fruit and seed production in flowering plants. Therefore, pollen fertility knowledge of any plant species is essential for plant breeders and commercial growers. They determine pollen fertility status using pollen viability tests in-vitro by

aceto-carmine staining technique in thirty two species of economic and environmental importance.

Quality of pollen are crucial for pollen storage studies, pollen-pistil interaction studies, understanding incompatibility and fertility studies, for breeding and crop improvement and for seed industry (Shivanna and Rangaswamy, 1992). Pollen viability and vigor decide the quality of pollen (Ottaviano and Mulcahy, 1989). Pollen fertility and viability have a paramount importance in hybridization programme. Successful pollination is a prerequisite for fertilization and seed set in most plants, and the insight knowledge on pollen biology, including pollen viability, pollen germination and pollen tube growth, is required for any rational approach to increase productivity (Shivanna, 2003). To differentiate aborted and non-aborted pollens, a simple and hasty technique is required for high throughput. An unambiguous procedure involves pollen deposition on receptive stigma followed by evaluation of seed set. These methods, however, are indirect and sluggish methods, associated with physical and physiological features of pollen with its ability to fertilize the ovule, are universally preferred (Rodriguez-Riano and Dafni, 2000). Pollen germination assays frequently require optimization as well as being time consuming and difficult to reproduce (Boavida, 2007).

Reproduction is a critical step in the life cycle of all organisms and pollination is one of the decisive stage (Jha and Dick, 2010) of reproduction process. The male gamete, pollen is surrounded by sporoderm, which consists of the inner layer intine (which is composed of pectin, cellulose and protein) and outer layer exine (synthesized and secreted by the tapetal tissue of the anther and is composed mainly by sporopol-protein). The glycoprotein present on the pistil generally interact with pollen coat protein (Zhang *et al*., 2008). During the pollination process pollen grain deposited on and adhere to the stigma, the pollen hydrates and germinate by

emitting pollen tube, which penetrate the cuticle of the stigma cells and grows through the extracellular matrix of the style. The process culminates in the discharge of male gametes into the embryo sac. In bitter gourd, the pollen tube penetrate papillae tissue within 1 hour of pollination and reaches to the ovule about 6 hour after pollination and fertilization is completed within 18- 24 hours after pollination.

In spite of taking proper care during pollination, breeder fail to get fertile seed during artificial pollination. Unless sterility is the main cause the failure of seed setting may be the result of slow growth of the pollen tube or its early degeneration in the style. To overcome these difficulties it is necessary to study the pollen viability, physiology of pollen germination and pollen tube growth.

There are numerous techniques are available to study pollen viability and the method choice depends on crop (species) and on establishing relationship between test and the fertility (Hanna and Towill 1995). The most accurate method of pollen viability is the ability of pollen to effect fertilization and seed set (Shivanna and Johri 1989). There are different methods to evaluate pollen viability; some noteworthy tests are dye and *in vitro* germination. The dye test have advantages as indicator of pollen viability because they are faster and easier compared with pollen germination, but they do tend to overestimate the viability and real germination of pollen grains. On the other hand, in vitro pollen germination depends on the genotype, environmental conditions, pollen maturity, composition and pH of the medium; thus it is necessary to determine optimum conditions for pollen germination. It is recommended simultaneously use several tests to reflect pollen performance.

The literatures on different tests of pollen viability was reviewed by Dafni and Firmage (2000) and they explained the advantage and disadvantages of each test. The ability to use

chemical staining to discriminate aborted from non-aborted pollen grains has well-known practical applications in agriculture. Pollen staining method some time not reliable for most of the species (Barrow, 1981), hence *in vitro* pollen germination is more practical used to determine the pollen viability. The ability of pollen germination play an important role not only on fruit set but also the flower-flower and flower-pollinator interaction. Pollen germination and pollen tube growth very crucial for fertilization and seed development. Due to involvement of the pistillate tissue in the nature, physiological and biochemical investigations on pollen germination and pollen tube growth *in vivo* are rather difficult. Hence *in vitro* germination techniques generally been used extensively on a variety of pollen systems. Such studies have provided considerable information on the physiology and biochemistry of pollen germination and pollen tube growth (Shivana and Johri 1989).

To reach pollen tube to micropyle region, it passes through four stages, a) imbibition phage, b) Lag phage, c) tube initiation phage and d) rapid tube elongation phage. Pollen tube growth proceeds through tip extension and can be affected by many factors, including temperature, medium osmolarity and the availability of sucrose, calcium, zinc and boron.

Effective pollination is a prerequisite for fruit- and seed-set in most plants, and information on pollen biology, including pollen viability and pollen tube growth, is required for any rational approach to increase productivity (Shivanna, 2003). Pollen viability can be evaluated by: (1) staining techniques; (2) in vitro and in vivo germination tests; or (3) analyzing final seed set. The choice of method depends on the crop or species (Dafni *et al.,* 2005). Rodriguez-Riano and Dafni (2000) recommended the use of heat-killed pollen as a control to check the potential of the dye for testing pollen viability. In many species, in vitro pollen germination is dependent on the addition of key substrates such as calcium nitrate to the

germination media (Steer and Steer, 1989). Indole-3-acetic acid (IAA) plays an essential role in plant sexual reproduction by controlling the development of stamens and ovaries; promoting the maturation of egg cells; and inducing the axial polarity and polar development of the embryo (Wu *et al.*, 2008). It has also been suggested that IAA promotes pollen tube growth in growing long and straight. Pollen tube growth can affect the outcome of self- versus cross-pollination, as pollen tubes from self-pollen may grow slower or have higher rates of attrition than those from cross pollen (Aizen *et al.*, 1990).

REVIEW OF LITERATURE

The development of palynology, a comparatively modern branch of science is closely associated with the improvement in the field of microscopy. The study of pollen grains began in the middle of the seventeenth century, when Nehemiah Grew, later known as the father of Plant Anatomy, microscopically observed pollen in the first time of history. In the 18th century various people studied pollen and spores especially with reference to their function in fertilization of ovule.

Pollen grains of angiosperms represent a highly reduced male gametophyte. They contain a vegetative cell enclosing either the generative cell or two male gametes. Pollen grains offer several advantages to study many basic problems in physiology, morphogenesis, genetics and cell biology. They are comparatively simple, haploid organisms having limited function. In many taxa they can be easily collected and stored for a considerable length of time (with – out loss of viability), and can be cultured on a simple nutrient medium. The growth of pollen tubes following germination is rapid, and is limited for a few hours. Thus, stored pollen grains can be used for studies throughout the year, and there is no need to maintain aseptic conditions in routine investigations. Pollen grains show many interesting morphogenetic features such as the presence of a thick ornamental wall made up of sporopollenin, restriction of the tube growth to the extreme tip, and deposition of callose plugs across the tubes.

Since long, pollen grains have been the objects of extensive investigations. Classical studies of Amici, Schleiden, Strasburger, and Nawaschin, during the last century, demonstrated the role of pollen grains in sexual reproduction. Since the embryologists have carried out meticulous studies on details of pollen development and fertilization in numerous taxa. As the

pollen wall architecture is generally consistent for a given taxon, studies on pollen morphology have been an important aspect of palynology. Besides embryologists and morphologists, pollen grains have attracted the attention of plant breeders, physiologists, biochemists, geneticists, and cell biologists. Many, amongst these, study pollen from the point of view of understanding its main function, namely, pollen germination, pollen tube growth, and the discharge of male gametes. Some others study pollen from the point of view of utilizing the knowledge on the biology of pollen in practical application mainly in plant breeding programme. Yet others use it as a system to understand many basic problems of morphogenesis, and cell biology. Recently, pollen grains are also being used in experiments concerned with genetic engineering (Hess *et al.*, 1976).

PHYSIOLOGICAL AND BIOCHEMICAL STUDIES

Physiological and biochemical studies have largely been confined to a few taxa having large anthers, and showing reasonable synchrony in meiotic divisions (amongst different anthers of the flower buds) such as *Lilium, Trillium, Allium*, and *Tradescantia*. Because of the technical difficulties. Most of these studies are carried out using the whole anthers and hence, are not aimed at understanding the differential activity of different layers of the anther tissue sporogenous tissue, tapetum, and wall layers. As these layers differ markedly in their morphogenetic pathway, this is a serious limitation in understanding the physiology and biochemistry of pollen development.

In many of the above studies the technique of Anther culture has been profitably used. Anthers of many taxa have been successfully cultured from zygotene stage onwards. It has not yet been possible to induce meiosis in anthers cultured before the initiation of meiosis. Recently, Reznikova and Bogdanov (1972) have studied, in detail, the responses of anthers cultured at

different stages of development in *Lilium candidum* Anthers cultured before the onset of meiosis showed many abnormalities such as lack of pairing of chromosomes, sysnapsis, and lack of chiasma formation, depending on the stage at culture. Also, callose formation did not occur around microspore mother cells. Normal meiosis was observed only when the anthers were cultured at zygotene. All these studies have led to the concept that the stimulus for the induction of meiosis in pollen mother cells originates at some other part of the plant, and is transmitted to the anther.

Detailed biochemical studies on the nature of synthesis of DNA, RNA and proteins during meiosis have been carried out. Although most of the DNA in microspore mother cells is synthesized during the premeiotic interphase, detailed studies of stern and his associates have demonstrated conclusively that a very small amount of DNA (0.3%) is a synthesized during zygotene and pachytene stages of meiosis. This was found to be essential for the orderly progress of meiotic events. Inhibition of prophase DNA synthesis resulted either in zygotene arrest, or fragmentation of chromosomes. Initiation of meiosis is characterized by the appearance of a distinctive lipoprotein complex, and a DNA binding protein, termed r-protein. These probably have a role in chromosomes pairing (Hecht and Stern, 1971).

The DNA synthesized during zygotene (when chromosome replication occurs) is typically semiconservative, and represents a delayed replication. The DNA synthesized during pachytene (when chiasma formation occurs) has the characteristic of a repair type of replication (Hotta and Stern, 1971). These studies strongly indicate that the synthesis of DNA during meiotic prophase has a functional role in crossing over process. The replication of zygotene DNA (Z- DNA) occurs in the lipoprotein complex in the presence of r-protein. The pachytene

interval is marked by the activation of endo nucleolytic activity (Howell and Stern, 1971). Synthesis of DNA during meiotic prophase requires simultaneous synthesis of nuclear proteins.

Synthesis of RNA and proteins has been studied by autoradiographic, biochemical, and cytochemical methods. Remarkable changes in RNA and protein content in meiocytes occur during meiosis. A prominent RNA and protein synthesis at premeiotic stage has been reported by many investigators, using different techniques. Autoradiographic studies have revealed a continuous drop in RNA synthesis during prophase. From metaphase I onwards until the completion of meiosis there is hardly any incorporation of label into RNA (Sauter, 1969). However, Hotta and Stern (1963) found transient peaks of RNA synthesis during meiotic prophase (pachytene – diplotene), interkinesis, and tetrad stage. They also showed, by the use of inhibitors and nucleotide analysis that at least part of the RNA synthesized during meiosis is MRNA. The drop in RNA synthesis during prophase is correlated with the cytochemical and ultrastructural studies in which a continuous drop in RNA content from preleptotene stage onwards, and a steady depletion of ribosome population in the cytoplasm, have been reported (Mackenize *et al.*, 1967). A Similar increase in RNA content has been reported by Linskens and Schrauwen (1968). Thus, although there is no disagreement between the results of different techniques in the fall of RNA synthesis during meiotic prophase, there are discrepancies in the minor details and the stage from which the rate of RNA synthesis starts increasing. Part of this discrepancy is probably due to the presence of a callose wall around meiocytes, which is reported to prevent the entry of some labels.

Many investigators have studied the rate of RNA synthesis following meiosis. In *Tradescantia paludosa* the period of highest ribosomal RNA (5s, 16s and 25s) synthesis was found to occur during microspore interphase, and no rRNA was synthesized during the last 48 hr

of pollen maturation (Peddada and Mascarenhas, 1975). However, many other investigations have shown an increase in the total RNA after microspore mitosis, during the terminal stages of pollen development (Sauter, 1969).

Protein synthesis also follows more or less a similar pattern as that of RNA. Autoradiographic studies have shown that the incorporation of the label is prominent in premeiotic stage, decreases as the meiocytes approach leptotene, and remains low through meiotic prophase and subsequent stages of meiosis (Albertini, 1967). Hotta and stern (1967) reported the presence of a unique histone, tic histone, which is absent in the somatic cells, is synthesized during premeiotic histone synthesis and persists tone is not known. Hotta and Stern (1963), by measuring the uptake of labelled amino acids in cultured meiocytes, peaks of incorporation in leptotene – zygotene, late pachytene, and early diakinesis. They have also collected evidences by the use of inhibitors to indicate that part of the proteins synthesized during prophase are coded by newly formed mRNA. Based on these studies, it has been inferred that meiosis is accompanied by a set of phase specific gene transcriptions, which are essential for the completion of normal meiosis. As pointed out earlier, synthesis of proteins during prophase was essential for the orderly progress of meiosis (Parchman and Stern, 1969).

Inhibition of protein synthesis during prophase resulted in meiotic abnormalities such as arrest of meiosis, change in chromosome morphology and lack of chiasma formation, depending on the stage of inhibition. However, unlike inhibition of prophase RNA synthesis, inhibition of protein synthesis does not induce fragmentation of the chromosomes (Parchman and Stern, 1969), nor inhibit the formation of synaptinemal complex (Sen, 1969). These results have been interpreted to indicate that protein synthesis is involved in structural organization of the chromosomes.

POLLEN-WALL PROTEINS

It has been known, since long, that pollen grains release enzymes into the germinating medium (Stanley and Linskens, 1965). It was generally interpreted in terms of active secretion from pollen grains. T singer and Petrovskaya-Baranova (1961) were the first to report the presence of proteins in the wall of pollen grains. Both wall layers of pollen, the intine and exine, contain considerable amount of proteins. The intine proteins are present in the form of radially-arranged tubules, or tangentially-oriented leaflets. There are generally concentrated near the germpore. The exine proteins are present in the cavities between the baculae (in tectate grains), or in the surface depressions (in non-tectate grains). A part of these proteins are enzymes, mostly hydrolytic enzymes such as esterases, acid phosphatase, amylase, and ribonuclease. The proteins responsible for pollen allergy are also present in the pollen wall.

Esterases occur predominantly in the exine, and acid phosphatases in the intine. Esterases and acid phosphatases can, therefore, be used as marker enzymes for exine and intine proteins, respectively (Vithanage and Knox, 1976). Amongst aquatic plants, and many members of Iridaceae, Cannceae and Zingiberaceae there is a progressive loss in the exine. In *Canna* the exine is reduced into a few isolated spinules, and there is no transfer of tapetal material. *Amphibolis and Thalaspodendron* (seagrasses) in which pollen grains are dispersed under submerged conditions, there is no recognizable exine. No esterase activitiy is found in the wall of mature pollen, but acid phosphatase activitiy is present throughout the wall. The pollen-wall proteins are readily released into the adequous medium within seconds of pollen hydration. In seagrasses an adhesive substance seems to be present on the pollen surface. This covering probably prevents the release of wall proteins into the water. A similar coating on the pollen surface may be present in other aquatic plants in which pollen dispersal takes place in water.

Heslop-Harrison and his associates in 1973, worked out the details of the origin of wall proteins. As the deposition of intine progresses, following the release of micropores from the tetrad, the plasmalemma of the pollen cytoplasm puts out radially-oriented tubules into the developing intine, Eventually, these tubules with their protein inclusions become cut off from the plasmalemma, and are sealed off from the cells surface by the deposition of a layer of intine free from tubules. In aperturate pollen the intine proteins are largely concentrated in the region of germpore, whereas in non-aperturate pollen the proteins are distributed throughout the intine. Similar tubules, apparently derived from plasmalemma, have been described in many other earlier fine structural studies. In *Cosmos bipinnatus* the incorporation of intine proteins is rather unique. Instead of tubular evaginations, the leaflets of plasmalemma with their protein inclusions become separated from the cytoplasm and get incorporated in the intine as a series of tangential lamellae.

The exine proteins, on the other hand, originate in the cells of the surrounding tapetum, a sporophytic tissue. During meiosis in the microspore mother cells, proteins and lipids accumulate in the tapetal cells, the former in single membrane-bound vesicles apparently derived from the rough endoplasmic reticulum, and the latter in the plastids. When the tapetal cells breakdown towards the end of pollen development, these proteins and lipids are released into the thecal cavity and, eventually, they become deposited in the surface depressions of the exine. In tectate grains the protein fraction passes through the microspores of the tectum and accumulates in the spaces between the baculae, and the lipids remain on the surface of tectum.

The intine proteins of pollen cytoplasm, the male gametophyte, and the exine proteins are the products of the tapetum which is a sporophytic tissue. Cytophotometric studies on the origin of esterases and acid (phosphatase in the pollen have been made in *Brassica oleracea*

(Vithanage and Knox, 1976). Acid phosphatase activity was confined to pollen grains, and the tapetum showed very little activity. Pollen showed two periods of acid phosphatase activity, the first associated with intine deposition and the second during pollen maturation. Esterase activity was rather insignificant in the pollen, but the tapetum showed a rapidly increase in enzyme activity until its breakdown. The breakdown of the tapetum was correlated with the dramatic increase in the esterase activity in pollen wall, indicating by the transfer from the tapetum to exine, cavities. These studies confirm gametophytic and Sporophytic origin of indicine and exine proteins, respectively. This differential origin of the pollen-wall proteins has important implications in controlling the breeding behavior of the species.

VIABILITY AND STORAGE

Generally, the pollen grains are shed under dehydrated condition and, hence, the metabolism is very low. The pollen in Gramineae, however, shed under hydrated condition, and the metabolism also remains high. After shedding, viability of protein grains varies significantly from species to species. In many fruit trees the viability is maintained for months (under laboratory conditions). In the members of Gramineae, and Compositae the viability is lost very rapidly, often within minutes after shedding. In most other taxa the period of viability falls between these two extremes. The environmental factors, particularly the temperature and humidity, greatly affect viability. There is a close correlation between the cytology of pollen (2 or 3 celled) and viability. The 2-celled pollen retains viability for a longer period than 3 celled pollen (Brewbaker, 1957).

Our knowledge of the physiological and biochemical changes, associated with the loss of viability, is fragmentary. In *Lilium longiflorum* loss of pollen germinability upon storage was correlated with a decrease in endogenous proline level. Such stored pollen showed increased

germinability when exogenous proline was supplied in the medium. The investigators of Hoekstra and Bruinsma (1975), on the respiration and longevity of 3-and 2-celled pollen, provide direct information on viability. The comparative studies on 3-celled pollen of many taxa belonging to Compositae and Gramineae with pollen of 6 species with 2-celled pollen have demonstrated that 3-celled pollen respire 2-or 3-times more than 2-celled pollen. Temperature and humidity affected respiration rate as well as germinability in both 2 – and 3-celled pollen. Although higher temperature increased the respiration rate in both the systems, respiration of 3-celled pollen was always higher than that of 2- celled pollen. Reduced humidity decreased respiratory activity, but the prolongation of viability at lower humidity was only marginal in 3-celled systems. In members of Gramineae relative humidity below 70% was highly detrimental to pollen viability. Based on these investigators, Hoekstra and Bruinsma suggested the high respiratory activity to be the cause for the rapid loss of viability in 3 – celled pollen.

TESTS FOR VIABILITY

One of the investigators has been the lack of a quick, simple, and dependable test for determining pollen viability. Staining and dependable test for determining pollen viability. Staining with acetocarmine, or cotton blue, which has been used by some of the early investigators (although suitable for determining sterility), are not valid for testing viability. Viability tests in terms of seed – set following manual pollination, although accurate, are time-consuming can be used only for a limited period, until the end of the flowering season of a given species. Also, this is more a qualitative test rather than quantitative, particularly in systems with fewer ovules, as a germination of a limited number of pollen tubes growing in the style following compatible pollination is often used as a indication of viability. In *Brassica oleracea* pollen sample producing at least 70 pollen tubes in the style is considered to be fully viable (Ockenden,

1974). This is again a cumbersome method, and may not be suitable for other systems. The production of instant tubes, in the acid medium (Stanley and Linskens, 1974), is suitable only for a few systems having aperturate pollen.

A few other techniques based on demonstrating the activity of particular enzymes are also available. Peroxidase test (King, 1960) is based on the oxidation of benzidine by peroxidase in the presence of hydrogen peroxide. Tetrazolium test (Hauser and Morrison, 1964) is based on the reduction of soluble, colourless tetrazolium salt by succinic dehydrogenase into red or purplish insoluble formazan. Following the incubation of pollen in tetrazolium solution, able pollen becomes coloured. Although the utility of tetrazolium test has been demonstrated in a few systems, often does not show correlation with other tests. For example, over 95% of stored pollen of *Simmondsia* developed colour action in tetrazolium solution even from samples which is not show any germination (Beasley and Yermanos, 1976). Also, different tetrazolium salts show variation in the response of pollen samples. This test is very efficient for testing seed viability; its utility for testing pollen viability is so far been confined only to a limited number of taxa.

Another test standardized by Heslop-Harrison and Heslop-Harrison (1970) is based on the enzymatically induced fluorescence, which can be readily observed under a fluorescent microscope. When the pollen grains are immersed in fluorescein diacetate solution, the compound, being non-polar, readily enters the pollen cytoplasm. Fluorescein diacetate is hydrolyzed by the esterases; resulting fluorescein, being polar, does not pass through the plasma membrane. It accumulates in the cell and give bright fluorescence under the fluorescent microscope. |the test is based works well members of Gramineae and Compositae, and is also been used in testing viability of isolated protoplasts. Additional systems need to be tested before

it can become a generally test for pollen viability. It is important to emphasize that a given sample of pollen may not respond equally well to all the tests mention above, it is inevitable to try different tests for a give species, and to standardize the one which is responsive a consistent.

GERMINATON

Germination is the first critical morphogenetic event in the pollen ion fulfilling its ultimate function of discharging the male gametes in the embryo sac. It is important to understand the physiology and biochemistry of pollen germination. In vivo the stigma provides a suitable site for pollen germination. However, studies in vivo are not easily feasible because of the complications involving pistillate tissue. It is possible to germinate pollen grains of a large number of taxa using rather a simple nutrient medium, and to achieve a reasonable length of tube growth. Therefore, extensive studies have been made on cultured pollen grains. The present knowledge on the physiology and biochemistry of pollen germination and the tube growth comes largely from in vitro studies.

GERMINATION REQUIREMENTS

The role played by boron in pollen germination and tube growth is not clear. Many hypotheses have been put forward, based on circumstantial evidences, to implicate boron in carbohydrate translocation and/or metabolism. By forming ionizable sugar-borate complex (boron ions react with hydroxyl-rich compounds), boron may facilitate the entrance of sugars into the cell. In markedly stimulates carbohydrate metabolism. May evidences indicate that boron affects enzymes involved in carbohydrate biosynthesis.

Methlene-myoinositol, antagonis t of myo-inositol, inhibits pollen germination and tube elongation (Chen *et al.,* 1977). In ositol path way in which myo-inositol acts as an intermediate

in converting hexose to wall pentoses and uroids is an important route for the synthesis of wall polysaccharides. It is suggested that boron plays a role in pectin synthesis in germinating pollen by stimulating the conversion of myoinositol to wall polysaccharides (Stanley and Loewus, 1964).

Boron is reported to have a role in partitioning metabolism between the glycolytic and pentose-shunt pathways. Boron complexes with 6-phosphogluconic acid, the initiation substrate in the pentose-shunt pathway and inhibits the action of 6-phosphogluconate dehydrogenase. In the absence of boron, the enzyme operates in greater capacity producting additional erythrose 4-phosphate through pentose-shunt pathway, which is the initial substrate for the biosynthesis of complex phenolic compounds. Another hypothesis put forward is that boron counteracts the toxic effects of IAA (Bohnsack and Albert, 1977). Mc Leod (1975) tested the responses to tomato pollen to IA and boric acid over a wide range of concentrations. The toxicity of IAA at higher concentrations could not be overcome by higher levels of boron, indicating that boron has no role in IAA mediated responses.

By manipulation of carbohydrate concentration, boron and calcium, it has been possible to achieve satisfactory in vitro germination and tube growth in a large number of taxa having 2-celled pollen. It is rather difficult to achieve satisfactory results in 3-celled taxa. Even in those 3-celled taxa in which germination has been achieved, the tube length hardly exceeds 500µm (Johri and Shivanna, 1977).

Optimal conditions for achieving germination and tube growth have been standardized only for a few 3-celled taxa. In *Zea mays* a nutrient medium comprising sucrose (15%) + bactoagar (0.6%) + calcium nitrate (0.03%) +_ boric acid (0.01%) produces maximal germination and longest tubes in most of the genotypes, although different inbred lines show

variation in their optimal requirements for calcium and born (Pfahler, 1970). In *Chrysanthemum cinerariaefolium* sucrose (1.32 M) + boric acid (100 ppm) + calcium hydroxide (2mM) was the best medium for germination and tube growth (Hoekstra and Bruinsma, 1975). In this taxon liquid medium was better than semi-solid medium, and equilibration of pollen grains in humid air for 15 min at 30C before germination gave better results. High humidity and temperature at the time of pollen shedding, drastically reduced germination.

Ferrari and Wallace (1975) studied the details of pollen germination in different genotypes of *Brassica oleracea* in liquid media. On conventional medium (containing sucrose, boric acid and calcium chloride) pollen grains failed to germinate. Consistent pollen germination was obtained when purified polyethylene glycol (PEG is not known. None of the other compounds tested, such as yeast extract, amino acids, glycerol, and paraffin, promoted pollen germination. Although per cent pollen germination and tube growth, even in the presence of PEG, were limited in most of the genotypes tested (30% 500 µm), in one genotype the maximum germination of 84% and the tube length 3.5 mm was obtained.

There are a few 3-celled taxa such as *Pennisetum lyphoideum* (Vasil, 1960). *Brassica napus* (Kubo, 1960). *Asckeouas* (Gabil and Zeroni, 1969) and *Plumeria* (Shivanna, 1977) in which germination and tube growth are comparable to 2 – celled systems, on simple nutrient media.

RELEASE OF PROTEINS

It is known since long that pollen grains release proteins into the germinating medium. As early as 1894. Green observed that pollen grains of *Lilium longiflorum* could metabolize starch present in the medium. In *Oenothera* Stanely and Linskens (1965) analyzed proteins

(having absorption peak at 285 nm were apparent in the germinating medium within 6 min. culturing the pollen, although germination occurred only after 30 min. In none of the studies was the origin of these proteins analyzed.

Recent histochemical and immunofluorescent studies of J. Helsop-0Harrison and his associates have clearly shown that proteins diffusing into the medium are located in the pollen grain wall (Knox *et al.,* 1975). By depositing pollen grains on an agarose film for varying periods and staining the agarose film by protein stains (thus printing the proteins released on to the medium within a seconds of pollen hydration. The exine proteins are released first through the surface of pollen, and intine proteins are released later through germ pores.

These proteins are heterogeneous and include enzymes such as esterases and phosphatases, and many antigens responsible for pollen allergy. In *Cosmos bipinnatus* polyacrylamide gel electrophoresis of pollen wall diffuses after partial purification revealed more than 7 bands of proteins of which 2 were glycoproteins. Immunological analysis revealed the presence of 6 antigens. Although these proteins are thought to play an important role in pollen germination and tube growth, experiments studies have so far been confined only to a few taxa In *Pyrus communis* Stanley (1971) studies on the effect of elution of pollen (for 15 seconds, 1 to 4-times) on germination. Germination was not affected by one elution, but subsequent elution reduced germination significantly and by fourth elution the per cent germination was reduced from 54 (fresh pollen) to 90. However, in *Lilium longiflorum*, removal of pollen-wall proteins did not affect germination and tube growth, both in vitro and in vivo (Fett *et al.,* 1976). In *Crotalaria juncea* elution of pollen, 3-times successively, at 5 min interval (0C), did not affect in vitro germination and tube growth (Shivanna, unpublished). Apparently, there is a great deal of variation between taxa, in the nature of pollen-wall proteins and their role in germination and

tube growth. More important functions of pollen wall proteins appear to be in controlling the breeding behaviour of species, and this aspect is covered later under germination in vivo.

Recently, Tupy *et al.* (1974) provided evidences to show that, in cultured pollen grains of *Nicotiana tabacum,* RNA and even ribosomes are released into the medium. The amount of RNA rises rapidly in the medium, and remains constant after 2-8 hr. On the other hand, cytoplasmic RNA decreases steadily, and is practically absent after 16 hour of culture. Tupy *et al.* (1974) suggested that the loss in cytoplasmic RNA and ribosomes may be the cause for cessation of tube growth. The significance of the release of RNA and ribosomes is not clear. If the ribosomes retain protein-synthesizing activity, it may have an important implication in pollen tube nutrition and incompatibility reaction. Reports of the release of amino acids (Chen and Huang, 1975) and t-RNA (Tupy *et al.,* 1974) from the pollen tube suggest this possibility.

POLLEN TUBE GROWTH

Pollen germination and tube growth are generally divided into 4 phases; imbibition phase, lag phase, tube initiation phase, and rapid tube elongation phase (Linskens and Kroh, 1970). The time taken for different phases varies greatly, from species to species, depending on the type of reserve food material in the pollen and the external factors. Pollen grains of *Impatiens balsamina,* which mainly contain glucose, initiate tubes within 2-3 min. whereas though of *Lilium* which largely contain sucrose, require 30-40 min (Iwanami, 1959). In both taxa starch formation takes place in the pollen even in the absence of any metabolizable carbohydrate in the medium (in about 10 min in Impatient and 50 min in *Lilium*. According to Iwanami (1959) the sugar-starch equilibrium is important in maintaining the osmotic pressure of the pollen above that of the nutrient medium, enabling a continuous uptake of water for tube growth.

In *Lilium longiflorum* Dickinson (1965) has shown that pollen grains germinating in vitro show 3 phases of respiration (a) an initial high rate shown 10 to 30 min coincide with rapid starch formation, (b) a lower rate from 30 to 60 min coinciding with tube initiation and (c) a high rate from 60 to 120 min coinciding with tube elongation. There are evidences to indicate that the alternations in the turn-over of high energy Phosphates determine the changes in respiration patterns, such studies need not be extended to other systems to get a better understanding on the metabolism of pollen during germination and tube growth.

The growth of the pollen tube is unique in many respects it is exclusively confined to a few microns in the extreme tip. This growth-zone is rich in RNA, Proteins, and PAS tip positive material. Electron microscopic studies have shown that the tube-tip growing in vitro is covered with a compartmental cap. The growth-zone possesses numerous vesicles, presumably formed from the ends of dictyosome cisternae, and an elaborate network of smooth membrane. Cytoplasmic organelles are generally absent, and RNA resides in the smooth membrane. The vesicles coat or with one another and, eventually, contribute their membrane and contents to the compartmented cap. The cap and vesicles contain pectin. The cytoplasm behind the tips contains cells organelles and amyloplasts.

Earlier studies had indicated the lack of microtubules tip pollen tubes (Crang and Miles, 1969) However, present detailed ultrastructural studies of Franke *et al.* (1972) on pollen tubes of *Lilium* and *Clivia* have clearly demonstrated 3 types of longitudinally oriented structures in the growing pollen tubes, which could involve in cytoplasmic streaming and directional movement of cell wall materials. These are microtubules, microfilaments and endoplasmic reticulum cisternae. Colchicine (did not affect pollen germination, cytoplasmic streaming, or pollen tube growth. Cytochalasin B, which is believed to interfere with the function of microfilaments,

prevents streaming within minutes. Based on these studies, Franke et *al.* (1972) suggested that microtubules are not involved in cytoplasmic streaming and directional secretion of cell surface materials, but microfilaments may play a role in these processes. In *Tradescantia* also cytochalasin B inhibits pollen germination, tube growth, and cytoplasmic streaming.

Comparative studies carried out on the growth of cells characterized by overall surface growth, and of pollen tubes and root hairs characterized by tip growth, have shown that the two types of growth are independent phenomena with different chemical sensitivity (Sawhney served in other cells, therefore, cannot be interpolated to explain the growth of pollen tubes.

The factors responsible for the movement of generative cell and vegetative nucleus in the pollen tube are not clear. In many taxa the presence of fibrillar material in the pollen tube has been reported. In *Petunia hybrida* the fibrillar material, in which microfilaments are scernible, is confined near the vegetative nucleus and generative cell (Cresti *et al.*, 1976). It is suggested that these filaments represent local contractile centers which we responsible for the movement of generative cell and vegetative nucleus, in the pollen tube.

Germination is characterized by the conversion of quietent Golgi apparatus to an active, vesicle-producing form, and the formation of vacuoles in the cytoplasm (Larson, 1965). In the pollen tubes of *Petunia* x-ray diffraction studies have clearly demonstrated the presence of cellulose type of polymer in Golgi bodies and, thus, the involvement of Golgi bodies in the synthesis and transport of cellulose (Engels and Kreger, 1974). The tube wall is largely made up of cellulose and pectin. In Petunia cellulose microfibrils in the tube-tip are directed at random, but in older regions they are oriented in two directions at about 45 degrees to the main axis of the tube (Sassen, 1964). In *Lilium* the orientation of microfibrils in older region of the tube is similar to that in Petunia, but microfibrils were not visible in the growing region (Rosen, 1971).

Another feature of pollen tubes in the deposition of considerable amount of callose, a B-1, 3-glucose polymer. Callose is absent at the tip-, and forms a layer inside the tube-wall a little behind the tip. As the tube elongates, it also forms callose plugs transversely sealing off successively the older part of the pollen tube (having scanty cytoplasm) from the younger parts with dense cytoplasm). Ultrastructural details of callose deposition have been dense have been obtained to implicate rough endoplasmic reticulum in the formation and accumulation of callose in the pollen tube. Deposition of callose plug is initiated by the formation of numerous spherical structures, apparently loaded with callose. These bodies get appressed or fused resulting in callose plug. Callose plug deposition is accompanied by strong staining of plasma matric, presence of lipd bodies, absence of dictyosomes and plastids, and discontinuity in the plasma membranes, indicating dip organization and disintegration of the cytoplasm involved in plug formation.

In *Lilium*, although peripheral callose was present, of the cytochemical techniques revealed the presence transverse callose plugs (Reynolds and Dashek, 1976). Analysis of callose localization following various enzyme treatments spectrophoto fluorometric study has given additional evidence for the glycol-protein nature of callosae (Reynolds and& Dashek, 1976).

The presence of callose in the pollen tube has made it impossible to localize pollen tubes easily in the pistil, through presence microscope technique. Callose shows bright presence when the pistils are stained with decolorized aniline blue and observed under fluorescence microscope. However, Reynolds and Dashek 1976) reported variable results in localization of callose in *Lilium* pollen tubes, and doubted the validity of this technique as a general method of detecting callose. The feasibility of localizing callose in pollen tubes by tannic acid iron alum has also been shown (Reynolds, 1975).

Detailed electrophysiological studies have been carried out in growing pollen tubes of *Lilium* (Weisenseel and Jaffe, 1976). Growing pollen tube invariably drives a steady current (flow of positive charges) through itself. Before germination, the current enters through the germ pore and leaves the pollen through the opposite end. Following germination, it enters through most of the tube surface and leaves through the whole grain. The current continues to flow as long as the tube grows. Blockage of cytoplasmic streaming by cytochalasin B does not inhibit the flow of current.

Initiation and localization of growth at one point, found systems such as plant eggs, pollen grains and Jafee *et al.* (1974) put forward a hypothesis based on flow of current through the system. According to this accept, plasma membrane of the growth region, or the assumptive growth region, becomes relatively leaky to and cations such as Ca^{2+}, K^{+} and Na^{+}, resulting in the entry into the cytoplasm. Cement of this cation flux, through the resistance of the ctyoplasm will generate a positive field under the leaky part of membrane. This will tend to pull vesicles and cytoplasmic constituents with a negative surface charge towards the leaky membrane region. The movement create further leak and, thus, initiate and subsequently maintain localized growth. According to this concept self-electrophoresis as a result of the flow of microcurrent an important mechanism in controlling intracellular localization.

Subsequent studies (Weisenseel and Jafee, 1976) showed the requirement of K+ and Ca2+ ions in the medium for maintaining the flow of current through lily pollen tubes. Increase in K+ ions inhibited growth. Based on the detailed analysis of the data Weisenseel and Jafee (1966) concluded that the major growth current enters as K+ and leaves H+. A minor but controlling component of inward current consists of calcium ions. This probably explains the requirement of potassium and calcium in pollen germination and tube growth reported for many

systems. Reports of satisfactory tube growth in the absence of potassium is possibly due to the contamination of other chemicals with potassium (Weisenseel and Jaffe 1976).

Pollen grains accumulate proline in considerable amounts. The significant of proline in the biology of pollen is not clear. Proline converted into hydroxyproline by hydroxylation. Plant cell walls contain a structural glycoprotein, termed extension. Extension is rich in hydroxyproline and has been proposed as a regulator of cell wall extension. Hydroxyproline arises as a result of hydroxylation peptide -bound proline. Britikov *et al.* (1964) proposed proline is used by pollen in wall protein formation. Proline supplied exogenously is incorporated in the production of carbohydrates (Thomas and Dnyanasagar, 1975). The presence of proline dehydrogenase has also been rated in the pollen grains of crotalaria. It is suggested that proline is converted into gluconic acid which is further metabolized through the citric acid cycle. Thus, proline may also have a role in the proline of carbohydrates.

The germination of pollen is not followed by any sub initial increase in the volume of protoplasm. A large vacuole, initiated in the pollen cytoplasm, extends into the tube pushing most of the cytoplasm to the tip leaving only a thin lining in the older region of the tube. A great deal of pushing additional membrane has to be incorporated into the tonoplast, as well as the plasmalemma, to keep pace with the extension of the vacuole. The germination of pollen is a rapid process; in many taxa it occurs within a few minutes of hydration. The growth of tubes is also rapid. It is, therefore, unlikely that the pollen grain can activate the machinery for so rapid a synthesis of enough membranes of all materials.

The *Haemanthus katherinae* Sanger and Jackson (1971) considered a large number of electron- dense deposits associated with the tonoplasts of small vacuoles, and presumed and they are lipids providing precursors for the synthesis membrane following pollen germination. In

*Epidendru*m, Cocucci (1973) reported the presence of a large of small vacuoles exhibiting localized electron transport material attached to the membrane. Some of these dense aggregates were conducted to the intra-vacuolated membrane system organized in a concentric manner Cocucci (1973) suggested that the electron -dense bodies are the precursors of the intra-vacuolar concentric-membranous bodies (ICMB), and the ICMB represent the membrane reservoir for use during initial stages of pollen germination. The incorporation of ICMB with the tonoplast and plasma lemma, at certain places, and their extension, would provide a rapid method of surface enlargement of the membrane.

The pollen grains of Impatiens are studded with a large number of spherical vesicles (surrounded by unit membrane) showing electron -opacity similar to the intine. The fusion of these vesicles with the plasma membrane would provide additional membrane (similar to ICMB), and the contents of the vesicles should provide precursors for wall materials required of the cell rapid growth of pollen tubes. Such inactivators of membranes and wall materials required for reservoirs of membranes and wall materials/precursors probably present in other systems also, and utilized for the initial growth of tube. Synthesis of additional membranes and wall materials would, no doubt, occur during later stages of tube growth.

Malik and his associates have investigated the effect of various growth regulators on pollen germination and tube growth, and changes in amino acids, proteins, and enzymes associated with germination and tube growth. These studies have been reviewed by Malik *et al*. (1977). Involvement of phytochrome in pollen germination and tube growth has been reported in Arachis hypogea, and a few other taxa. Lectins have been shown to stimulate the germination of lily pollen (Southworth, 1975).

In *Tradescantia paludosa* (Malik *et al.*, 1976) and *Calotropis procera* (Balasimha and Tewari, 1977) cycling has been reported to promote pollen germination and a tube growth. Although the role of c-AMP in the metabolism of animals has been well established, its involvement of plant system is still controversial. It would be need to carry out detailed investigations on these lines, and to extend them to other taxa. If the involvement of c-AMP is established in the pollen system, it would open up new avenues in our understanding of the physiology of pollen.

CHEMOTROPISM

In the pistil pollen tubes grow through a predetermined path, and reach the embryo sac. It was generally believed that this directional growth in the pistil was in response to a chemotropic response in cultured pollen tubes (Rosen, 1961). None of these tests fully distinguish chemotropic effect from growth-stimulation- effect. Surface test in which pollen grains are arranged around the test material on an agar medium is the simplest one. The depression test in which the test substance and pollen grains are put in the wells dug out in the agar medium is probably more accurate.

In Antirrhinum majus, Mascarenhas and Machlis (1962), using depression test showed that calcium elicited strong chemotropic activity. They also found an increasing gradient in the distribution of total calcium in the pistil from stigma to the ovary, and suggested that calcium may be universal chemotropic factor in the pistils of angiosperms. However, calcium has no effect on chemotropic response of pollen tubes of *Lilium* (Rosen, 1964). Also, subsequent studies of Mascarenhas (1966) showed that the concentration of ionic calcium in the pistil of *A. majus* was almost constant throughout the length of style, and was slightly higher in the stigma, placenta and inner region of ovary wall. Consequently, neither there was any gradient in the

concentration of soluble calcium in the pistils, nor higher concentration of calcium in the ovule or the micropyle which is to be expected if calcium could not be the chemotropic substance even in Brewbaker (1971). These include dehydrogenases, oxidases, transferases, hydrolases, lyases, and ligases. Pollen grains generally lack enzymes associated with plastids and plant pigments. Zymograms of many of the enzymes from pollen pigments. Zymograms of many of the enzymes from pollen often reveal unique bands when compared to enzymes from other parts of the plant body. Although a few of the enzymes are reported to show increased activity following pollen germination (Brewbaker, 1971), it appears to be largely due to activation or release and not due to net synthesis.

Dickinson and his associates studied many enzymes involved in sugar and polysaccharide metabolism in *Lilium longiflorum* (Dickinson *et al.*, 1973). These studies have shown that all the enzymes involved in polysaccharide metabolism are present in ungerminated pollen, and no synthesis or activation of enzymes involved in nitrogen metabolism, such a nitrate reductase, proline dehydrogenase and glutamate synthetase have been reported (Kapur and Malik, 1976).

OBJECTIVES

The major objectives of this work are summarized as below:

♦ The present investigation carried out to compare different staining method to study pollen viability and i*n-vitro* pollen germination studies in selected members of plants in Thoothukudi District.

♦ Identifying coastal species, which are reproducing freely and are therefore most suitable for coastal reforestation and rehabilitation.

MATERIALS AND METHODS

Study site and plant species

Eight different plants namely *Datura metel, Solanum nigrum, Solanum trilobatum, Solanum surattense, Solanum melongena, Lycopersicum esculentum, Vinca rosea* and *Hibiscus rosa-sinensis* were selected for the study. Freshly opened male flowers were studied for their pollen viability and pollen germination test.

Datura metel L.

Synonym: *Datura alba* Nees.

Family: Solanaceae

Phytography: Coarse annual herb, may be 2 m tall, stem scented; leaf alternate, petiolate, simple; flowers axillary, peduncled, white or nearly so, 17.5 cm long and may be 12.5 cm in diameter across the mouth; capsules subglobose, 3 cm in diameter, equally spinous on all sides.

Phenology: Flowering: throughout the year. Fruiting: with blunt spines.

Distribution: Throughout India; occasionally grown in gardens.

Solanum nigrum

Synonym: Black nightshade

Family: Solanaceae

Phytography: Leaves are alternative, ovate and are carried on short stalks, 2-8cm long, and very between plants from smooth edged to shallowly lobed. Stems are round or angular 0.2-1.0m tall. Star shaped flower; Fruits are globular.

Phenology: Flower bud appearance; fruit of black nightshade.

Distribution: Throughout India.

Solanum trilobatum L.

Synonym: *Solanum canaranum*

Famaily: Solanaceae

Phytography: A slender prickly scrambling shrub, prickles curved, broad- based , yellowish and numerous along the stems , otherwise almost glabrous .Leaves rounded – ovate in outline , obtusely 3- lobed , 2-3 cm long, 1-4 cm wide , 3-9 flowered. Corolla 12- 16mm long.

Phenology: Flowers are purple, star- shaped flowers are arranged singly or in pairs in the leaf axils. Fruits are small, round berries are initially dark green, turning white and then purplish black at maturity.

Distribution: Throughout India.

Solanum surattense

Synonym: *Solanum virginianum*

Family: Solanaceae

Phenology: It is a pereninal herb. Both stem and leaves have dharp straight pickles also pubescent. Leaves pinnatified. Flowers distinct and deep blue in few flowered receme. Calyx lobes recurved

Phenology: Flowering peaks from December-March and from July -September.

Distribution: Throughout India

Solanum melongena

Synonym: *Solanum ovigerum*

Family: Solanaceae

Phytography: The plant may grow 2 to 4 feet tall and is multi- branched. The leaves and stems have star shaped hairs, and the small violet flowers are also star- shaped.

Phenology: The flowers are solitary, star shaped, and usually violet on color. The fruit is a large fleshy smooth berry. The fruit has many pale brown kidney shaped seed.

Distribution: Throughout India

Lycopersicum esculentum

Synonym: *Solanum lycoperiscum L.*

Family: Solanaceae

Phytography: Stem are light green to purplish green; more or less terete and glandular short pubescent. Alternate compound leaves occur along these stems that are widely spreading; they are 4-18 long and 2-6 across. These compound leaves are odd- pinnate with 3-5 pairs of lateral leaflets are smaller secondary leaflets. The petioles of the compound leaves are 1-4 long, light green to publish green, and glandular short- pubescent.

Phenology: Some flowers will fail to set fruits; they are self-fertile to some extent. **Distribution:** Throughout India

Vinca rosea

Synonym: *Catharanthus roseus*

Family: Apocynaceae

Phytography: *Catharanthus roseus* is an evergreen subshrub or herbaceous plant growing 1m tall. The leaves are oval to oblong, 2.5-9cm long and 1-3.5 cm wide, glossy green, hairless, with a pale midrib and a short petiole 1-1.8cm long. The flowers are white to dark pink with a darker red centre. With a basal tube 2.5-3cm.

Phenology: Flowering throughout the year; Fruiting throughout the year.

Distribution: Throughout India

Hibiscus rosa-sinensis

Synonym: *Hibiscus arnottii* Griff.

Family: Malvaceae

Phytography: Stem woody, branches densely or sparsely pubescent or tomentose with simple, stellate hairs or glabrous. Leaves simple, palmilobed or palmiparted, alternate, midrib usually with obscure.

Phenology: Flowering and fruiting; throughout the year

Distribution: Throughout India

Pollen viability tests

The literature was reviewed to determine the types of tests used in the past for pollen viability and to determine what problems had been reported for these tests (Dafni and Firmage, 2000) for a list of tests reviewed and the advantages and disadvantages of each test. Base on the literature and facility availability at laboratory condition, three staining methods for pollen viability experiment.

Iodine- potassium iodide Test (I2KI):

The technique described indicates viability and starch content of pollen grains. Iodine broke up in a watery arrangement of potassium iodide the tri-iodide-anion edifices with starch, creating blue black color.

Procedure:

1. Dissolve 1 g potassium iodide and 0.5 g iodine in distilled water to make a final volume of 100 mL.

2. Put 1 or 2 drops of the dye over pollen and mix thoroughly.

3. Place a cover slip and after 5-10min count the number of darkly stained (viable) pollen grains under the microscope.

Aceto-carmine test (2%):

Carmine show the presence of cytoplasm. The pollen nucleus is rich in chromatin material and viable pollen stains pink to deep red with acetocarmine, while sterile (mostly shriveled) pollen does not take any stain and thus remains almost white and transparent (Marutani, *et al.,* 1993).

Procedure:

1. Weigh 2 g of carmine powder, dissolve it in 95 mL of glacial acetic.

2. Add distilled water to make a total of 100ml solution.

3. Boil it, cool and filtered and stored in a refrigerator.

4. Two to three drop of stain was placed on slide and pollen grains were dusted on it followed by covered with coverslip and pollen viability was recorded after 5-10 min.

5. The dark red colored grains are counted as viable pollens.

Saffranin:

The pollen viability was obtained one hour following sowing in the safranin medium.

Procedure:

Safranin dissolved in 95% alcohol (40 mL) – 1 g

Distilled water - 100 mL

One part safranin was mixed with two parts glycerol and one part distilled water (1:2:1). To determine viability, about three hundred pollen grains of each replicate from four different areas were counted under a light microscope.

The percent pollen viability was calculated using formula

$$\text{Pollen Viability (\%)} = \frac{\text{Number of stained pollen grains}}{\text{Total number of pollen grains on slide}} \times 100$$

***In vitro* pollen germination test**:

In vitro pollen germination test were carried out using hanging drop method (Shivanna and Rangaswamy, 1992) with three different Brewbaker-Kwack germination media with media composition of 5 percent, 10 percent, 15 percent and 20 percent sucrose with boric acid 100 mg/L, 300 mg/L of Calcium Nitrate, 100 mg/L of potassium nitrate and a control treatment with 10 percent sucrose. The culture was maintained in a humidity chamber to prevent evaporation. The freshly opened male flower are collected between 7.30-8.00 A.M. were used for pollen viability and in *vitro pollen* germination studies. Germination of pollen from open male flowers was counted after incubation for 24 h at 25ºC on germination medium. Percent pollen germination was computed using formula given bellow.

$$\text{Pollen Germination (\%)} = \frac{\text{Number of germinated pollen grains}}{\text{Total number of pollen grains on slide}} \times 100$$

RESULTS

In the present study pollen viability was tested with acetocarmine, I2KI stain and safranin against pollen of *D. metel, S. nigrum, S. trilobatum, S. surattense, S. melangina, Lycopersicum esculentum, Vinca rosea* and *Hibiscus rosa-sinensis* (Table 1, Figure 1). Results of the current study reveals that *Datura metel* and *Vinca rosea* showed 100% viability of the tested pollen grains. Whereas acetocarmine stain showed higher percentage of pollen viability. Highest pollen germination was recorded in *S. nigrum, S. trilobatum* and *S. surattense.* Lowest pollen viability was recorded in the pollen of *Hibiscus rosa-sinensis.*

Invitro pollen germination using different concentration of sucrose for 24 hours reveals varying degree of pollen germination. Pollen germination was 100% in *Datura metel* and *Vinca rosea.* Among the four different concentrations tested maximum pollen growth was observed in 10% concentration of sucrose (Table 2, Figure 2). Either increase or decrease in the concentration of sucrose has resulted in decline in pollen germination percentage.

Pollen released during early hours (4 hour after anthesis) had the highest viability (100%). Pollen viability gradually decreased after early hours and after flower opening, pollen was no longer viable. There was significant difference in pollen germination among plants. Pollen germination began within 20 minutes of being placed in Brewbaker's solution. Pollen-tube growth was fast and increased significantly each hour from 0 to 8 h. Within the first 2 h, the average rate of tube growth was about 160 μm h^{-1}. During the 3 to 4 hour period it was 280 μm h^{-1}. Overall, the rate of tube growth within the first 8 hour varied

from 60 to 280 μm h^{-1} with an average of 140 μm h^{-1}. After 8 h, there was no significant

increase in pollen tubegrowth. The rate of pollen-tube growth varied among plants.

Of the 8 flowers studied for pollen germination 8 to 24 h after pollination, the number of flowers in which pollen tubes germinated varied with species. Studies on *in vitro* pollen germination at different time intervals after anthesis indicated that 100% germinating pollen with a mean of 101 µm long pollen tube development was observed in 10% sucrose solution. The maximum 96% pollen germination along with 1105 µm long pollen tube developed after 3 hours in 10% sucrose solutionsupplemented with boric acid.

DISCUSSION

In the present study the three staining methods, acetocarmine, I2KI stain and safranin methods could easily differentiate the viable and non-viable pollen grains. Results of the present study confirms 100% viability of pollen in *Vinca rosea* using all the three tested stains. Whereas in *Datura metel* 100% viability was recorded using acetocarmine and safranin stain. The least viability has been recorded in *Hibiscus rosa-sinensis* using all the three tested stains. The viability percentage was less using I2KI stain. The loss of viability is usually attributed to the deficiency of metabolites, as the metabolic activity in the pollen would continue even after shedding. Stored pollen grain normally require a higher sugar concentration for germination in vitro, than fresh pollen grains.

An analysis of viable and non-viable pollen of 4 species of *Pinus*, stored for 15 years, showed that low molecular weight sugars and organic acids were invariably higher in viable pollen than in non-viable pollen (Stanley and Poostachi, 1962). Substantial decrease in some of the vitamins following storage has also been reported in many taxa (Nielsen, 1956). Studies of Linskens and Pfahler (1973), on the quantitative changes of free amino acids following storage, in pollen grains of *Zea mays*, have shown significant changes in all the 16 amino acids investigated. Some of the amino acids such as aspartic acid, isoleucine, leucine, phenylalanine, ethanolamine, and aminobutyric acid showed consistent increase, and some other such as glutamic acid, proline, glycine, and alanine showed consistent decrease following storage.

The most commonly used test for pollen viability is in vitro germination test. A major limitation of this test has been the difficulty of achieving suitable germination in many taxa, particularly in 3 celled systems. Moreover, the medium which is optimal for fresh pollen may not be optimal for stored pollen. Hence, the differences obtained in percentage germination between

fresh and stored pollen may not indicate true viability, as many of the stored pollen, which fall to germinate in optimal medium, may germinate on the stigma. Nonetheless, this is more dependable than other techniques.

Pollen grains of only a few taxa germinate in distilled water; they generally require a carbohydrate source. Sucrose is the most commonly used carbohydrate, although other forms such as glucose, fructose, raffinose are effect give in many taxa (Hrabetova and Tuppy, 1964). It is presumed that sugars incorporated in the germinating medium serve two functions; (a) in maintaining osmotic pressure, and (b) as a substrate for the metabolism of pollen. Incorporation of exogenously supplied sugars in the metabolism of pollen has been demonstrated by using labelled compounds (Stanley and Linskens, 1964). Optimum concentration of sucrose varies from species to species and while 2 – celled pollens require 10-20%, 3-celled pollens require up to 60%.

Besides carbohydrates, two other important substances, which are usually required for pollen germination and tube growth, are boron and calcium. Stimulation of pollen tube growth by boron was reported as early as 1933 by Schmueker in *Nymphea*. Since, then its stimulatory role has been demonstrated in many taxa. Boron (10-150 ppm) is now routinely incorporated in the medium; boric acid is generally used as the boron source. Many other boron compounds such as butyl borate, borax, potassium tetraborate are also reported to be effective (Stanely and Lichtenberg, 1963). In the absence of boron pollen grains show poor germination, and a high proportion of bursting. In *Yucca aloifolia* incorporation of boron in the medium not only improved pollen germination but also led to optimal pollen germination over a wide range of sugar concentrations (Portnoi and Horovitz, 1977). Further, fructose, which could not induce pollen germination, when used along with boron was as effective as glucose or sucrose in

achieving pollen germination. Pollen grains are considered to be deficieted in boron, which is compensated by the high level of boron present in the stigma and style.

Calcium is another inorganic substance which has a remarkable effect on pollen tube growth. The effect of calcium was discovered as a result of studies concerning the crowding effect of pollen grains. As early as 1924, Brink showed that culturing of pollen grains in larger populations resulted in better germination and tube growth as of smaller populations. The crowding effect termed population effect was shown to be a general phenomenon in the pollen of many taxa. It was thought that this effect is due to the release of some substances, required for pollen germination and tube growth, from the pollen grains into the medium, the effective concentration of which is attained only when pollen grains are present in larger numbers. Brewbaker and Majumder (1961) reported that the population effect was due to a heat stable, water soluble, and highly diffusible substance termed pollen growth factor (PGF). Subsequently, Brewbaker and Kwack (1964) identified PGF to be calcium ion, and demonstrated that calcium could replace PGF. Pottasium and magnesium ions enhanced the effect of calcium ions. Based on their studies, Brewbaker and Kwack (1964) formulated a medium which was suitable for pollen germination and tube growth of 86 species they tested. The medium comprises sucrose (10%) + boric acid (100 ppm) + calcium nitrate (300 ppm) potassium nitrate (100 ppm). However, pollen grains of many taxa do not require calcium for germination and tube growth. This may be due to the presence of higher levels of endogenous calcium in the pollen of these taxa. In *Juglans nigra* although pollen grain showed population effect, calcium could not overcome (Hall and Farmer, 1971).

The mechanism of action of calcium is also not understood. It is thought that calcium binds pectic carboxyl groups in the tube wall giving rigidity to pectin (Kwack, 1967). Studies of

Dickinson (1967) have shown that calcium plays a role in controlling the permeability of pollen medium results in an increase in the membrane permeability leading to the loss of internal metabolites. In *Lilium* calcium has been shown to accumulate in the growing tips of pollen tubes (Jaffe *et al.,* 1975). This accumulation appears to be a result of faster entry of calcium at the tip than the other regions, and is probably related to the localized secretion of vesicles at the tip. K+ and Ca2+ions were shown to be essential for the steady flow of current through *Lilium* pollen tubes, which is a normal feature of growing tubes (Weisenseel and Jaffe, 1976). Thus, the role of calcium and potassium may be in maintaining the flow of current through the pollen tubes. Other systems need to be investigated on these lines.

Besides the carbohydrate source, boron and calcium, the effect of many other substances – auxins, gibberellins, cytokinins, amino acids, vitamins, steroids, fungicides, insecticides and pesticides – has been tested on pollen germination and the tube growth. Except in a few systems, the effect of these chemicals is not pronounced. Their effect is highly variable and, often, affects germination differently from tube growth (Johri *et al.,* 1977). As with any other physiological process, temperature and PH also affect pollen germination and tube growth. The optimal range of PH and temperature varies from species to species.

Although pollen viability had not previously been correlated with the type of pollination, Ottaviano and Mulcahy (1989) observed that pollen must be programmed

to have high viability in the habitat of the species. Previous studies correlated the decrease in viability with the stage of development of the male gametophyte, binucleate pollen generally surviving longer than trinucleate (Stanley and Linskens, 1974). Among the latter, however, at least in the Graminaceae, some differences had been noted (Chaudhury and Shivanna, 1986).

The pollen grain withstands changes in volume due to variations in water content, relative humidity and temperature by virtue of harmomegathy (Lisci *et al*., 1994). Pollen resistance to dehydration is thought to be due to cytoplasmic disaccharides and oligosaccharides that act as membrane stabilizers (Speranza *et al.,* 1997). *Cucurbita pepo,* the species with the shortest pollen viability of those considered here, has none of these carbohydrates, unlike *Mercurialis annua* (Speranza *et al*., 1997). Another facet of pollen dehydration/ rehydration is plasma membrane structure; this aspect has only been investigated in angiosperms. Shivanna and Heslop-Harrison (1981) suggested that in the partly dehydrated grain at the time of dispersal the membranes are largely dissociated and do not form an osmotic barrier. Barrow (1981) suggested that some time staining techniques could not discriminate between aborted and non-aborted pollen grains, hence *in vitro* pollen germination study has to follow.

In bitter gourd, Saoji (1975) reported *in vitro* pollen germination media with sucrose @10 % was best for pollen germination studies, however in or study sucrose @15 % + Boric acid @0.25% + Calcium nitrite @300mg found to be superior. Off the staining methods tested, none was very satisfactory as accusatory predictor of the in vitro pollen germination studied (Rathod *et al.,* 2018). The pollen germination results are not accurately resemble to pollen viability tested with different method, hence different staining methods could be useful for estimation of pollen viability accomplished with in vitro pollen germination. Similar observation was reported by Sedgley *et al*., (1993) in *Acacia*.

Current investigation reveals that pollen grains of *Vinca rosea* exhibited higher pollen germination percentage with maximum pollen tube growth. Whereas *Datura metel* also exhibited 100% germination with pollen tube growth. Sucrose at 10% concentration promoted the pollen germination and growth of pollen tubes. Sucrose in combination with boric acid

promoted pollen germination as well as tube development, because boron makes a complex with sugar and this sugar-borate complex is known tobe capable of better translocation than non- borate, non-ionized sugar molecules (Sidhu and Malik, 1986). *In vitro* germination measures pollen germinability under the specific conditions of the medium and temperature conditions reveals the state of the reserves, the condition of the membranes and the sub sequent rate of reserve conversion (Heslop- Harrison *et al*. 1984).

Shivanna and Johri (1989) stated that the externally supplied sucrose maintains the osmotic pressure and acts as a substrate for pollen metabolism. The role of boron has been confirmed in germinating pollen and growing pollen tubes in vascular plants (Sidhu and Malik, 1986). The studies of Stanley and Loewus (1964) indicated that boron is directly involved in pectin synthesis and thus indirectly involved in development of pollen tube membrane. Scott (1960) suggested that boron could exert a protective effect in preventing excessive polymerization of sugars at sitesof sugar metabolism. In nature water, sugar and amino acids are supplied by the style to nourish the growing pollen tubes. Boron is also provided by stigmas and styles and facilitates sugar uptake and play a vital role in pectin production in the pollen tubes (Richards, 1986). Boric acid is known to be crucial for pollen germination and tube growth and it is required at concentration of 100 ppm for most species (Brewbaker and Majumder, 1961).

Environmental factors and especially desiccation risks are considered a main selective force leading to better protection ofthe pollen grain and from the evolutionary ecology view-point, the possible relation between pollen longevity and pollination chances, pollen competition, and breeding system is noteworthy. Even if pollen is delivered successfully into the properreceptive stigma, there is no guarantee that itis still viable and one may point out

thatpollen longevity on the vector body even at the right location to meet the stigma may also be a crucial factor in pollination efficiency (Dafni and Firmage 2000).

The pronounced effect of sucrose and boric acid on germinating pollen might be reflected with the views of Johri and Vasil (1961). The induced role of Calcium andboron on *in vitro* pollen germination wasreported by Brewbaker and Kwack (1964). The role of boron in flowering and fruiting process has been established (Brown *et al.,* 1994) and its deficiency resulted in low pollen viability, poor pollen germination and reduced pollen tube growth (Nyomora and Brown, 1997). Boron takes part in pollengermination and style tube formation andtherefore has a vital function in fertilization of flowering crops. Boron added in the form of boric acid, is also essential for the *in vitro*culturing of pollen from most species and it is also reported that elimination of boric acid from the culture medium often leads to tube bursting (Acar *et al*., 2010).Wang *et al.*, (2003) studied the effect of boron on thelocalization of pectins and callose in the wall of pollen tubes in *Picea meyeri*. Acar *et al*. (2010) also reported the stimulatory effect of boron on *in vitro* pollen germination of *Pistacia vera*.

Salts of Calcium Nitrate, Potassium Nitrate and Magnesium Sulphate were used to study the effect of Ca, K, and Mg ions on *in vitro* pollen germination. The role of all the salts were well marked where Calcium Nitratewas most effective. The results also indicate that Calcium ion was the effective to influence the pollen germination. Calcium isone of the most important Cations involved in cell metabolism. It is also known to be important in maintaining membrane integrity and permeability (Brewbaker and kwack 1964). According to Kwack (1967) Calcium propabaly gives rigidity to the pollen tube wall by binding pectic carboxyl groups and also induced pollen germinations. Miller *et al.* (1992) demonstrated that calcium concentration play a critical role in maintaining the tube growth.

According to Brewbaker and Kwack (1964) Magnesium ions enhance the effect of Calcium ions result in the growth of pollen tube. The role of K^+ was established in pollen germination and tube elongation in *Arabidopsis* and Both the Ca^{++} and K^+ are interdependent on each other because the inward K^+ channel are greatly regulated by Ca^{++} while the external supply of K^+ also enhanced the rate of pollen germination as well as pollen tube growth in *Arabidopsis* (Fan *et al,* 2001). Choudhury *et al* (2013) studied the role of sucrose, boric acid and difference salt like Calcium nitrate, Potassium nitrate and Magnesium sulphate on *in vitro* pollen germination. Thus, the present findings corroborate the findings of Olaymi *et al*. (2011).

SUMMARY AND CONCLUSION

Eight different plants from Thoothukudi District like *Datura metel, Solanum nigrum, Solanum trilobatum, Solanum surattense, Solanum melongena, Lycopersicum esculentum, Vinca rosea* and *Hibiscus rosa-sinensis* were selected for the study. Freshly opened male flowers were studied for their pollen viability and pollen germination percentage.

Results of the current study reveals that pollen germination was 100% in *Vinca rosea* followed by *Datura metel.* Maximum pollen viability was recorded with all the tested pollen except *Hibiscus rosa-sinensis*. Moreover the pollen viability percentage observed was high with acetocarmine followed by safranin whereas potassium iodide stain showed poor pollen viability percentage. As a result, these stain tests may be used to determine pollen viability in these species to provide only a rough estimate of viability. However, the exact amount of viable pollen may be determined in vitro by pollen germination.

Pollen germination studies reveal that 100% pollen germination was observed with *Datura metel* and *Vinca rosea* in 10% sucrose concentration. Best growth of pollen was recored in 10% concentration of sucrose. The decrease or increase in sucrose concentration has led to the decline in the germination percentage of pollen which clearly indicates that the concentration of sucrose affects pollen germination. Moreover most of the members of the family Solanaceae showed poor germination of pollen also poor germination of pollen was recorded in *Hibiscus rosa-sinensis.*

For a long time, pollen germination, tube elongation and double fertilization have been fascinating research topics for plant biologists. Reception or rejection of pollen by the female stigma is an evolutionary strategy that plants have adapted to ensure successful sexual reproduction, species preservation and biodiversity generation. Despite immense efforts during

the past few decades to understand the genes involved in these processes, considerable information is still needed to elucidate the full molecular mechanisms of fertilization, aspects that are crucial for plant breeding. However, the availability of the advanced tools and resources opens a new era for investigation of the genes and mechanisms regulating pollen germination and tube growth. A good understanding of these processes provides critical information that will contribute greatly to plant breeding and will catalyse further translational innovations within the life sciences.

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PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL ACTIVITY OF *RICINUS COMMUNIS*

A Short Term Project Work Submitted to St. Mary's college (Autonomous) affiliated to Manonmaniam Sundaranar University in Partial Fulfillment for the Degree of

BACHELOR OF SCIENCE IN BOTANY

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THOOTHUKUDI-628 001

2021 - 2022

CERTIFICATE

It is certified that this short term project work entitled **"PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL ACTIVITYOF *RICINUS COMMUNIS*"** submitted to **St. Mary's College (Autonomous)** affiliated to **Manonmaniam Sundaranar University** in partial fulfillment of the requirements for the degree of **Bachelor of Science in Botany** and is a record of work done in the Department of Botany, St. Mary's College (Autonomous), Thoothukudi during the year 2021 - 2022 by the following students.

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CONTENT

INTRODUCTION

Plants are considered as God's gift to human beings in various forms, especially medicinal plants have a vital role to preserve the human life. In the last few decades there has been an exponential growth in the field of herbal medicines. The food, clothes and shelter are three basic necessity of human beings and an important one necessity is good health, which provided by plant kingdom. Plant kingdoms are the rich source of organic compounds, many of which have been used for medicinal purposes. It is well-documented in the scientific literature that plants have been used for medicinal purposes for the past 5000 years ago (Solecki, 1975). Still today, millions of people around the world depend on medicinal plants for their well-being (WHO, 2002). In the tropics, medicinal plants are often used on a regular basis in rural communities where pharmaceuticals are hard to obtain or even unavailable. This is in contrast to westernized societies where medicinal plants are typically used as an alternative or supplement to prescribed medicine (WHO, 2002). Medicinal plants are important for people, not only as a primary source of medicines but also as phytochemical building blocks for development of new drugs (Fabricant and Farnsworth, 2001). It is estimated that 67% of drugs used in chemotherapy are derived from natural products (Wangkheirakpam, 2018). This applies to the discoveries of active compounds such as vincristine (Raviña, 2011), taxol (Fischer *et al.,* 2010), and artemisinin (Tu, 2011). Moreover, medicinal plants also offer an opportunity for rural dwellers to generate a cash income (EL-Hilaly *et al.*, 2003).

Ethanobotany has been deeply rooted in the Indian tradition and culture. Many medicines and their formulations have been based on rich ayurveda of ancient India. Many plants have been utilized to cure uncommon disorders. The large family Euphorbiaceae contains nearly about 300 genera and 7,500 species. Generally they are the flowering plants. In traditional medicine, there are many natural crude drugs that have the potential to treat many disease like fever, burning

sensation and ulcer. Among all, *Riccinus communis* L or castor bean plant has high traditional and medicinal value towards a disease free community like anticancer, antioxidant and antidiabetic It belongs to the family Euphorbiaceae which is popularly known as 'castor plant' and commonly known as 'palm of Christ'. The plant is widespread throughout tropical regions as ornamental plants is widespread throughout tropical regions as ornamental plants. This plant is cultivated for leaves, flowers or oil production and it grows wild in waste places. It is also used as a lubricant, lamp fuel, a component of cosmetics, and in the manufacture of soaps, printer's ink, plastics, fibers, hydraulic fluid, brake fluid, varnishes, paints, embalming fluid, textile dyes, leather finishes, adhesives, waxes, and fungicides. In the Indian system of medicine, the leaf, root and seed oil of this plant have been used for the treatment of inflammation and liver disorders. Traditionally this plant is used as laxative, purgative, fertilizer and fungicide etc. whereas the plant possess beneficial effects such as anti-oxidant, antihistamic, antinociceptive, antiasthmatic, antiulcer, immune modulatory, antidiabetic, hepato protective, antifertility, anti inflammatory, antimicrobial, central nervous system stimulant, lipolytic, wound healing, insecticidal and larvicidal and many other medicinal properties. This activity of the plant possess due to the important phytochemical constituents like flavonoids, saponins, glycosides, alkaloids and steroids etc.

The plant has been found to be useful hepatoprotective, antifilarial, antioxidant, antiasthmatic and antimicrobial activities. The leaves are used for skin diseases and kidney; urinary bladder infections. The root of this plant is also useful as an ingredient of various prescriptions for nervous diseases and rheumatic affections such as lumbago, pleurodynia and sciatica. Roots of this plant showed anti-inflammatory and free radical scavenging, anti-fertility, anti-diabetic, and antimicrobial properties.

The seeds of *Ricinus communis* have several traditional applications. They are used with arguable success in the treatment of warts, cold tumors' indurations of the mammary glands,

corns and moles. Further more the toxic organic substance present in the plant. These compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, phenol compounds, flavonoids resins, fatty acids and gums, which are capable of producing definite physiological action on body. *Ricinus communis* is used medicinally in different countries and are a source of many potent and powerful drugs. The seed contains 46-60 % oil and the only commercial source of ricinoleic acid. The oil is used as industrial lubricants, paints, coatings and plastics. According to WHO (1993), 80% of the world's population is dependent on the traditional medicine and major part of the traditional therapies involves the use of plant extracts or their active constituents. With the continuous use of antibiotics, microorganisms have become resistant. This has created immense clinical problem in the treatment of infectious diseases. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of various infectious diseases. However, it is essential to work on locally available resource to bring out their pharmaceutical values and antimicrobial in properties. Hence the present investigation was undertaken with following objectives

- To identify the phytochemicals present in the different parts of *Riccinus communis*
- The effectiveness of medicinal plants in human pathogenic bacteria such as *Bacillus cereus, Escherichia coli* and *Staphylococcus aureus*
- The phytochemical screening in different plant parts (leaf, stem, root and seeds) of *Riccinus communis* using various solvents (ethanol, acetone, chloroform, benzene, aqueous).
- It can be concluded that presence of various pharmacological compounds in plant samples using FT - IR analysis.
- The result of the study also emphasize the cost effective, bio friendly resources which would be tapped for development of effective drug in future

REVIEW OF LITERATURE

In the last few decades there has been an exponential growth in the filed of herbal medicine. Medicinal plants have a vital role to preserve the human healthy life. It is truth that without nature human being life is not possible. The food, clothes and shelter are three basic necessity of human beings and an important one necessity is good health, which provided by plant kingdom. Plant kingdoms are the rich source of organic compounds, many of which have been used for medicinal purposes. (Trease, G.F and Evans2002). All parts of plants are important viz Root bark, leaves, flowers, additional and medicinal value for maintain the disease free life. Traditionally the plant is used as laxative, purgative, fertilizer and fungicide etc. (S E Princea 2011).

The large family of Euphorbiaceae contains nearly about 300 genera and 7,500 species. This plant is widespread throughout the tropical regions as an ornamental plant (Maman *et al.*,2015). Amongst all, the *Ricinus communis* or castor plant has high traditional and medicinal value for maintain the disease free healthy life. This plants was cultivated for leaf and flower colors and for oil production. Leaves are green or reddish in colour and about 30-60 cm in diameter. The leaves contain 5-12 deep lobes with coarsely toothed segments which are alternate and palmate. The stems are varying in pigmentation. The flowers are monoecious and about 30-60 cm. long. The fruit is a three-celled thorny capsule. The capsule of fruit covered with soft spins like processes and dehiscing in to three 2-valved cocci. The seeds are considerable differences in size and colour. They are oval, somewhat compressed, 8-18 mm long and 4-12 mm broad. The testa is very smooth, thin and brittle. Castor seeds have a warty appendage called the caruncle, which present usually at one end from which runs the

raphe to terminate in a slightly raised chalaza at the opposite end of the seed (Kadri *et al.*,2011).

Traditionally the plant is used as laxative, purgative, fertilizer and fungicide etc. whereas the plant possess beneficial effects such as anti-oxidant, antihistamic, Antinociceptive, antiasthmatic, antiulcer, immunemodulatory, Antidiabetic, hepatoprotective, Antifertility, anti inflammatory, antimicrobial, central nervous system stimulant, lipolytic, wound healing, insecticidal and Larvicidal and many other medicinal properties (Encyclopedia Britanica 2000). This activity of the plant possess due to the important phytochemical constituents like flavonoids, saponins, glycosides, alkaloids and steroids etc.

The castor oil obtained from the seed of the plant is still widely used traditionally and herbally as a medicine. The seed of the plant is used as fertilizer after the oil was extracted cooked to destroy the toxin and incorporated into animal feeds. The principal use of castor oil is as a purgative and laxative (Singh Ramesh Kumar 2010). Ricinus *communis* or castor plant is a widely traditionally used and potent medicinal plant amongst all the thousands of medicinal plants. The pharmacological activities reported in the present review confirm that the therapeutic value of *Ricinus communis* is much more.(Isharma S., Singh T. and Vijayvergia R. 2009). It is important source of compounds with theirs chemical structures as well as pharmacological properties. The presence of phytochemical constituents and pharmacological activities proved that the plant has a leading capacity for the development of new good efficacy drugs in future. (Bandaranayake W.M. 2006).

Lin *et al.,* (1972) found that, *R.communis* have the strong protective effect of abrin and ricin against Ehrlich ascites tumor cells in mice and some certain protective effect against leukemia was also reported. In preliminary studies,

the toxins have also been used in the treatment of certain forms of human cancers. The castor beans are known for their high toxicity for centuries. In ancient times, farmers knew to keep their livestock away from the castor plant or else they would risk losing them. Their seeds have been also used in folk medicine against a wide variety of diseases (David *et al.*, 2007). Castor oil is still produced in large quantities throughout the world and the toxin which remains in the castor meal after the oil has been extracted with hexane or carbon tetrachloride is easily removed through a simple salting-out procedure (David *et al.*, 2007).

The commercial production of oil is generally processed in a number of ways and then used for different purposes. The treated oil can also be used as paints, enamels and varnishes, oiled fabrics, linoleum, patent leather, fly-paper, typewriting and printing inks, greases and special lubricants. The leaves have also been recommended in the form of a decoction or poultice and as an application to the breasts of women to increase the secretion of milk (Bentley *et al.*, 2007).

The powdered leaves are used for repelling aphids, mosquitoes, white flies and rust mites. Leaves are used in the form of a poultice or fomentation on sores, boils and swellings. Oil derived from the leaves is commonly applied over the abdomen to give relief in the flatulence in the children (The Wealth of India, 1972).

Chouhan et al., (2021) reported *R. communis* leaf shown the presence of major phenolic compounds. Whereas roots test has presented the Indole-3-acetic. This findings reflect that, the plant contains high medicinal benefits, which offer a solution for several kinds of diseases like cancer, diabetes, ulcer and anthelmintic, etc. Also, the plant is anti-inflammatory property of the plant facilitates therapeutic use. Therefore,

all parts of the medicinal plant *Ricinus communis* are considered highly beneficial in the medicinal field (Govindasamy C and Kannan R 2012).

ANTIBACTERIAL ACTIVITY

Rabia Naz *et al.,* (2012) have done the Antimicrobial potential of *Ricinus communis* leaf extracts in different solvents against pathogenic bacterial and fungal strains Methanol leaf extracts were found to be more active against Gram positive bacteria (Bacillus subtilis: ATCC 6059 and Staphylococcus aureus: ATCC 6538) as well as Gram negative bacteria (Pseudomonas aeruginosa: ATCC 7221 and Klebsiella pneumoniae) than ethanol and aqueous leaf extracts. Antifungal activity of methanol and aqueous leaf extracts were also carried out against selected fungal strains as Aspergillus fumigatus and Aspergillus flavus. Methanolic as well as aqueous leaf extracts of *Ricinus communis* were effective in inhibiting the fungal growth.

X Siwe Noundou *et al.,* 2014 have done the Antibacterial activity of the roots, stems and leaves of Alchornea floribunda. Generally, the ethanol (EtOH), methanol (MeOH), ethyl acetate (EtOAc) and chloroform (CHCl3) extracts demonstrated the best activities, with the leaves exhibiting the highest average activity for six of the eight pathogens. Of these, the ethanolic leaf extract was the most active against Staphylococcus aureus with an MIC value of 50µg/mL. Some other notable activity was observed for the ethyl acetate and chloroform root extracts against Staphylococcus aureus (50µg/mL), and for selected stem extracts against Staphylococcus aureus (50µg/mL), Klebsiella pneumoniae (63µg/mL) and Staphylococcus saprophyticus (63µg/mL).

Jennifer Suurbaar *et al.*, 2017 have done the Antibacterial and antifungal activities and phytochemical profile of leaf extract from different extractants of *Ricinus*

communis against selected pathogens. The aqueous, methanol and ethanol extracts were shown to contain most of the phytochemicals analyzed. All solvents extracts exhibited inhibitory activity against the growth of all microorganisms. The methanol extract showed highest zones of inhibition and was found to be statistically significant ($P < 0.05$) compared to other solvents extracts. All solvents extracts exhibited both bacteriostatic and bactericidal effects on the test organisms at varying concentration, with MIC values ranging from 3.13 to 25.0 mg/ml and MBCs were from 200 to 400 mg/ml. MFCs of Candida albicans was between 200 and 400 mg/l. Our data confirm the anti-bacterial and anti-fungal properties of *R. communis* and showed that the biologically relevant phytochemicals from the leaves of this plant can be extracted with the solvents aqueous, methanol and ethanol.

Asaad Khalid *et al.*, 2022 have done the The antibacterial activity of all three plant extracts was tested against three standard Gram-positive, four standard Gram-negative, and two clinical bacterial strains. Among the extracts examined, *R. communis* plant extract was most effective against various isolated bacteria. This study, interestingly, sheds light on the bioactive components found in plant extracts that can be utilized for cytotoxic and antibacterial purposes.

MATERIALS AND METHODS

Morphology of *Ricinus communis L*

SYSTAMATIC POTISION

Class	:	Dicotyledons
Order	:	Euphorbiales
Family	:	Euphorbiaceae
Genues	:	Ricinus
Species	:	communis

Description

Ricinus communis is a robust shrub or small tree with a softly, woody stem,growing up to 4m high.The stem are smooth,round,and frequently red, with clear sap.The leaves are simple and alternate.Long purple leaf stalks are attached near the centers of the leaf blades. These are large,star-shaped with sharply -toothed margins;there colour is shiny dark green or reddish and they are paler below; they have an odour when crushed .The flowers are clustered in terminal spikes with creamy male flowers 3-5 in the lower position and reddish females flowers 1-7 at

the top of the inflorescence The fruit is a spiny,greenish to reddish purple three-lobed capsule containing large,oval,shiny,bean-like,highly poisonous seed with variable brownish mottling . the whole plant is poisonous

Collection and processing of materials

The various part of *Ricinus communis* were collected from in and around Thoothukudi district in Tamilnadu during February 2022. The collected samples were washed carefully with water to remove dust and foreign materials. The samples were dried under shade dried and coarsely powered using blender. The final uniform powder was used for extraction of active constituents of the plant materials.

Preparation of solvent apparatus extracts – check the solvent what u used

10 gm power sample (leaf, stem, root, seed) was sequentially extracted with 200 ml of Ethanol, Methanol, Chloroform, Petroleum ether, Aqueous solution using in soxhlet apparatus. The prepared extract was used for further analysis.

Phytochemical Analysis

The phytochemical tests were done for analysing different chemical groups present in the extracts. These were done find out the presence of bioactive chemical constituents such as Alkaloids, Indole alkaloid , Phenol, Flavonoids, Terpenoids, Cardiac glycosides, Quinone, Steriode, Tannins, Saponins.(methanol, ethanol, petroleum ether, chloroform, aqueous).

Preliminary Phytochemical Analysis

Alkaloid

A few ml of test solution in acetic acid was treated with 2 drops of dragen droff reagent red or orange precipitation indicated the presence of alkaloid excess reagent was avoided.

Indole Alkaloids

Methanolic extract add conc. H_2SO_4 add potassium dichromate was taken in flask. Colour change is confirmation for the presence of indole alkaloids

Phenols

Fecl$_3$ Test

About 2ml of plant extra was taken and warmed at 45 to 50℃. Then 2ml of .3% $Fecl_3$ was added. Formation of green or blue colour indicate the present of phenols.

Flavonoids

Aqueous extract was added in 10% Ferric chloride. A green precipitate indicates positive test. Secondly methanol extract add 10% NaOH add dil. HCl were added in test tube. Yellow solution turned colourless on addition of dil. HCl which indicates positive test.

Terpenoids

Salkowski Test

About 2 ml of chloroform was added to 1 ml of the extract. Then 3ml of conc. H_2SO_4 was carefully added to form a layer. A reddish-brown colour reaction of the interface indicates the presence of terpenoids.

Cardiac Glycosides

Methanol extract (2ml)add 3.5 % of $FeCl_3$ +Glacial acetic acid add2ml of conc. H_2SO_4 was taken in beaker, reddish brown ring at inter phase is indicator of positive test.

Quinone

A few ml of the test solution was treated with cons H_2SO_4 or aqueous NaOH. Colourization indicated the presence of quinoid compound

Steroid

Libermann-Burchard's test

A few ml of test solution in chloroform was treated with a few drops of acetic acid acetic anhydride 2 drop of conc. H_2SO_4 and heated gently blue or green colour show the presence of steroid.

Tannins

2-3ml extract was taken in test tube and 10% $FeCl_3$ (ferric chloride solution) was added in it. Dark blue or greenish grey coloration was observed. This is confirmation test for tannin.

Saponins

About 0.5 ml of extract was taken with 5 ml distilled water and then heated to boil frothing (appearance of creamy miss of small bubbles) shows the presence of saponin.

Antibacterial activity – Disc diffusion method

The test organisms were obtained from the Department of Microbiology, St. Mary's College (Autonomous), Thoothukudi. The two gram positive bacteria viz.

Bacillus subtilis, *Staphylococcus aureus* (G +ve) and two gram negative bacteria *Escherichia coli, Vibriyo cholerae* were used in the present study. *Bacillus* responsible for food borne gastroententis. *E. coli, Staphylococcus aureus* cause diseases like mastitis, abortion and upper respiratory complications, while *Vibrio cholera* cause disease like cholera.

The media used for anti-bacterial test were Nutrient broth. The test bacterial strains were inoculated into nutrient broth. The test bacterial strains were inoculated into nutrient broth and incubated at 37°C for 24 hrs. After the incubation period, the culture tubes were compared with the turbidity standard.

Disc diffusion assay

Anti-bacterial activity was evaluated by agar disc diffusion method. Test solutions were prepared with known weight of different solvent extracts dissolved in 5% dimethyl sulphoxide (DMSO). What man No.1 sterile filter paper discs (5mm) were impregnated with 20 µl of these extracts and allowed to dry at room temperature. The spread plates were prepared by proper concentration of inoculum. Each sample loaded disc was placed in the seeded agar plate. After 24-48 hours of 37°C incubation, the diameter of the inhibition zone was measured. For positive control, streptomycin disc (100µg/ml) was used, whereas for negative control, respective solvents were loaded on the sterile disc.

Determination of relative percentage inhibition

The relative percentage inhibition of the test extract with respect to positive control was calculated by using the following formula.

Relative percentage inhibition of the test extract = $\frac{100 \times (X-Y)}{(Z-Y)}$

Where,

X: total area of inhibition of the test extract

Y: total area of inhibition of the solvent

Z: total area of inhibition of the standard drug

The total area of the inhibition was calculated by using area=πr^2; where, r=radius of zone of inhibition.

FT-IR (fourier transforms infra – redspectroscopy) spectroscopic analysis

(vijayabaskar and shiyamala, 2012)

Ten milligram of *Riccinus communis* leaf, stem, root and seed powder was mixed with 100mg of dry potassium bromide (FT-IR grade) and then compressed into a pellet using hydraulic press (5000- 10000pis). The pellet was immediately put into the sample holder and FT-IR (systronics 166) spectra were recorded in the range of 400 to 4000 cm^{-1}.

RESULT AND DISCUSSION

Phytochemical constituents in plants samples are considered to be biologically active compounds with a variety of functions including antioxidant, antiamicrobial, antifungal, hypoglycaemic, anti-diabetic, anti-inflammatory, anticarcinogenic, ,antimalarial, anticholinergic properties. (Hossain and nagooru, 2011, Suresh and Nagarajan, 2009).

The presence of different phytochemical constituents in aqueous, chloroform, ethanol, methanol, petroleum ether of various part of *Riccinus communis* are evaluated qualitatively and present in Table 1& 2. The phytochemical such as alkaloids, indole alkaloids, phenol, flavonoids, terpenoids, cardiac glycoside, quinone, steroid, tannin and saponin were analysed.

Alkaloids are found in chloroform, petroleum ether and aqueous leaf extracts although it is absent in ethanol methanol extracts (Table 1&2). Alkaloids which make up one of the most diverse classes of phytochemicals found in plants, have remarkable effects on humans, leading to the production of effective pain relievers (Suresh and Nagaraj, 2009). However, phenols were detected in ethanol, chloroform, petroleum ether and methanol likewise cardiac glycosides were found in chloroform leaf extracts. Flavonoids are reported in ethanol, methanol, aqueous leaf extract (Parekh and Chanda,2009). Herbs are those that contain tannins as one of their main components and are used to treat intestinal problem like diarrhea and dysentery. (Dharmananda and Gallnuts, 2003). In our study tannin was reported in chloroform, petroleum ether leaf extract.

In the present investigation, the leaf extracts of *R. communis* showed the prescence of quinone in ethanol, methanol, petroleum ether, chloroform and the saponin content was absent in ethanol, methanol, chloroform and aqueous leaf extract while

saponin anti inflammatory, anti-hepatotonic, wound healing, veinotonic, expectorant, spasmolytic, hypoglycemic, antimicrobial and antiviral properties (Rahaman Onlike, 2010).

Indole alkaloid are present in chloroform, petroleum ether and aqueous leaf extract. Steroids were found in chloroform, petroleum ether, ethanol and methanol. Terpenoids were present in ethanol, methanol and aqueous leaf extract. Petroleum ether had a high number of secondary metabolites with a higher degree of precipitation than all other solvent extracts. Aqueous leaf extracts of plant showed the less variety of these secondary metabolites. Alkaloids, glycosides, tannin, phenols, flavonoids, steroid, saponin are these secondary metabolites found in *R. communis.* They have a high therapeutic value and are commonly used in the pharmacy and drug industries

The phytochemical analysis of different stem extracts (aqueous, chloroform, ethanol, methanol, petroleum ether) of *Riccinus communis* were shown in Table 2. Most of the phyto chemicals such as alkaloids, cardiac glycosides, phenols, saponins, tannin ,terpenoids, steroid, indole alkaloids, flavonoids and quinone were exhibited in stem part. Alkaloids are found in chloroform, petroleum ether, ethanol, methanol and aqueous stem extract. Alkaloid are huge group of naturally occur in organic compounds which contain nitrogen atom ore atoms (amino or amido in some cases) in their structures. However, phenols & flavonoids were detected in ethanol, aqueous and methanol, chloroform and petroleum ether. Tannin are polyphenolic substances found in many plant product of secondary metabolism. In this investigation, tannin, quinone and steroids are showed in ethanol, methanol and chloroform extract.

The phytochemical compound of *Riccinus communis* root extracts (aqueous, chloroform, ethanol, methanol, petroleum ether) were shown in Table 2. Result were

obtain in alkaloids, cardiac glycosides, phenols, saponins, tannin, terpenoids, flavonoid, steroid, indole alkaloid and quinone. Alkaloids are found in chloroform, petroleum ether, ethanol, methanol and aqueous root extract of *R.communis.*. However, phenols were detected in chloroform and petroleum ether extracts. In addition to this, saponin content was present in ethanol, petroleum ether and chloroform. Terpenoids are found in ethanol, methanol and petroleum ether. Terpenoids are the largest group of plant specialized (secondary) metabolites. These naturally occurring chemical compounds are highly diverse in chemical structure.

The phytochemical investigation of *R.communis* seed extracts (aqueous, chloroform, ethanol, methanol, petroleum ether)were shown in Table 2. It clearly visualized that alkaloids, cardiac glycosides, phenols, saponins, tannin, terpenoids, steroid, indole alkaloid, flavonoid and quinone are showed in various extracts. Alkaloids are found in chloroform, petroleum ether, ethanol, methanol and aqueous seed extract of *R. communis.*. However, phenols were detected only in chloroform seed extract. Flavonoids are present in methanol and petroleum ether. Quinone was showed in methanol, chloroform petroleum ether and aqueous extracts which plays an important role in oxidative stress and have a diverse role in medicine, including anti- cancer agents and anti -aging and arteriosclerosis (Damodar *et al.*, 2011). In *Ricinus communis*, saponin content was occur in methanol, petroleum ether, and aqueous seed extract. Indole alkaloid are present in methanol, chloroform, petroleum ether and aqueous seed extract. Steroids were found in petroleum ether and aqueous seed extract. Terpenoids were present in chloroform and aqueous seed extract. Alkaloid had a high number of secondary metabolites with a higher degree of precipitation than all other solvent extracts. Phenol had a plant showed the less variety of these secondary metabolites. It is observed that the stem extracts has more phytochemicals as compared to other parts

(Jamdhade *et* al., 2010).Therefore it is concluded that stem extracts of *Ricinus communis* could be effectively processed to be utilized as a source for antibacterial therapeutic drug preparations.

Antibacterial activity

In the present study, antibacterial activity of medicinal plant leaf, stem, root and seed extracts *Ricinus communis* of five different solvents (aqueous, Ethanol, petroleum ether, methanol and chloroform) were tested against human pathogenic bacteria (*Escherchia coli*) and were presented in Table 3,4,5 & 6. The diameter of the inhibition zones against these species ranged from 10 mm to 25 mm. The study revealed that all extracts inhibited the growth of *E.coli* was tested, in which ethanol extracts of *Ricinus communis* exhibited maximum activity against *E. coli* (25 mm, 22 mm, 24 mm, 25mm). Next to that petroleum ether and chloroform extract of *R. communis* inhibited the growth of *E.coli*. The Petroleum ether extract of *Ricinus communis* showed less antibacterial activity (10 mm, 11 mm, 13 mm, 11 mm). This research gives a scientific validation to the fact that bioactive components in the plant *R.communis* are extracted substantially in ethanol and exhibited highly promising antibacterial and antifungal inhibitory activity.

The high antibacterial activity in the ethanolic leaf extract of plants may be due to the presence of flavonoids, alkaloids. Antibacterial activity is elicited by these medicinally bioactive components through a variety of mechanisms. Flavonoids are known to be synthesized in response to microbial infection by plants and have been shown to be effective antimicrobial substances against a wide range of microorganisms in *vitro*. They can form complexes with extra cellular and soluble proteins, as well as bacterial cell walls (Marjorie,1999). Steroids are also known for their antibacterial

activity, which is related to membrane lipids and induces liposome leakage (Epand *et* al., 2007).

Fourier Transform Infra – red Spectroscopy analysis

Fourier Transform Infra – red Spectroscopy measurement spectrum were carried out to identify the possible biomolecules responsible for the antimicrobial properties. The FTIR spectroscopy analysis of *R. communis* values are depicted in table 7 – 10, and Fig 5-8 . The representative spectrum of *Ricinus communis* leaf showed absorption peaks 3414.73, 2924.85, 2851.56, 2361.67, 2226.66, 1741.6, 1542.95, 520.74. These absorption peaks are known to be associated with stretching vibration for N-H amine, amides, C-H stretch alkanes stretch, C-H alkanes stretch H-C=O aldehydes stretching -C=N alkynes stretching C=O saturated aliphatic stretching N-O nitro compound stretching asymmetric C-O stretching alcohols carboxylic acid ester ethers C-Br alkylhali .

The representative spectrum of *Ricinus communis* stem showed absorption peaks 2924.85, 238.92, 2308.63, 1743.53, 1622.02, 1510.16, 1424.33, 1319.22, 1266.18, 1033.77, 780.15, 518.82. These absorption peaks aare known to be associated with C-CI alkenes stretching, C-H aldehydes stretching, H-C-O aliphatic amine stretching, C=O aldehydes stretching, N-H amines, C-H-O aromatics stretching, C-H aromatics stretching, C-O carboxylic acid stretching, C-N aromatic amine stretching C-N aliphatic amine stretching, C-Br alkynes bend, C-Br alkylkalides stretching.

The representative spectrum of *Ricinus communis* seed showed absorption peaks 3770.58, 3312.51, 3009.71, 2927.74, 2854.45, 2371.32, 2282.60, 1744.49, 1460.98, 1169.75, 1081.03, 724.22, 627.79. These absorption peaks aare known to be associated with 0-H alcohol phenol stretching, -C=C-H alkynes stretching, =C-H alkanes stretching, C-H alkanes stretching, =C-H alkanes stretching, $-NH_2^+$ secondary

amines salts stretching, C=N nitriles stretching, C=O saturated aliphatic esters stretching, C-H alkanes bend, C-N aliphatic amines stretching. C-H alkanes bend, C-H alkanes rock, C-I indo compound stretching.

The representative spectrum of *Ricinus commuinis* root showed absorption peaks 2300.92, 1680.85, 1623.95, 1596.95, 1510.16, 1374.19, 1318.25, 1277.75, 1196.75, 1021.24, 895.87, 778.22, 719.4, 549.71, 494.71, 469.63. C=N nutriles stretrching, -C=C- alkenes stretching, N-H amine bend stretching C-C aromatic stretching, N-O asymmetric stretching, N-O asymmetric stretching, N-O nitro compound stretching, C-N aliphatic aromatic stretching, C-N aliphatic amine stretching, C-N aliphatic amine stretching, C-H alkaylhalides stretching, C-Br alicyclicaxial C-1 indo compounds stretching. S-S sulphides stretching. The result of the present study revealed that the whole plant of *R. communis* contain considerable amount of secondary metabolites and it may considered in future to be used human disease management.

Summary and conclusion

India is richly endowed with natural resources that can fuel its future growth, with a wide variety plants having medicinal value. These plants are commonly used by people from all walks of life, either as folk remedies or as medicinal preparations for modern medicine. Phytochemical with biological active compounds have a lot of applications in term of pharmaceuticals and pharmacological effects. In the present phytochemical studies exposed to the presence of phytoconstituents such as terpenoids, flavonoids, alkaloids, phenols, steroids, saponins, quinones, cardic glycosides, indole alkaloid and tannin. The result of the present study revealed that, the whole plant of *R.communis* contain considerable amount of secondary metabolites. Alkaloids, steroids and tannin were reported in all five extracts of *Ricinus communis*. Hence this plant should be evaluated further to assess its phytotherapeutic properties.

The different plant parts of *Riccinus communis* have the antibacterial activity. As shown in the figure 1 -4, the study revealed that all extracts inhibited the growth of *E.coli* was tested, in which ethanol extracts of *Ricinus communis* exhibited maximum activity against *E. coli* (25 mm, 22 mm, 24 mm, 25mm). Next to that petroleum ether and chloroform extract of *Ricinus communis* inhibited the growth of *E.coli*. Over all, the present study concluded that, this plant is wealthy resources of active phytochemical and antibacterial activity that is strongly emphasized the further pharmacological evaluations, toxicological studies and possible isolation of the therapeutic antibacterial from this plant are the future challenges.

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ISOLATION IDENTIFICATION OF PHYTOPLANKTON AND WATER QUALITY OF POND WATER

A short term project work submitted to **ST. Mary's College (Autonomous)**

Re accredited with A^{+}Grade by NAAC affiliated to **MANONMANIAM SUNDARANAR UNIVERSITY** in partial fulfilment of the requirements for the Degree of Bachelor of science in Botany.

By

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DEPARTMENT OF BOTANY

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2021-2022

CERTIFICATE

It is certified that this short term project work entitled "**ISOLATION IDENTIFICATION OF PHYTOPLANKTON AND WATER QUALITY OF POND WATER** submitted to St. Mary's College (Autonomous) affiliated to MANONMANIUM SUNDARANAR UNIVERSITY in partial fulfilment of the requirements for the degree of Bachelor of science in Botany and is a record of work done in the Department of Botany, St.Mary's College (Autonomous) Re accredited with A+Grade by NAAC Thoothukudi during the year 2021-20122 by the following students.

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CONTENT

INTRODUCTION

INTRODUCTION

Human society relies on freshwater for domestic, industrial, agricultural and other goods and services. These needs have subjected the ecosystems which include rivers, streams, lakes and ponds to increasing contamination by a variety of mineral, agrochemicals and organic pollutants due to higher frequency of allochthonous input from anthropogenic activities. The rapid pollution of water resources and risk of extinction is placed at the top of these environmental issues. Increasing population, unplanned industrialization and urbanization are also accelerating this process day by day.

Natural lake systems represent essential reservoirs for domestic water supply, fish production, and recreational activities. At the same time, however, lakes are among the most vulnerable ecological systems and, therefore, should be continuously monitored (Woolway *et, al,*. 2020). Recently eutrophication of freshwater environment and occurrence of algal blooms received a great of deal of attention. More than 60% of fresh water habitat have been eutrophicated and suffered from algal blooms. They have caused a lot of social, environmental and economic problems.

Phytoplankton are the plant-like components of the plankton community ("phyto" comes from the Greek for *plant*). They are autotrophic (self-feeding), meaning they generate their own food and do not need to consume other organisms.

Phytoplankton performs three crucial functions: they generate nearly half of the world atmospheric oxygen, they regulate ocean and atmospheric carbon dioxide levels, and they form the base of the marine food web. When conditions are right, blooms of phytoplankton algae can occur in surface waters. Phytoplankton is r-strategists which grow rapidly and can double their population every day. The blooms can become toxic and deplete the water of oxygen. However, phytoplankton numbers are usually kept in check by the phytoplankton exhausting available nutrients and by grazing zooplankton.

Phytoplankton consists mainly of microscopic photosynthetic eukaryotes which inhabit the upper sunlit layer in all oceans. They need sunlight so they can photosynthesize. Most phytoplankton are single-celled algae, but other phytoplankton are bacteria and some are protists. Phytoplankton include cyanobacteria (above), diatoms, dinoflagellates, euglenoids, coccolithophorids, cryptomonads, chlorophytes, prasinophytes, and silico flagellates. They form the base of the primary production that drives the ocean food web, and account for half of the current global primary production, more than the terrestrial forests.

Phytoplankton is a polyphyletic group with utmost variation in size, shape, colour, type of metabolism, and life history traits. Phytoplankton species composition and biomass are fundamental parameters in food web ecology. Therefore much attention has been focused on resource competition and predator – prey interaction in both fresh water and Marine ecosystem. Microalgae is unicellular or multicellular organisms from photoautotrophic water, and is a microscopic organism that require inorganic nutrients for photosynthesis (Biondi and Tredici, 2011). The phytoplankton in aquatic ecosystem has the potential to be developed as a biological feed through isolation of phytoplankton from aquatic ecosystem

The availability of food is one of the factors that determine population size, growth, reproduction and population dynamics and the condition of the fish in the waters (Tugiyono and Master, 2015). Besides, as a natural feed phytoplankton, micro algae play an important role in outlining the waste, improve soil structure and increase fertility and produce methane and fuels to energy. As well as grown for animal feed including the fisheries, human food, and pharmaceutical (Sivakumar *et, al.*, 2012).

The availability of natural feed (phytoplankton and zooplankton) under normal conditions in the natural aquatic ecosystem is available in enough even abundant. This feed (phytoplankton and zooplankton) can be used as a natural feed by each trophic level efficiently

in food web, especially fish that occupy the highest trophic level. Problems with feed requirements usually only appear when the organism is in a farming environment. The quality of phytoplankton is outstanding and has been a key factor for hatchery culture success especially with delicate species (Bluebiotech, 2017).

The information can serve as tool for advocacy of policies to protect the ecological system for sustainable utilization. The objective of this study was to determine the pollution index by assessing the water quality and phytoplankton structure of the ecosystem.

SCOPE

AND

OBJECTIVES

SCOPE AND OBJECTIVES

The distribution of the plankton shows that they are sensitive to changes in levels of nutrients and other interactions with one another and with other factors. As such the plankton can be good indicators of water quality. Based on this the present study was designed with following objectives.

1. Collection of water samples
2. Isolation of Planktons
3. Identification of planktons
4. To determine the physico – chemical characteristics of water samples.

REVIEW

OF

LITERATURE

REVIEW OF LITERATURE

Inland waters represent only about 0.02% of all water in the biosphere, and nearly 90% of this total is contained within only about 250 of the world's largest lakes (Wetzel, 1983a). Nonetheless, it is fresh water that is most important for human consumption and is most threatened by human activities. Algal ecologists play an important role in the understanding of aquatic ecosystems, their productivity, and water quality issues (Dow and Swoboda, 2000; Oliver and Ganf, 2000)

Lentic environments include standing waters from the smallest ponds (a few square meters) to enormous bodies of water (e.g., Laurentian Great Lakes: 245,000 km2). Their formation, geography, limnology, and conservation have been covered in several texts (Abel *et, al.,* 2000). This section summarizes some features of lentic environments as they pertain to the ecology and distribution of freshwater algae. Sizes and shapes of water basins (their morphometry) have profound effects on the physics, chemistry, and biology of lake ecosystems, and influence the composition of algal communities and their productivity.

Tugiyono *et,al,*. 2017 carried out the study on Isolation and identification of phytoplankton from aquatic ecosystems of Lampung Mangroves Center (LMC) as biological feed. The analysis of sea water quality parameters in the location of the study consisting of pH, salinity, brightness, water temperature, nitrate, nitrite and phosphate, revealed that all the parameters meet the quality standard, except for nitrates and nitrites. Isolation and identification of Phytoplankton taken from aquatic ecosystem of the LMC using net plankton ± 15 was carried out to obtain pure cultures in Live Feed Laboratory of the Center for Marin Culture Lampung, Indonesia. The results showed that there were 14 genera of phytoplankton identified. Six types of 14 genera of phytoplankton identified have potential to be developed as a biological feed: Thalassiosira, Cyclotella, Chaetoceros, Tetraselmis, Nannochloropsis, and Isochrysis, The isolates of phytoplankton produced can be stored in the refrigerator as pure

stock cultures and can last for up to 6 months before being used in a medium culture or laboratory culture scale.

Mary Jacintha *et,al,*. 2020 studied the Water Quality and Phytoplankton as an Indicator of Pollution in a Mambazhathurayar Reservoir, Kanyakumari District, Tamil nadu, S. India. Water plays major role in biodiversity conservation hence the need for its protection. The integrity of an aquatic ecosystem can be accessed through the physico-chemistry and phytoplankton structure. Samples were collected monthly from the study site for 12 months from July 2016 to July, 2017. Nutrient concentration (phosphate) was comparatively higher in the dry season than south west and North West monsoon. Four phytoplankton divisions including Bacillariophyceae (29species), Chlorophyceae (19 species), Chrysophyceae (12 species), and Myxophyceae (09 species) were identified. The most dominant among the pollution indicators were Pleurosigma directum, Synedra nana and Euglena granulate. Phytoplankton count also registered higher value during non-rainy months. The distribution of the plankton shows that they are sensitive to changes in levels of nutrients and other interactions with one another and with other factors. As such the plankton can be good indicators of water quality. The seasonal and spatial distribution of the plankton in this study shows that, they are sensitive to changes in levels of nutrients and other interactions with one another and with other factors. As such the plankton can be good indicators of water quality.

Phytoplankton is a polyphyletic group with utmost variation in size, shape, colour, type of metabolism, and life history traits. Due to the emerging knowledge in nutritional capabilities of microorganisms, our view of phytoplankton has drastically changed (Flynn *et, al.*, 2013). Phagotrophy is now known from all clades except diatoms and cyanobacteria. At the same time, ciliates, which have not been considered as part of 'phytoplankton', span a gradient in trophic modes that render the distinction between phototrophic phytoplankton and heterotrophic protozoa meaningless. This complexity has been expressed in the high diversity

of natural phytoplankton assemblages. Diversity can be defined in many different ways and levels. Although the first diversity measure that encompassed the two basic components of diversity (i.e., the number of items and their relative frequencies) appeared in the early forties of the last century (Fisher *et,al.*, 1943), in phytoplankton ecology, taxonomic richness has been used the most often as diversity estimates. Until the widespread use of the inverted microscopes, phytoplankton ecologists did not have accurate abundance estimation methods and the net plankton served as a basis for the analyses. Richness of taxonomic groups of net samples, and their ratios were used for quality assessment (Thunmark, 1945, Nygaard, 1949). The study of phytoplankton diversity received a great impetus after Hutchinson's (1961) seminal paper on the paradox of the plankton. The author not only contrasted Hardin's competitive exclusion theory (Hardin, 1960) with the high number of co-occurring species in a seemingly homogeneous environment, but outlined possible explanations. He argued for the non-equilibrium nature of the plankton, the roles of disturbances and biotic interactions, moreover the importance of benthic habitats in the recruitment of phytoplankton. The 'paradox of the plankton' largely influenced the study of diversity in particular and the development of community ecology in general (Naselli-Flores & Rossetti, 2010). Several equilibrium and non-equilibrium mechanisms have been developed to address the question of species coexistence in pelagic waters (Roy and Chattopadhyay, 2007). The paradox and the models that aimed to explain the species coexistence in the aquatic environment have been extended to terrestrial ecosystems (Wilson1990). Wilson reviewed evidences for twelve possible mechanisms that potentially could explain the paradox for indigenous New Zealand vegetation, and found that four of them, such as gradual climate change, cyclic successional processes, spatial mass effect and niche diversification, were the most important explanations. By now, the paradox has been considered as an apparent violation of the competitive exclusion principle in the entire field of ecology (Hening and Nguyen, 2020).

Although Hutchinson's contribution (Hutchinson, 1961) has given a great impetus to research on species coexistence, the number of studies on phytoplankton diversity that time did not increase considerably partly because in this period, eutrophication studies dominated the hydrobiological literature. Understanding the drivers of diversity has been substantially improved from the 70 s when laboratory experiments and mathematical modelling proved that competition theory or intermediate disturbance hypothesis (IDH) provided explanations for species coexistence. Many field studies also demonstrated the role of disturbances in maintaining phytoplankton diversity, and these results were concluded by Reynolds and his co-workers (Reynolds *et, al.,* 1993).

From the 2000 s a rapid increase in phytoplankton research appeared which might be explained by theoretical and methodological improvements in ecology. The functional approaches - partly due to Colin Reynolds's prominent contribution to this field (Reynolds *et, al.,*2002) - opened new perspectives in phytoplankton diversity research. Functional trait and functional 'group'-based approaches have gained considerable popularity in recent years (Ye *et, al.,* 2019).

Traditional approaches assessing phytoplankton diversity, distribution, and abundance of phytoplankton taxa, based on morphological characteristics obtained by light microscopy (Maurer 2000, Soares *et,al.,*2011) have a number of limitations: (Patrick *et,al.,* 1981) labor intensity that limits the size of the quantified sample to hundred(s) of cellular events and a relatively low number of samples to be processed; (Dokulil et, al., 2000) accurate diagnostics of taxa and their abundances are hampered by undifferentiated morphologies, unidentified early-life algal stages and numerous cryptic species and incomplete description of the changes in biodiversity based on a limited number of morphologically identified taxa. During the last two decades, cytometric methods (flow cytometry (FCM) and imaging flow cytometry (IFC)) have been recognized as a powerful tool to study seasonal and spatial trends of phytoplankton

(Peperzak *et,al.*,2000, Dashkova *et,al.*, 2017) It is noteworthy, however, that conventional cytometry may not be allowed to isolate and characterize all plankton species and colonial forms identified by traditional microscopy due to size limitations of flow cells typically within a 150 μm limit (Dubelaar and Jonker 2000).

Hong Zhang (2011) studied the Isolation, identification and characterization of phytoplankton-lytic bacterium CH-22 against *Microcystis aeruginosa*. A bacterial strain named CH-22 showing phytoplankton-lytic activity against bloom-forming *cyanobacterium Microcystis aeruginosa* was isolated from Lake Chaohu of Anhui Province, China. The isolated strain was identified as Pseudomonas putida by morphology and homology research based on 16S rDNA. Comparative analysis of freshwater phytoplankton communities in two lakes of Burabay National Park using morphological and molecular approaches was carried out by Dmitry *et,al,*. 2021. They analyzed phytoplankton assemblages' variations in oligo-mesotrophic Shchuchie and Burabay lakes using traditional morphological and next-generation sequencing (NGS) approaches. The total phytoplankton biodiversity and abundance estimated by both microscopy and NGS were signifcantly higher in Lake Burabay than in Lake Shchuchie. NGS of 16S and 18S rRNA amplicons adequately identify phytoplankton taxa only on the genera level, while species composition obtained by microscopic examination was signifcantly larger.

MATERIALS

AND

METHODS

MATERIALS AND METHODS

STUDY AREA

A small fresh water pond located in the Botany Garden of St' Mary's College (Autonomous) Thoothukudi. District of Tamilnadu. The sampled location lies between 8.812^0 latitude and 78.161^0 longitude was selected for the present study. (Plate 1)

COLLECTION OF WATER SAMPLES

Water samples are collected from four corners after stirring. To 1L of sample add 10ml of Lugol's iodine solution. Leave it undisturbed for 24h then decant water. Collect the settled planktons in small bottles and preserve for further studies.

ISOLATION OF PLANKTONS AND IDENTIFICATION OF PLANKTONS

On coming to the laboratory, the phytoplankton samples were condensed by centrifuging 100ml of the sample to10mI. The concentrated sample was taken, separated carefully stained and temporarily mounted in glass slide with glycerine. Identified with the help of Identification hand book of Fresh water Planktons By. Edmondson.

PHYSICO – CHEMICAL CHARACTERISTICS OF WATER SAMPLES

TEMPERATURE

Temperature of the water sample is measured by using Thermometer.

pH

pH is measured using pH meter, which comprises a detecting unit consisting of a glass electrode, reference electrode, usually a calomel electrode connected by KCl Bridge to the pH sensitive glass electrode and an indicating unit which indicates the pH corresponding to the electromotive force is then detected. Before measurement, pH meter should be calibrated by using at least two buffers.

DETERMINATION OF ACIDITY

Principle

The presence of excess carbon dioxide in water depends on its concentration in water and atmosphere that is it tends to be in and atmosphere and water. If the level exceeds in atmosphere a part of it enters in to surface waters by absorption and therefore it is a normal component in the aquatic system. Acidity is caused by CO_2 in water with pH<4.The main source of CO_2 in water is atmospheric diffusion even though a small amount is produced by the biological oxidation of organic matter.

Water sample when titrated with sodium hydroxide using phenolphthalein as indicator the CO_2 dissolved in water forms carbonic acid and when the hydrogen (H+) ions are neutralized, the excess of NaOH turns the solution basic. The phenolphthalein turns faint pink at this point. The amount of alkali needed to produce the pink colour indicates the amount of CO_2 in solution in the sample.

Requirements:

- N/44 Sodium hydroxide (N=equivalent weight. 40/44=0.91g
- **Phenolphthalein indicator:**

  Dissolve 1g Phenolphthalein in 100 ml ethyl alcohol. Dilute with 100ml of boiled and cooled distilled water.
- **Glassware:**

  Burette, burette stand, conical flask pipettes and measuring cylinders

Procedure:

Take 50 ml of water sample in a conical flask. Add 10 drops of phenolphthalein indicator. If the sample turns pink and remains so on addition of the indicator solution there is no free CO_2. If the sample remains colourless titrate against standard NaOH. Swirl gently to

mix. Continue titration until a persistent pink colour appears in the sample. Note the initial and final volume of NaOH. Repeat the procedure to attain concordant value.

Calculation:

The number of milliliter of NaOH solution requires to reach the end point multiplied by 20 gives the amount of CO_2 in ppm which when multiplied by 2.272 give acidity in ppm ($CaCO_3$).

Estimation of alkalinity in water samples

Principle:

Alkalinity is a measure of the capability of water to absorb H^+ ions without significant change of pH. In other words, alkalinity is a measure of the acid buffering capacity of water. Alkalinity of a sample of water is due to the presence of OH^- (hydroxide ion), HCO_3^- (bicarbonate ion) and CO_3^{2-} (carbonate ion) or the mixture of two ions present in water. The possibility of OH^- and HCO_3^- ions together is not possible since they combine together to form CO_3^{2-} ions.

$$OH^- + HCO_3^- \longrightarrow CO_3^{2-} + H_2O$$

The alkalinity due to different ions can be estimated separately by titration against standard acid solution, using selective indicators like phenolphthalein and methyl orange.

i) $OH^- + H^+ \longrightarrow H_2O$

ii) $CO_3^{2-} + H^+ \longrightarrow HCO_3^-$

iii) $HCO_3^- + H^+ \longrightarrow H_2O + CO_2$

The neutralization reaction up to phenolphthalein end point shows the completion of reactions (i) and (ii) (OH^- and CO_3^{2-}) and (CO_3^{2-} and HCO_3^-) only. The amount of acid used thus corresponds to complete neutralization of OH^- plus half neutralization of CO_3^{2-}. The titration of water sample using methyl orange indicator marks the completion of the reactions (i), (ii) and (iii). The amount of acid used after phenolphthalein end point corresponds to one

half of normal carbonate and all the bicarbonates. Total amount of acid used represent the total alkalinity due to all ions present in water sample

Requirements:

Reagents:

- **Phenolphthalein indicator solution:**

0.5g of phenolphthalein is dissolved in 50ml of ethyl alcohol and to which 50ml of distilled water is added.

- **Methyl orange indicator solution:**

0.05g of methyl orange is dissolved in 100ml of distilled H_2O.

- **0.02 N sulphuric acids:**

0.56 ml of sulphuric cid in 1000 ml of distilled water.

Glassware:

Burette, burette stand, conical flask, pipette, measuring cylinder.

Procedure:

Take 50ml of water sample in a conical flask and add 5 drops of phenolphthalein indicator. If the solution turns pink, it is due to the presence of Hydroxide or carbonate. Titrate against with standard sulphuric acid solution until the pink colour of the sample disappears.

If there is no pink colour on addition of phenolphthalein add 1 to 2 drops of methyl orange indicator and proceed with the titration till the orange colour of methyl orange gets turn to pinkish orange. Note the initial and final volumes of 0.02N sulphuric acid.

Calculation:

- Phenolphthalein alkalinity or carbonate alkalinity is calculated as follows.

$$\text{Phenolphthalein alkalinity} = \frac{\text{ml of titrant used} \times 1000}{\text{Volume of sample [ml]}}$$

Where,

ml of titrant =Volume of acid used in titrating to the phenolphthalein end point.

- Methyl orange alkalinity or Bicarbonate alkalinity is calculated as follows

$$\text{Bicarbonate alkalinity} = \frac{\text{ml of titrant} \times 1000}{\text{Sample volume [ml]}}$$

Where,

ml of titrant=Volume of acid used in titrating to the methyl orange end point.

Total alkalinity [mg/I] = phenolphthalein alkalinity+ methyl orange alkalinity

Determination of Dissolved Oxygen in water (winkler's method)

Principle

Manganous sulphate reacts with potassium hydroxide to give a white precipitate of manganous hydroxide. In the presence of oxygen manganic oxide is formed. Addition of sulphuric acid dissolves manganic oxide yielding manganous sulphate which reacts instantly with Iodine to yield Iodine. In effect, oxygen oxidizes Mn^{2+} to Mn^{4+} and oxidizes I to I_2, Iodine is determined titrimetically with sodium thio sulphate and starch as indicator.

Materials required

Conical Flasks, burette, pipettes, measuring cylinder and water samples.

Reagents

- Manganous Sulphate

  48g of Manganous sulphate dissolved in 100ml of water.

- Alkali Mixture

  70g of Potassium hydroxide and 15g Potassium iodide are dissolved in distilled water and diluted to 100ml

- Starch indicator

  1g of starch in 100ml of boiled distilled water.

- Con. H_2SO_4

- Sodium thiosulphate (0.025N)

3.102g of sodium thiosulphate is dissolved in previously boiled distilled water and made up to 500ml and stored in brown bottle.

Procedure

The sample collecting bottles (250 ml) must be below the surface of the water body while filling. The water sample was allowed to overflow in order to avoid entrapping of air bubbles and stoppered.

The bottle is opened and 1ml of manganous sulphate and 1ml of alkali mixture are added.

The bottle is restoppered and carefully tilted when a white precipitate of manganous hydroxide is formed and settled at the bottom.

The stopper is removed and 1ml of con. H_2SO_4 is added. The bottle is tilted carefully until a clear straw coloured solution is formed due to the liberaration of iodine.

100ml of the solution is removed from the bottle and is transferred to 250ml conical flask.

This is titrated with 0.025N sodium thio sulphate until a pale yellow colour is formed. At this stage 3-4 drops of starch indicator is added and the titration is continued until the disappearance of the blue colour. Note the final volume of sodium thio sulphate solution

Calculation

Amount of dissolved oxygen in sample water (mg/l)

$$= \frac{\text{Titrant value x 0.025N x8x1000}}{\text{Volume of the sample}}$$

Where,

0.025 = normality of titrant sodium thiosulphate

8= molecular weight of oxygen

Estimation of Phosphate

Principle

Phosphorus is one of the major nutrients which is necessary for the production of nucleic acids, phospholipids and a variety of Phosphorylated compounds. It has a limiting or regulating effect on productivity. In nature phosphorus does not occur in a free state but in the form of phosphorus. In aquatic system phosphates are present in the form of soluble phosphates and organophosphorus. It is of great importance in determining the

Productivity. Phosphate reacts with molybdate to form phosphomolybdic acid which on being reduced by amino naptho sulfonic acid (ANSA) produces blue colour , the intensity of which is directly dependent on the amount of phosphates present.

Requirements:

- Glassware: Conical flask, pipettes, measuring cylinder.
- Reagents: ANSA (0.25%)

Dissolve 1, 2, 4, amino naptho sulphuric acid (250mg) in 95ml of 15% sodium bisulphate (15g/100ml) . Add 5ml of 20% (1g/5ml) sodium sulphite shake well and store in cold.

- Ammonium molybdate(5%)
- Perchloric acid (70%)

Stock phosphate sodium:

Dissolve 4.39g potassium dihydrogen phosphate (KH_2PO_4) IN 1liter of distilled water. This solution contains 1g p/l.

Std.phosphate solution:

Dilute 1ml stock solution to 1litre. This solution contains 1mg Po_4 p/l.

Procedure:

Take 5ml of water sample mix with 0.4ml perchloric acid, 0.4ml of ammonium molybdate (5%) and 0.2ml of reducing agent (ANSA). The contents of the tubes are shaken between each addition. The tubes are allowed to stand for few minutes. Measuring the OD of the blue colour developed against a reagent blank at 650nm. Read the amount of phosphate present from the standard graph.

Estimation of Nitrate.

Principle:

The four primary elements which make up plant tissue are carbon, oxygen, hydrogen and nitrogen. Plant has to obtain their nitrogen from compound such as nitrites, nitrates and ammonia. Normally only a small quantity of this compound occurs in solution in natural waters and these are usually derived from organic decomposition. Nitrogen compound and other essential nutrients may become critically scarce due to this depletion by plant growth. The non-availability of this compound has a limiting effect on plant growth and thus, indirectly affects the animal population. An outburst of algal production is usually preceded by high nitrate and phosphate availability. Nitrates present in the water sample react with salicylic acid to form nitrosalt and when this is converted into an alkaline salt a yellow colour is produced. Since the intensity of this colour is directly proportional to the amount of nitrate present, measurement of the colour indicates the amount of nitrates.

Requirement:

- Glassware: conical flask, pipettes, measuring cylinder, test tubes

Reagents:

- 5% (w/v)salicyclic acid .Dissolve 5 gm of salicylic acid in 100 ml con. H_2SO_4
- 2N NaOH. - Dissolve 8 gm of NaOH in 100ml distilled water.(Need 400 ml Std etc.).

- Standard potassium nitrate (KNO_3) solution : Dissolve 0.722 g of potassium nitrate in distilled water and make upto 1liter (10 mg NO_3 N/l).From this stock solution prepare appropriate dilution with distilled water preferably in the range from 0.1 to 1 mg NO_3/l.

Procedure:

Take 25ml of water sample in a conical flask. Heat it until it is dried. Add 0.8 ml of 5% salicylic acid to the residue. Swirl the flask to completely wet the residue. Allow to stand for 20 minutes. Now add 19 ml of 2N NaOH slowly with pipette to rise to the pH12. Then it is cooled down to room temperature and extinction was read at 410 nm.

A STUDY ON THE GROWTH OF JASMINUM AURICULATUM CALLUS CULTURES IN MS MEDIA WITH VARIOUS CONCENTRATIONS OF GROWTH REGULATORS

A Short Term Project Work Submitted to St. Mary's college (Autonomous) affiliated to Manonmaniam Sundaranar University in Partial Fulfillment for the Degree of

BACHELOR OF SCIENCE IN BOTANY

By

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DEPARTMENT OF BOTANY

ST.MARY'S COLLEGE (AUTONOMOUS)

THOOTHUKUDI-628 001

2021 - 2022

CERTIFICATE

It is certified that this short term project work entitled **"A study on the growth of *Jasminum auriculatum* callus cultures in MS media with various concentrations of growth regulators "** submitted to **St. Mary's College (Autonomous)** affiliated to **Manonmaniam Sundaranar University** in partial fulfillment of the requirements for the degree of **Bachelor of Science in Botany** and is a record of work done in the Department of Botany, St. Mary's College (Autonomous), Thoothukudi during the year 2020 - 2021 by the following students.

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CONTENT

INTRODUCTION

Conservation of natural resources is the wise use of the earth's resources by humanity. **Usher (2000)** defined conservation as the maintenance of genetic, species, and ecosystem diversity in the natural abundance in which they occur. **Thomas (2003)** sees conservation as the sacrifice of immediate rewards in return for delayed ones. As the global human population approaches eight billion, there is growing concern about the rate of extinction of other species that inhabit our planet (**Barnosky et al., 2011; Dirzo et al., 2014)**. Compelling evidence points to humans as the direct or indirect cause of most modern extinctions. The primary threats to these species include residential and commercial development; overexploitation via fishing, hunting, or collecting; disturbance by humans during work and recreational activities; pollution; and the introduction of exotic species (**Venter *et al.*, 2006; Prugh *et al.*, 2010; McCune *et al.*, 2013**).

The two main methods of conserving biodiversity are: ex-situ and in-situ conservation. Ex-situ conservation is the process of protecting or preserving an endangered species of either plants or animal outside of its natural habitat either by removing whole or part of that population from the threatened habitat and placing it in a new environment which may be a wild area or within human control environment. Methods of ex-situ conservation are Biological Gardens, Seed bank, Gene bank, Germplasm bank and In-vitro storage. While in-situ conservation involves the conservation and preservation of species in their natural habitat in places where the species naturally occurs. Under this method the entire ecosystem is protected and maintained so that all the constituent species, both known and unknown are conserved. The main methods of in-situ conservations are: Strict nature reserve (SNR), Games Reserve and National Park. In-situ conservation has been made almost impossible due

to the disappearance of large wild areas as a result of natural disasters, pests, pathogens and threats from changing government policies and urban development. Ex-situ Conservation is very difficult to carry out due to the following problems: an adequate sample has to be taken for the conservation of genetic diversity, land space requirement is very important, particularly in the case of forest trees which are very large, whereas land availability drastically decreases, labour costs and trained personnel (**Engelman, 1991**).

Plant tissue culture is a modern method of propagation and conservation of plant species. Apart from their use as a tool of research, plant tissue culture techniques have in recent years, become of major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites. Small pieces of tissue (named explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis (**Akin-Idowu *et al.*, 2009**). In-vitro organ culture offers an alternative source for the conservation of endangered genotypes (**Sengar *et al.*, 2010**), which can be achieved using slow growth (short and medium term conservation) procedure or cryopreservation (long-term conservation) (**Scherwinski-Pereira *et al.*, 2010**).

Tissue culture protocols can be used for preservation of plant tissues or organ when the targets for conservation are clones instead of seeds. This helps to keep the genetic background of a crop and to avoid the loss of the conserved patrimony due to natural disasters, whether biotic or abiotic stress (**Tyagi *et al.*, 2007**). The plant species which do not produce seeds (sterile plants) or which have 'recalcitrant' seeds that cannot be stored for long period of time can successfully be conserved via in-vitro

techniques for the maintenance of gene banks. The method also reduced storage space for maintaining a large number of explants in an aseptic environment, the stored materials are readily available for use and it can be easily seen to be alive.

Plant tissue culture is the in vitro aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions (**Thorpe, 2007**) often to produce the clones of plants. The resultant clones are true to type of the selected genotype. The controlled conditions provide the culture an environment conducive for their growth and multiplication. These conditions include proper supply of nutrients, pH medium, adequate temperature and proper gaseous and liquid environment. Plant tissue culture technology is being widely used for large scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have in recent years, become of major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites. Small pieces of tissue (explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis (**Akin-Idowu *et al*., 2009**). Endangered, threatened and rare species have successfully been grown and conserved by micropropagation because of high coefficient of multiplication and small demands on number of initial plants and space. In addition, plant tissue culture is considered to be the most efficient technology for crop improvement by the production of somaclonal and gametoclonal variants.

The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well-adapted high yielding genotypes with better disease resistance and stress tolerance capacities (**Brown and Thorpe,**

1995). Certain type of callus cultures give rise to clones that have inheritable characteristics different from those of parent plants due to the possibility of occurrence of somaclonal variability (**George 1993**), which leads to the development of commercially important improved varieties.

Commercial production of plants through micropropagation techniques has several advantages over the traditional methods of propagation through seed, cutting, grafting and air-layering etc. It is rapid propagation processes that can lead to the production of plants virus free (**Garcia-Gonzales *et al.*, 2010**). *Coryodalis yanhusuo,* an important medicinal plant was propagated by somatic embryogenesis from tuber-derived callus to produce disease free tubers (**Sagare *et al.*, 2000**). Meristem tip culture of banana plants devoid from banana bunchy top virus (BBTV) and brome mosaic virus (BMV) were produced (**El-Dougdoug and El-Shamy, 2011**). Higher yields have been obtained by culturing pathogen free germplasm in vitro. Increase in yield up to 150% of virus-free potatoes was obtained in controlled conditions (**Singh, 1992**).

Plant tissue culture medium contains all the nutrients required for the normal growth and development of plants. It is mainly composed of macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in case of solid medium. Murashige and Skoog medium (MS medium) is most extensively used for the vegetative propagation of many plant species in vitro. The pH of the media is also important that affects both the growth of plants and activity of plant growth regulators. It is adjusted to the value between 5.4 - 5.8. Both the solid and liquid medium can be used for culturing. The composition of the medium, particularly the plant hormones and the nitrogen source has profound effects on the response of the initial explant. Plant growth regulators (PGR‟s) play an essential role in determining the development pathway of plant cells and tissues in

culture medium. The auxins, cytokinins and gibberellins are most commonly used plant growth regulators. The type and the concentration of hormones used depend mainly on the species of the plant, the tissue or organ cultured and the objective of the experiment (**Ting, 1982**). Auxins and cytokinins are most widely used plant growth regulators in plant tissue culture and their amount determined the type of culture established or regenerated. The high concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. A balance of both auxin and cytokinin leads to the development of mass of undifferentiated cells known as callus.

SCOPE AND OBJECTIVES

Plant tissue culture as an important tool for the continuous production of active compounds including secondary metabolites and engineered molecules. Novel methods (gene editing, abiotic stress) can improve the technique.Humans have a long history of reliance on plants for a supply of food, shelter and, most importantly, medicine. Current-day pharmaceuticals are typically based on plant-derived metabolites, with new products being discovered constantly. Nevertheless, the consistent and uniform supply of plant pharmaceuticals has often been compromised. One alternative for the production of important plant active compounds is in vitro plant tissue culture, as it assures independence from geographical conditions by eliminating the need to rely on wild plants. Plant transformation also allows the further use of plants for the production of engineered compounds, such as vaccines and multiple pharmaceuticals. This review summarizes the important bioactive compounds currently produced by plant tissue culture and the fundamental methods and plants employed for their production.

Objectives:

- Development of protocols for induction of callus from explants using a range of hormonal combinations in the nutrient media
- Development of protocols for in vitro Micro-propagation of this plant
- Identification of biochemical changes during callus induction and Micro-propagation.

Plant tissue culture is the most effective approach for propagating rare, endangered medicinal and commercially important plant species on a wide scale. The conservation of rare, endangered, medicinal, and commercially valuable plant species must be prioritised. However, for a few medicinal plants, the success rate of propagation using this strategy is variable. Researchers are also having difficulty propagating plant tissues and acclimating in vitro produced plants to their natural environment. There are numerous causes for these issues. This review covers all of the obstacles in this technique, from laboratory setup through field adaption of tissue grown plants, as well as solutions to all of the problems.(Chinnappan.,2018)

Medicinal plants are still the most common source of treatments for a variety of illnesses, and only a small percentage of herbal products come from produced biomass. Wild harvesting is the primary source of therapy ingredients, and such activities jeopardise species diversity as well as the quality and safety of the finished goods. This study aims to demonstrate the importance of incorporating medicinal plants into crops and the utilisation of micropropagation as a strategy for mass-producing high-demand biomass, hence addressing therapeutic natural substance supply challenges. The overview provides instances of in vitro processes and their importance in pharmaceutical, phytomedicinal, and functional food crop development. It also explains how to make high-yielding genotypes, uniform clones from highly heterozygous plants, and identify mutants. It also goes over how to make high-yielding genotypes, uniform clones from highly heterozygous plants, and identify elite phenotypes with bioassays as a selection tool. Finally, we consider the following implications of micropropagation

techniques: a)pharmaceutical crops for the production of small therapeutic molecules (STM), b)phytomedical crops for the production of standardised therapeutic natural products, and c)plant micropropagation for the production of large therapeutic molecules (LTM), such as fructoligosaccharides, which are classified as prebiotic and functional food crops. **(Rita *et al*., 2021)**

Micropropagation is a type of vegetative propagation that takes place in a controlled and aseptic environment within the culture vessel. Which meet all of a plant's growing requirements in natural settings. Different propagation strategies have recently been discovered, which could help with large-scale plant production and species improvement. An overview of meristem culture in vitro propagation. Here you will find information on callus culture, protoplast culture, and other related topics. Micropropagation techniques are being used to grow vast numbers of new high-quality plants in a short amount of time and space at a low cost that may also be preserved. **(Nikita *et al*., 2020)**

Since ancient times, medicinal aromatic herbs have been employed for healing and various uses as sources of secondary metabolics. People have recently preferred medicinal and fragrant herbs to stay healthy. As an alternative to traditional agriculture, plant tissue culture methods offer the potential to develop therapeutic chemicals such as secondary metabolics. The demise of medicinal plants in natural ecosystems is caused by increasing population and desire for herbal remedies, as well as unwitting collection and illegal trading. To protect biodiversity and endangered species, it is critical to produce medicinal and aromatic plants. As a result, plant tissue culture technologies can be used to propagate medically and commercially essential plants, as well as produce

bioactive components for the pharmaceutical sector. generation of therapeutically significant secondary metabolic products (Beutul.,2020)

Since the dawn of human existence, medicinal plants have piqued man's interest. The vast majority of the world's population continues to rely on traditional medicines for their basic health care needs; yet, demand for herbal medicines has increased, making it extremely difficult to meet demand from field plants. As a result, in vitro plant production by tissue culture can be employed to meet the demand for medicinal plants. Because tissue culture procedures are costly, we attempt to summarise the data for a low-cost strategy for in vitro plant micropropagation using tissue culture. This will aid researchers who are developing tissue culture methods for medicinal plant in vitro growth. (Sahu *et al* .,2013)

Agriculture, horticulture, forestry, and plant breeding have all made extensive use of plant tissue culture. It is a type of applied biotechnology that is utilised for plant in-vitro cloning, viral eradication, and secondary metabolite production. Plant tissue culture has recently been employed for short and medium term conservation, also known as slow growth, and cryopreservation, also known as long term conservation, of endangered plant species. These strategies were effectively employed to protect plant species with resistant seeds or dormant seeds, and they outperformed traditional conservation methods. (Ojo *et al* .,2018)

Many countries and cultures use medicinal plants as a source of medicine. Tissue culture is an important biotechnological tool for medicinal plant genotype selection, multiplication, and conservation. Furthermore, in-vitro regeneration is critical in the development of high-quality plant-based therapy.

Plant tissue culture techniques provide an integrated approach for the mass production of consistent plant material for physiological characterization and analysis of active components, resulting in standardised grade phytopharmaceuticals. Several medicinal plants have been known to regenerate in vitro from various parts, although few are grown in soil, and mass micropropagation has seldom been achieved. Several micropropagation procedures for cloning medicinal plants have been devised employing various amounts of Murashige and Skoog medium variants with plant growth regulators (Murashige and Skoog, 1962). Organogenesis and embryogenesis were used to regenerate in response to auxins and cytokinins. Tissue culture for medicinal usage is also becoming familiar with the synthesis of secondary metabolites. Culture systems' integrated approaches will serve as the foundation for the future development of safe, effective, and high-quality consumer products. **(Lemma *et al.*, 2020)**

Growers can now use well-developed procedures to suit the demands of the pharmaceutical sector in the twenty-first century. Micropropagation of medicinal plants is widely employed in the herbal and pharmaceutical industries to produce active chemicals. Overexploitation and habit destruction of wild medicinal plant supplies are being caused by population increase, urbanisation, climate change, and unrestricted gathering of medicinal plants from nature. Culturing techniques are also used to conserve genetic elements of several fragile medicinal plants. Micropropagation procedures for a wide range of therapeutic plants, including endangered and vulnerable plant species, have been created. Only the role of in vitro propagation techniques in medicinal plants is discussed in this review. **(Chatterjee and Ghosh,2020)**

Bioactive substances are found in Rejasa. Callus cultures can be used to manufacture bioactive chemicals. The growth of rejasa callus in various types and quantities of growth regulators will be investigated in this study. The types and amounts of growth regulators (2,4-dichlorophenoxyacetic acid (2,4-D) and Picloram) are the study's independent variables. Callus growth is a dependent variable. The percentage of explants that formed callus, callus formation period, and callus morphology were used to calculate callus growth. Young petioles were used as explants, which were cultivated on Murashige and Skoog solid medium with different concentrations of 2,4-D and Picloram. The results revealed that explants grown on media with 3.5 ppm 2,4-D had the lowest proportion of callus formation (27 percent). The explants that were grown on the medium with 3.5 ppm picloram added had the highest callus growth percentage (93 percent). Explants grown on MS media supplemented with 3.5 picloram demonstrated the fastest callus induction, with an average of 29.9 days. The callus is generally brown, but certain treatments result in green callus. According to the findings of this investigation, MS medium with 3.5 ppmpicloram is the optimal medium for inducing rejasa callus. (Habibah *et al.*,2019)

The medicinal tree Crataevatapia L. is harvested in large quantities by pharmaceutical businesses (Family Capparaceae). This tree is generally propagated by seeds, however this is hampered by poor seed germination caused by the presence of a hard seed coat and significant insect infestation. Plant cultivation must be encouraged in order to supply the ongoing need for raw materials. A callus induction technique was designed employing leaf, stem, petiole, and petiolate explants from field grown Crataevatapia L. and inoculated on MS, B5, and WPM media fortified with two PGRs, 2,4-D (0.5-2.5 mg/l), BAP (0.5-2.5 mg/l),

and CM (5-15 percent) for callus induction. The greatest of the three media used on MS medium, callus response was obtained using leaf and stem explants, as well as BAP (0.5-2.5 mg/l) and 2,4-D (0.5-2.5 mg/l) in combination with 10% CM. The results of this study can be exploited for micropropagation and in vitro production of commercially important secondary metabolites employing callus. This could aid in preventing uncontrolled overexploitation of this tree species, allowing the plant to be preserved in its native habitat. (Priyanka *et al.*, 2017)

Due to overexploitation by pharmaceutical firms, Atropa acuminata, an essential medicinal plant belonging to the Solanaceae family, is facing extinction in its natural environment. The goal of this work is to establish callus cultures of this essential medicinal plant, as callus has a lot of potential as a substitute for producing secondary metabolites for industrial application, thus lessening pressure on natural populations. Atropa acuminate leaf and root explants were used to create callus cultures. Callus induction was performed using Murashige and Skoog (MS) media with various concentrations and combinations of 6-Benzyl Amino Purine (BAP), Naphthalene Acetic Acid (NAA), Kinetin (Kn), and 2,4- Dichloropheoxyacetic acid (2,4- D). Different phytohormonal combinations produced various callus forms and degrees. BAP and NAA in combination on the most effective combination and concentration for in vitro callus development from root explants was MS media supplemented with 0.5mg/l BAP in combination with 1.0mg/l NAA, and the most effective combination and concentration for in vitro callus development from leaf explants was 1mg/l of both BAP and NAA. The maximum callus fresh weight obtained from root explants was 33.13 mg per explant, while the maximum fresh weight obtained from leaf explants was 22.14 mg per explants. (S.A.Dar *et al.*,2021)

One of the tissue culture procedures is induction callus. Plant Growth Regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D) and coconut water can be used to encourage callus production by adding them to the media. The goal of this study was to find the optimal medium formulation for inducing and growing callus from a stem mangosteen explant (Garcinia mangostana L.). With 12 treatments and 3 replications, a completely randomised design was used with two factors: 2,4-D concentration (0, 1, 2, and 3 ppm) and coconut water (0, 15, and 30 percent). The callus development period (weeks), callus fresh weight (g), and callus shape were all measured in this study (colour and texture). After 12 weeks of observation, the results revealed that adding 2,4-D and coconut water to the media can cause callus growth in a stem mangosteen explant The optimum treatment combination for the production of mangosteen callus was 2 ppm 2,4-D and 15% coconut water, which produced yellow and compact callus. This concentration was suggested for growing the mangosteen (*Garcinia mangostana L.*) callus, and it could be useful for disease-free and healthy plant materials, as well as genetic transformation and secondary metabolite production of mangosteen (*Garcinia mangostana L.*) using biotechnological methods. **(N.H.Nasution and I.W.Nasution, 2019)**

Plants are essential to both human and animal life. When compared to manufactured chemicals, plants are a preferable alternative for medical uses, and nature has offered a variety of medicinal plants. The night blooming flowering plant Jasminum grandiflorum Linn. (family Oleaceae) is a major source of methyl jasmonates, which are used in plant fragrance plant Jasminum grandiflorum Linn. is native to tropical and warm temperate climates, and it has been found to have beneficial characteristics that can be utilised to treat a variety of diseases. The

plant's leaves are used in Ayurvedic medicine to treat wounds. The plant's blossoms are used to embellish women's hairstyles. This article contains a an attempt has been made to offer an updated review of this plant, with a focus on chemical constituent isolation and quantification, therapeutic potential, and patents on medicinal and cosmetic compositions including Jasminum grandiflorum Linn. **(Bharathi et al .,2020)**

The goal of this study was to figure out which auxins and cytokinins to use and at what concentrations to get good switchgrass culture initiation and plant regeneration (Panicum virgatum L.). Picloram or 2,4- dichlorophenoxyacetic acid (2,4-o) in conjunction with benzyladenine were used to culture whole caryopses and immature seedling explants on MS media. The best results from mature caryopses were from a combination of 11.3-45.0 pM 2,4-n and 15.0 or 45.0 pM benzyladenine (BA). After the second transfer to regeneration media, several treatments yielded more rhan i00 plunt per embryogenic callus (90 days after initiation of the cultures). Both auxins were used to induce regeneration in immature seedling explants. Picloram, on the other hand, was more effective than 2,4-D over a wider range of BA concentrations. Hundreds of plants were regenerated using protocols created during this work and were easily grown in the field. **(P.D.Denchew and B.V.Conger)**

Plant tissues and organs are grown in vitro on artificial media that provide the required nutrients. A comprehensive blend of nutrients and growth regulators is called a culture medium. Cultured plant cells have nutritional requirements that are extremely similar to those of entire plants. The nutritional requirements of plants growing in soil, and later nutrient solutions used for whole

plant culture, appear to have provided the initial clue for establishing a basic culture medium. Mineral salts are dissociated and ionised when they are dissolved in water. The ions of various sorts, rather than the molecules, are the active agent in the medium. More than one salt in the medium may provide one sort of ion. As a result, a valid comparison between On the basis of total concentrations of different types of ions, two media can be created. Murashige & Skoog's formulation is the most often used medium (1962). Murashige and Skoog (1962) (MS) or Linsmaier and Skoog (1965) (LS) salt compositions are the most extensively utilised, especially in operations involving plant regeneration. Introduction The genetic make-up, surrounding environment, and composition of the culture medium all influence an explant's growth, development, and morphogenic response in culture. The choice of culture medium is critical to the outcome of a plant tissue culture experiment. A culture medium contains all of the necessary nutrients and growth regulators. The key to creating a basic culture medium appears to have come first derive from the nutritional needs of soil-grown plants, and then from nutrient solutions utilised in whole-plant culture. The nutritional needs for optimum tissue growth in vitro differ by species. Even different tissues from different regions of a plant may have varied development requirements (Murashige and Skoog) (1962). Murashige and Skoog (1962) (MS) or Linsmaier and Skoog (1965) (LS) salt compositions are the most extensively utilised, especially in operations involving plant regeneration. (Emiru.,*2020*)

Modern biotechnology techniques, like as in vitro micropropagation, have enormous potential for producing high-quality plant-based medication. They also enable for large-scale disease-free plant multiplication, quicker cloning, and the preservation of desirable genotypes in a

short amount of time. Modification of both the genetic information of MAPs and the regulation of genes responsible for the synthesis of useful biologically active chemicals has also become possible via genetic transformation techniques, in either bigger levels or with superior qualities. Many plant species cultivated in vitro require micropropagation techniques to provide macro- and micronutrients, vitamins, a source of carbohydrates, proper environmental conditions (light intensity, photoperiod, and temperature), and plant growth regulators to achieve high regeneration rates. As They are intended to make economically viable micropropagation easier. To manufacture pharmacologically significant secondary metabolites, well-defined cell culture methods have also been devised. Transgenic therapeutic plants and metabolites are created using genetic engineering. This chapter provides a quick overview of the current state of MAP biotechnology. In vitro micropropagation and rapid clonal multiplication of selected elite genotypes, organogenesis and somatic embryogenesis regulation, exploitation of somaclonal variation advantages, and genetic engineering techniques for crop improvement and in vitro germplasm conservation have all received special attention. The generation of flavour and volatile elements in tissue cultures, as well as the use of other biotechnological methods, will be briefly reviewed. **(Akos.,2015)**

Ziziphora tenuior L. (Lamiaceae) is a fragrant herb with antifungal and antibacterial properties. Micropropagation can be used to increase the number of essential oil producing plants on a wide scale, preventing overexploitation of natural resources. The goal of this study is to create a viable in vitro propagation strategy for Z. tenuior and compare the antioxidant activity of in vitro grown and wild plants. After 45 days of incubation in the dark on medium

supplemented with 1.5 mg/L of NAA, the explants were sterilised and cultured on MS medium containing different concentrations of growth regulators naphthalene acetic acid (NAA) or indole-3-butyric acid (IBA) with 0.5 mg/L of kinetin (Kin) callus formation was 70.2 percent. After a month of callus cultivation on medium containing 2 mg/L BA, the For the multiplication step, the shoot number was 5.12. On medium supplemented with 1 mg/L Kin + 0.1 mg/L NAA, the shoot number was 4.21 and the length was 6.17 cm. The antioxidant activity was determined using the DPPH reagent. The IC50 of aqueous and methanol extracts of in vitro plants treated with 1.5 and 1 mg/L of kin plus 0.1 mg/L of NAA was 0.307 and 0.369 mg/ml, respectively, whereas the IC50 of aqueous and methanol extracts of wild plants was 0.516 and 9.229 mg/ml, respectively. Plant growth regulators and in vitro culture conditions boosted antioxidant activity, according to our findings. **(A.Dakah *et al*.,2014).**

MATERIALS AND METHODS

Medicinal plants are commonly referred to as "Chemical Goldmines" because they contain natural compounds that are safe for humans and animals. All of these compounds are impossible to make in a lab. Many plant secondary metabolites are commercially relevant and are used in a variety of pharmaceuticals. Since the dawn of civilization, humans have relied on plants to meet their health-care needs. More than 80,000 of the 2,50,000 higher plant species on the planet are therapeutic. Approximately 5000 species are extensively used in traditional medical systems and are examined in depth. Over 95% of the plants utilised in the herbal and pharmaceutical industries are harvested from the wild. Given the worrisome rate of biodiversity loss owing to various well-known reasons, as well as the indiscriminate collecting of wild medicinal plants, many of our medicinal plant species are in grave risk of extinction. In the face of a significant danger to biodiversity, it is critical to take immediate actions to conserve and enhance medicinal plant genetic resources in all of our different agro-ecosystems, as well as their cultural roots.

For the present study *Jasminum auriculatum* is proposed to be used as experimental material. *Jasminum auriculatum* is a climber of tropical Asia and belongs to the family Oleaceae.

Plant Description:

It is an evergreen climber. Leaves are oppositely arranged along the stem, trifoliolate (sometimes simple), grey pubescent (hairy), petioled. Leaflets are ovate in shape, with rounded base and acute apex. Lateral leaflets are smaller than terminal leaflet. Softly hairy, or almost glabrous (smooth). Branches are tomentose (covered with densely covered with short wooly hairs). Flowers are borne on an inflorescence,

located terminally at side shoots, consisting of at least 5 flowers per cluster. Flowers are white, fragrant, measuring about 2 cm. Calyx are hairy, tubular with tiny lobes. Corolla tube is 1.5 cm long, with 5 - 7 elliptic lobes. Black berries, globose, measuring about 5 mm.

Standardization of micro propagation techniques:

Culture parameters

Glass wares and Equipment:

Culture vessels employed were 100ml, 250ml Borosil Erlenmyer flasks, culture tubes (150 x 25mm) and petri-dishes. Other glassware used for media preparations were measuring cylinders, pipettes, beakers etc. and equipments used were pH meter, electronic balance, hot plate, autoclave, forceps, needles, inoculation loops, scalpels, spatula and spirit lamp.

Chemicals

All the chemicals used for preparation of basal medium as well as for biochemical analyses, were procured from Qiteus chemicals, Tamil Nadu, India and vitamins and plant growth regulators from the same.

Culture medium

Murashige and Skoog's medium (1962) and Woody plant medium (Mc Cown and Llyod,1981) were used as basal medium. The composition of these medium are presented in the table-2&3. 3.18.4 Cleaning of glass ware All culture vessels used during experiments were cleaned with chromic acid solution and then cleaned with detergent (Teepol 0.1%) and thoroughly washed with running tap water, rinsed twice with double distilled water and sterilized in dry air oven at l800C for 24 hours.

COMPOSITION OF MURASHIGE AND SKOOG MEDIUM (1962)

ESSENTIAL ELEMENTS	CONCENTRATION IN MEDIUM (mg/l)	1000ML (g)	500ML (g)	250ML (g)
Macro elements MSA				
NH_4NO_3		33.000	16.5	8.25
KNO_3		38.000	19.0	9.5
$CaCl_2.7H_2O$		8.800	4.4	2.2
$MgSO_4.7H_2O$		7.400	3.7	1.85
KH_2PO_4		3.400	1.7	0.85
Micro elements MSB1				
KI		1.660	0.83	0.415
Micro elements MSB_2				
H_3BO_3		0.248	0.124	0.062
$MnSO_4.4H_2O$		0.892	0.446	0.223
$ZnSO_4.7H_2O$		0.344	0.172	0.086
$Na_2MoO_4.2H_2O$		0.010	0.005	0.0025
$CuSO_4.5H_2O$		0.001	0.0005	0.00025
$CoCl_2.6H_2O$		0.001	0.0005	0.00025
Iron source MSC				
$FeSO_4.7H_2O$		5.560	2.78	1.39
$Na_2EDTA.2H_2O$		7.460	3.73	1.865
Vitamins B5				
Myoinositol		20.000	10.0	5.0
Nicotinic acid		0.200	0.1	0.05
Pyridoxine – HCl		2.000	1.0	0.5
Thymine – HCl		0.200	0.1	0.05
Carbon source				
Sucrose		30.000	15.0	7.5
Agar		8.000	4.0	2.0

Preparation of culture media

Murashige and Skoog's medium was used as basal medium through out the present tissue culture studies. The component of medium is given in the table-2&3. Stock solutions like A, B, C, D, E, F and G were prepared according to composition of nutrient medium in double distilled water and stored at 10°C in dark in a refrigerator. The medium was supplemented with 3% w/v sucrose and 100mg/l of meso-inositol. Similarly, growth regulators were prepared as stock solutions in 20 ml of double distilled water. The auxins were dissolved first in few drops of absolute alcohol and final volumes were made add double distilled water to 20 ml stored at low temperature. For the preparation of semi solid medium 0.8%(w/v) agar was added as gelling agent into medium. The pH of all media was adjusted to 5.6-5.8 by using 0.1N HCl or 0.1N NaOH before dispensing a known volume of medium into culture vessels. The amount of medium dispensed into the various culture vessels was 20 ml/tube (15 and 25mm), 30ml/100ml flask for cultures. The culture vessels were plugged with non- absorbent cotton and wrapped with paper or aluminum foil. 3.19.2

Autoclaving:

The prepared media, glass ware, forceps, scalpels, filter papers, double distilled water were wrapped/covered with aluminum foil and carefully autoclaved at 121 oC for 20 minutes in a vertical autoclave at 1.05 kg/cm (15psi).

Surface sterilization of explant

Different explants were treated with 0.1% HgCl2 solutions for 2-3 minutes followed by rinsing 3-4 times with sterile double distilled water.

Inoculation

The working table of laminar airflow chamber was cleaned with 70% alcohol. Before carrying out operations the cabinet as well as media vessels, sterilized petriplates, instruments like scalpel, forceps, inoculation loop, spatula etc. were irradiated in U.V. light for 30 minutes. These implements were sterilized by flaming with 90%alcohol inside laminar airflow chamber before use, while not in use these were kept immersed in alcohol. Both incubation and inoculation chambers were sterilized by fumigation twice in a month from fumes obtained by potassium permanganate with formaldehyde and with the help of bromine liquid capsules. All inoculations were carried out in laminar airflow chamber.

Incubation of cultures

The cultures were incubated in culture room and provided with white light of 1400 lux intensity with 16/8 h light/dark cycles at 25 ± 20C (temperature maintained by automated photoperiod controlled device and air conditioner or room heater).

Callus Culture

Culture Establishment Medium

Explant In all the selected plants young leaf and stem were used as explants. The explants were inoculated for establishing callus culture. The callus induced from different explants was maintained on the same medium for minimum of 2-3 passages and was used for subsequent experiments callus was transferred on MS medium supplemented and woody plant medium, auxin viz. 2,4-D and cytokinin viz. Kn BAP in combination.

Qualitative analysis

1.Test for alkaloids

Wagner's test: About ten mg of extract was taken and few drops of Wagner's reagent was added and the formation of a reddish brown precipitate indicates the presence of alkaloids.

2.Test for flavonoids

To 2 ml of extract, 1 ml of 2N NaOH was added. The presence of flavonoids indicates the formation of yellow colour.

3.Test for tannins

Ferric chloride test: Five mg of extract was taken and 0.5 ml of 5% ferric chloride was added. The development of dark bluish black colour indicates the presence of tannins.

4. Test for steroids and sterols

Salkowski's test: Five mg of extract was dissolved in 2 ml of chloroform and equal volume of concentrated sulphuric acid was added along the sides of the test tube. The upper layer turns red and lower layer turns yellow with green fluorescence, indicating the presence of the steroids and sterols compound, in the extract.

5. Test for Carbohydrates

Fehling's test : Five ml of Fehling's solution was added to 0.5 mg of extract and boiled in a water bath. The formation of yellow or red precipitate indicates the presence of reducing power.

6.Test for protein

Ninhydrin test: About 0.5 mg of extract was taken and 2 drops of freshly prepared 0.2% ninhydrin reagent was added and heated. The appearance of pink or purple colour indicates the presence of proteins, peptides or amino acids.

7. Test for Glycosides

Glycoside test: 0.5 mg of extract was dissolved in 1 ml of water and then aqueous NaOH solution was added. Formation of yellow colour indicates the presence of glycosides.

8. Test for Saponins

Foam test: 0.5 mg of extract was diluted with 20 ml distilled water and shaken well in a graduated cylinder for 15 min. The formation of foam to a length of 1cm indicated the presence of saponins and steroids.

9.Test for quinine

To 1 ml of extracts and 1 ml of con. Sulphuric acid was added. Appearance of red colour indicates the presence of quinine.

10.Test for phenol

To 1 ml of the extract, 2 ml of distilled water was addedfollowed by few drops of 10% aqueous ferric chloride. Appearance of blue or green colour indicates the presence of phenols.

11.Test for coumarin

To 1 ml of extract, 1 ml of 10% sodium hydroxide was added. The presence of coumarin indicates by formation of yellow colour.

12. Test for Anthraquinone

Borntragers test : About 0.5 ml of the extract was taken into a dry test tube and 5 ml of chloroform was added and shaken for 5 minutes. The extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution. A pink violet or red colour in the lower layer indicates the presence of anthraquinone.

13.Test for terpenoids

To 0.5 ml of extract, 2 ml of chloroform and con. Sulphuric acid was added. Formation of red brown colour indicates the presence of terpenoids.

14.Test for betacyanin

To 2 ml of extract and 1 ml of 2% sodium hydroxide heated for 5 minutes at 100c. formation of yellow colour indicates the presence of betacyanin.

RESULTS AND DISCUSSION

Establishment of Callus Culture.

Callus growth time data and the percentage of callus explants are presented in Table 1 & 2. The time of callus induction and callus growth on the young petiole explants varied greatly depending on growth regulators. The results showed that the lowest percentage of callus formation observed in explants grown on medium supplemented with 3.5 ppm 2,4-D (27%). The explants grown on medium with the addition of 3.5 ppm picloram showed the highest callus growth percentage (93%). The time needed for the explants for callus formation ranges from 20-35 days after planting. The best average time of callus induction occurs in explants grown in 3.5 ppm picloram treatment ie 20.9 days after planting. Explants grown on medium with the addition of 2,4-D, 2.5 ppm showed the slowest growth These results indicate that the callus growth rate from *J.auriculatum* young petiole explants is influenced by the type and concentration of growth regulator.

This reinforces the results of Neibauret *et al.* 2008 who reported that the type and concentration of Plant Growth Regulator (PGR) had a significant effect on callus formation in *Paspalum vaginatum*. The same thing was also reported by Xu *et al*. 2009 in *Juncus effuss* L. The results of this studied reinforce that types of plant growth regulators (auxin and cytokinin) are important for increasing the percentage of callus formation and callus growth (Zerk, 1978). Auxin and cytokines also have an important role in the transition from the G2 to M phases because these hormones activate CDC25-like phosphatase involved in cell cycle transitions Pereira (2012).

Table 1: Effect of MS media with 2,4,D in callus initiation

S.No.	Growth Regulator concentration (ppm)	Days taken to form callus	Callus induction frequency (%)
1	1.5	31.4	60
2	2.5	20.9	47
3	3.5	32.0	27

Table 2: Effect of MS media with Picloram in callus initiation

S.No.	Growth Regulator concentration (ppm)	Days taken to form callus	Callus induction frequency (%)
1	3.5	20.9	93
2	5	40.9	53
3	7.5	34.0	33

Callus induction is regulated by a complex cell cycle regulation mechanism. In auxin-induced callus formation, auxin signals are transduced through transcription factors Auxin Response Factor (ARF), especially ARF7 and ARF19, which in turn activates transcription factors that play a key role in cell re-entry into the cell cycle. Cyclin-dependent kinases encoding genes are genes that play an important role in the entry of cells into the cell cycle by pushing cells in the G1 phase into the S phase and the cells in G2 enter the M phase. Growth factors such as auxin, cause the formation of CDKA/CYCD complexes which when active induces the transition from phase G1 to phase S (Inz'e D and Veylder, 2006: Ikeuch et al., 2012).

Callus morphology

Friable callus are the most produced in the treatment as shown in Table 2 and Figure 1. The callus is formed starting at the part that is injured. According to Ahmad *et al*. 2010 cell proliferation in the injured explant section is related to accumulation of auxin at the point of injury, which stimulates cell proliferation by the presence of growth regulating substances. Most callus formed are the yellowish white colour at the beginning of its formation, and it turns into brownish associated with an increased content of phenolic compounds on the callus The callus formed in the wound area is a wound covering cell that proliferates to form a yellowish white cell. The callus some are brown and some are greenish in color. The brown callus occurs because of an increase in the content of phenolic compounds or other secondary metabolites in the callus. Activity of peroxidase paralleled increased browning of callus (Chaudhary and Dantu, 2015). Phenolic compounds react with oxygen with the help of polyphenol oxidase enzymes producing highly reactive ortho-diquinones. Ortho-diquinones react spontaneously with proteins and other cellular components form dark pigments called melanin (Tang and Newton, 2004). According to Karimi et al. 2013, high light intensity

Table 3: Effect of MS media with 2,4,D on callus morphology and callus colour

S.No.	Growth Regulator concentration (ppm)	Callus morphology	Callus colour
1	1.5	Friable	White
2	2.5	Friable	Yellow
3	3.5	Friable	Yellow

Table 4: Effect of MS media with Picloram on callus morphology and callus colour

S.No.	Growth Regulator concentration (ppm)	Callus morphology	Callus colour
1	3.5	Friable	brown
2	5	Friable	brown
3	7.5	Friable	brown

will increase phenolic content. The older the callus age, the higher the phenolic accumulation.

Qualitative analysis:

Preliminary Phytochemical screening of *Jasminum auriculatum* was presented in Table 5. It showed that the plants contain various secondary metabolites including Tannins, Steroids, Coumarin, Alkaloids, Flavones, Anthroquinones, Phenols, Saponins and Quinones. In particular the presence of phenols was noted in all samples. Tannins were also observed to be present in all the plant extracts in this study. This group of metabolites has shown anti bacterial, antidiarrhoeal and antihelminthic properties (Tiwari et al., 2011). Interestingly quinine was found to be negative for all the plant extracts tested. The reason can be that the solvent used was not effective on extracting quinine compound. This was supported by Leksawasdi et al., 2008.

Table 5: Qualitative analysis of *Jasminum auriculatum* petiole and leaf

S.NO	EXPERIMENT	Extract	*Jasminum* leaf	*Jasminum* petiole
1.	Alkaloids	Aqueous	+	+
		Methanol	+	+
2.	Flavonoids	Aqueous	+	+
		Methanol	-	-
3.	Tannins	Aqueous	+	-
		Methanol	+	-
4.	Steroids	Aqueous	-	-
		Methanol	-	-
5.	Glycosides	Aqueous	+	+
		Methanol	-	-
6.	Carbohydrate	Aqueous	+	-
		Methanol	+	+
7.	Protein	Aqueous	+	+
		Methanol	+	+
8.	Saponin	Aqueous	+	+
		Methanol	+	+
9.	Quinine	Aqueous	+	+
		Methanol	-	-
10	Phenol	Aqueous	+	-
		Methanol	+	-
11.	Coumarin	Aqueous	+	+
		Methanol	+	+
12.	Anthraquinine	Aqueous	-	-
		Methanol	-	-
13.	Terpenoids	Aqueous	-	+
		Methanol	+	+
14.	Betacyanin	Aqueous	-	-
		Methanol	-	-

+ indicates presence

- indicates absence

SUMMARY AND CONLUSION

Plant tissue culture is an useful technique used to conserve rare and endangered plants by providing artificial media and environment suitable for the plants to grow. The media provide an ample way, a good replacement with soil nutrients so as to make them grow in the controlled environment.

For the present study, *Jasminum auriculatum*, an evergreen woody climber from the family Oleaceae was selected. It is an ornamental plants grown for recreation purposes. Also it is useful in perfume industries. It has slight medicinal properties too. The preliminary photochemical studies revealed the presence of important secondary metabolites so as they attribute to the medicinal value of the plant.

The tissue culture studies of this plant showed very minimal response when treated with various growth regulators. At the various concentrations, in Picloram and that too in 3.5 ppm only the callus initiation was at good level. And in 2,4,D the callus initiation was very slow. Same goes for the colour and nature of callus. This is all determined by the phenolic compounds present in this sample.

This work is the most primitive level of work where only the callus initiation was observed. This area needed further testing and more research can be done according to the need of the hour.

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STUDY ON THE METABOLITES OF SELECTED AMARANTHUS SPECIES AND ITS RESPONSE AGAINST SALT STRESS

A Short-Term Project Work Submitted to St. Mary's college (Autonomous) Affiliated to Manonmaniam Sundaranar University in Partial Fulfillment for the Degree of

BACHELOR OF SCIENCE IN BOTANY

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CERTIFICATE

It is certified that this short term project work entitled **"STUDY ON THE METABOLITES OF SELECTED AMARANTHUS SPECIES AND ITS RESPONSE AGAINST SALT STRESS"** submitted to **St. Mary's College (Autonomous)** affiliated to **Manonmaniam Sundaranar University** in partial fulfillment of the requirements for the degree of **Bachelor of Science in Botany** and is a record of work done in the Department of Botany, St. Mary's College (Autonomous), Thoothukudi during the year 2019-2020 by the following students.

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CONTENT

INTRODUCTION

Indigenous vegetables have a very significant role in the livelihood of rural people in emerging worlds. In developing worlds, many people in rural areas have less food for their families resulting in deficiency of important nutrients. These rural poverty stricken people depend on locally available indigenous vegetables for income and food, as staple food, during lean seasons. Therefore, indigenous leafy vegetables are valuable sources of both nutrients, as micronutrients and herbal medicines. Indigenous leafy vegetables play important role in being protective foods; used in human health maintenance and disease prevention. Plants produce organic compounds that are not directly used in primary growth and development metabolic processes of plants. These compounds are non nutritive plant secondary metabolites that are also called phytochemicals. These phytochemicals are antioxidant bioactive chemicals that prevent oxidative processes occurrences in animals and plants These essential phytochemicals include saponins, alkaloids, flavonoids, tannins and phenolic compounds fibres and vitamins (**Baang *et al.*, 2015**). They are absorbed by the human body to be utilized as energy sources, body building and protective materials **(Uwah and Ogugbuaja, 2012).** It has high fiber content compared to root vegetables and cereals **(Saidu and Jideobi, 2009).** The high fiber content has been reported to reduce cholesterol levels in the body resulting in low occurrences of cardiovascular diseases **(Chionyedua *et al.*, 2009).** Potassium from leafy vegetables is responsible for preventing body diuretic and hypertensive complications (George, 2003) while oils/fats from vegetables lower blood lipids thereby controlling incidences of coronary diseases **(Adenipenkun and Oyetunji, 2010).** But it is widely acknowledged that

indigenous leafy vegetables have been underutilized with limited knowledge on their nutritional values.

Amaranths are some of the earliest vegetables that have existed, globally, as grains, leafy vegetables, dye plants, ornamentals, and weeds, in tropical, subtropical, and temperate climates. *Amaranthus* is a plant genus comprised of about 74 annual species, with a wide morphological diversity, distinctly characterized by monoecy and dioecy. They are a promising group of plants that could deliver plant-based proteins, high-quality nutrients, unsaturated fatty acids, and other essential organic minerals derived from their leaves, seeds, and roots. Amaranths adapt easily to adverse environmental conditions because they manufacture food through the C4 photosynthetic pathway. They have evolved certain physiological characteristics that make them easily cultivated, allow them to survive attacks from pathogenic organisms, and enhance their phenotypic plasticity and genetic diversity.

Worldwide, soil salinity has adversely affected about 30% of the irrigated and 6% of total land area with a resultant monetary loss of USD 12 billion in agricultural production. High salt content affects plant growth by modifying their morphological, anatomical and physiological traits. Such growth impairment is due to osmotic effects and ionic imbalances affecting plant metabolism. The severity of salt damage has been found to be dependent on species, cultivar and growth stages of the plant **(Hoang *et al.*, 2019).**

Salt stress is a condition where excessive salts in soil solution cause inhibition of plant growth or plant death. On the world scale, no toxic substance restricts plant growth

more than saline stress. Salt stress presents an increasing threat to plant agriculture. **Jacob Levitt (1972)** suggested that biological stress is any change in environmental conditions that might reduce or adversely change a plant's growth or development Salt stress is one of the major environmental constraints limiting agricultural productivity. More than 900 million hectares are affected by high salt concentration in the substrate including half of irrigated areas. Plant growth is compromised by salinity at all developmental stages, but sensitivity varies greatly at different stages. Crop production in saline areas largely depends upon successful germination, seedling emergence and establishment and efficient reproductive phase. *Amaranthus*, collectively known as amaranth, is a cosmopolitan genus of annual, short-lived or perennial plants. Some amaranth species are cultivated as leaf vegetables. *Amaranthus* is a promising crop for semi-arid regions. It exhibits a high nutritive value but also a fascinating ability to adapt to diverse harsh environments. So the present study was focused to identify the phytochemical constituents of the selected *Amaranthus* species and its growth performance against salt stress.

SCOPE AND OBJECTIVES

Over the centuries, people have been dependent on natural resource for their subsistence as they are efficient and cheap sources of several important micronutrients. However, these plant resources and their indigenous use are in danger of being lost in areas where environmental and cultural transformations have led to changes in feeding practices. Many indigenous communities abandon or change their traditional customs and thereby lose their plant knowledge over time. Changes in land-use due to urbanization and habitat destruction, as well as the slash and burn system of traditional farming with its associated shifting cultivation, have been causing forest destruction and degradation. Following are the objectives of the present study.

- Collection of leafy vegetables from Pasuvanthanai
- Phytochemical screening
  - Qualitative analysis
    - Alkaloid
    - Flavanoid
    - Phenol
    - Tannin
    - Steroid
    - Carbohydrate
    - Saponin
    - Glycoside
    - Protein and Amino Acid

- Terpenoid
- Phlobatannin
- Coumarin
- Cardiacglycoside

➢ Quantitative analysis

- Primary metabolites
  - Carbohydrates
  - Proteins
  - Aminoacids
  - Lipids
- Secondary metabolites
  - Phenol
  - Flavanoid
  - Tannin

❖ Study the effect of NaCl stress on the growth performance of the selected *Amarathus* species

REVIEW

Amaranth is an edible plant that has been used by humans for over 4000 years. It is grown and consumed as grain or vegetable crop in Australia, Africa, and Asia. In India , amaranth is commonly consumed as a leafy vegetable. It is a valuable food source of nutrient with high quality of proteins, vitamins, minerals and bioactive compounds such as phenolics **(Gomes *et al.,* 2016).** The Phenolic compounds are well-described secondary plant metabolites, which can play an important role in absorbing and neutralizing free radicals, quenching singlet oxygen and decomposing peroxides. An enhancement of antioxidant activity and the amount of phenolic compounds can be observed under various environmental factors and stress conditions (**Valifard *et al.,* 2014**).

Dietary phytochemicals such as phenolic acids and flavonoids are rich in fruits and vegetables. These phenolic compounds are known as natural secondary plant metabolites that mainly participate in the defense mechanism in the plants and provide protection against abiotic or biotic stress.

The nutraceutical values of amaranths have been reported widely in scientific publications. Findings from different experiments have indicated that *Amaranthus* has a higher proximate composition than commonly consumed food crops, such as corn, buckwheat, rye, and rice **(Rastogi *et al.,* 2013)** comparable nutritive characteristics to commonly patronized vegetables, e.g., spinach (*Spinacia oleracea*) **(Unuofin and Lebelo, 2020)** equivalent nutrient content to some fodder crops, such as barley, maize, and wheat and is rich in extremely rare amino acids (e.g., lysine and tryptophan) that could replace animal protein and supplement human diets with

moderate-quality amino acids **(Salvamani *et al.*, 2016).** Metabolic diseases, such as diabetes, ulcers, congestive cardiac, liver, and renal failure, cancer, helminthic infections, and most degenerative diseases, such as ageing hypertension, atherosclerosis, obesity, and being chronically underweight are induced by damages done to cells and tissues by free radicals. Several species of *Amaranthus* are reported to play important roles in the regression of oxidative stress-induced disorders due to their ability to scavenge free radicals, thereby neutralizing their degenerating consequences **(Venskutonis and Kraujalis, 2013).**

Amaranthus tricolor linn plant commonly known as Lalshaak, tandalijo or tandaljobhaji. In telgu and Andhara commonly known as peruguthotakura" is an plant belonging to the family Amaranthcea. It is annual erect stem stout, diffuse herb Attaining 1-2 m high, usually much branched. roots having tap root shape cylindrical, yellow in colour with roots lets. stem purplish pink with ridges and furrous, fracture, short, leaf simple 4-11 cm long, 2 – 8 cm wide very variable in shape. rhomboid, ovate, lanceolate, obtuse apex, pentiolate, memberanous and purplish pink seed 1.5 mm in diameter biconvex, smooth, shiny black coloured, infloresene an axillary, globase cluster upto 2.5 cm in diameter, the upper cluster sometimes forming a terminal spike with male and female flower intermixed : brown or shiny black seed faintly reticulate **(Kumar *et al.*, 2019)**. *Amaranthus tricolor* have excellent nutritional value because of their higher essential micronutriernts such as iron, calcium, zinc, vitamin C and vitamin A. The plant is a laxative and used in the treatment of piles and blood disorders, stomachic appetizer, toothache antipyretic, dysentery, astringent, diuretic, haemorrhagic colitis and hepato-protective agent. Carbohydrates, protiens, Aminoacids, steroids, cardiac glycosides, alkaloids, tannins, and Flavanoids

phytochemicals was found in *Amaranthus tricolor L*. The Presence of tannins and flavonoids exhibited various biological activities such as antibacterial, antifungal, antioxidant and anthelmintic (**Sowjanya et al., 2017)**.

A number of phytochemicals including flavonoids, alkaloids, tannins, phenolics, saponins, glycosides have been isolated from various *Amanthus* sp. such as *A. caudatus, A. hypochondriacus,* and *A. cruentus* **(Pa´sko et al., 2009)**.

Extracts of all plant parts of *Amaranthus* seem to have medicinal benefits; hence the focus of recent studies has been to identify therapeutic constituents of *Amaranthus* from different bioparts. High antioxidant activity of most *Amaranthus* spp., along with anti-inflammatory property, has increased interest in investigating its nutraceutical and clinical potential as functional food. Phytochemical analysis of aerial parts of various *Amaranthus* spp. has established presence of active constituents like alkaloids, flavonoids, glycosides, phenolic acids, steroids, saponins, amino acids, vitamins, minerals, terpenoids, lipids, betaine, catechuic tannins and carotenoids **(Nana *et al.,* 2012)**. Also documented are amaranthoside, a lignin glycoside; amaricin, a coumaroyl adenosine, betalains such as betacyanins (amaranthine, isoamaranthin), betaxanthin; hydroxycinnamates, quercitin and kaempferol glycosides as flavonoids and phenolic acids (**Stintzinga *et al.,* 2004)**. Amaranth extracts isolated sequentiall by acetone and methanol/water from defatted plant leaves, flowers, stem and seeds yield rutin, nicotiflorin, isoquercitrin, 4- hydroxybenzoic and p-coumaric acids as major components (**Kraujalis etal., 2003)**. In species specific studies, evaluation of bioactive substances and phenolic contents of *A. tricolor* and *A. hypochondriacus* leaves revealed high content of betacyanins and betaxanthins while isoquercetin and rutin were the most abundant flavonoids; salicylic, syringic, gallic,

vanilic, ferulic, p-coumaric, ellagic and sinapic acid were the most common phenolic acid . In addition to the known betalains, red-violet amaranthin, a novel betaxanthin, methyl derivatives of arginine betaxanthin and betalamic acid were detected in A. tricolor leaves. Rutin and quercitin content was determined in individual plant parts of five Amaranthus spp. (**Biswas et al., 2013)**

Amaranthus leaves sampled at maturity stage contained quercetin or quercetin derivatives. *A. cruentus* was the best source of rutin while amount of rutin and quercetin in methanolic leaf extract of *A. viridis* was found to be 58.52 and 9.12% w/v respectively **(Kumar *et al.,* 2009)**. In an exclusive study, **Jhade *et al.* (2011**). reported alkaloids, glycosides, terpenes, and sugars as the major phytochemicals in roots of A. spinosus.

A study by **Dlamini *et al***. (concluded that A. cruentus is potentially a good dietary source of the pro-vitamin A carotenoid (bcarotene). Carotenoid content was highest in leaves, followed by seeds, stem and roots. The major carotenoid identified in the leaves was canthaxanthin (antitumor agent), followed by b-carotene and lutein (retardant for age related eye diseases). The level of bcarotene (28.5 mg/100 g) in *A. cruentus* was seven times higher than in tomatoes and thus may help to treat anaemia in African countries. In a comparative study, calcium content in dry leaves of *A. spinosus* was found to have higher value (4500 mg/100 g dry weight) followed by *A. tricolor, A. viridis* and *A. blitum*, while iron content was maximum in A. viridis (15 mg) followed by *A. spinosus*, *A. tricolor* and *A. blitum*. Thus *Amaranthus* spp. can be used as a source of biogenic calcium and in antacid preparations (**Srivastava, 2011).**

The studies mentioned above conclusively indicate that *Amaranthus* spp. contains appreciable amount of nutrients and can be included in diet to supplement daily

nutritional requirements to combat diseases, hence serving as a nutraceutical for fortification of food.

MATERIAL AND METHODS

Plant Collection

Five indigenous leafy vegetables of *Amaranthus* species such as were collected from Pauvanthanai village, Thoothukudi Distict, Tamilnadu. The leafy vegetables were collected during the month of February 2022. It was shade dried and made into powder and used for further analysis.

Sample I : *Amaranthus caudatus*

Sample II : *Amaranthus dubius*

Sample III : *Amaranthus crutens*

Sample IV : *Amaranthus viridis*

Sample V : *Amaranthus campestris*

DESCRIPTION OF THE PLANT

AMARANTHUS CAUDATUS

Kingdom: Plantae

Order : Caryophyllales

Family : Amarantheaceae

Genus : *Amaranthus*

Speceis : *caudatus*

Usefullparts: Whole plant

Large, erect annual herbs.

The whole plants red or purple suffused.

Leaves 3-15 x 2-7 cm, ovate-oblong or rhomboid-ovate, base cuneate to attenuate, apex obtuse to subacute, mucronate; petiole to 7 cm long. Flowers in axillary and terminal red or green panicled spikes, to 20 cm long; male and female flowers intermixed throughout the spikes. Bract ovate, caudate-acuminate, aristate.Flowers unisexual.Perianth 5, 2-3.5 mm long, oblong-elliptic, aristate. Stamens 5 in male.Stigma 3-lobed, erect or flexuose. Utricle c. 2 mm long, ovoid-globose, circumscissile. Seeds 1-1.5 mm across, lenticular, compressed, black, shining.

AMARANTHUS DUBIUS

Kingdom:Plantae

Order : Caryophyllales

Family : Amarantheaceae

Genus : *Amaranthus*

Species : *dubius*

Useful parts: Leaves

Erect annual herb, up to 150 cm tall; stems slender to stout, branched, glabrous or upwards, especially in the inflorescence, with short to rather long hairs.Leaves arranged spirally, simple, without stipules. petiole up to 8.5(–12) cm long; lamina ovate or rhomboid-ovate, 1.5–12(–22) cm × 0.7–8(–14) cm, cuneate at the base, blunt or retuse at apex, mucronate, entire, glabrous or shortly pilose, sometimes the centre of the lamina blotched red. Inflorescence spike like or paniculate, axillary and terminal, the terminal one up to 25cm long, consisting of glomerules more or less isolated at base of inflorescence and agglomerated towards apex. bracts up to 2.5 mm

long, awned. Flowers unisexual, subsessile, with (4–)5 tepals up to 2.5 mm long. Male flowers usually near apex of inflorescences, with 5 stamens c. 2 mm long; female flowers with superior, 1-celled ovary crowned by 3 stigmas. Fruit an ovoid-urceolate capsule c. 1.5 mm long, with a short inflated beak below the stigmas, dehiscing circularly, the lid strongly rugulose below the beak, 1-seeded.Seed lenticular, compressed, c. 1 mm long, black.

AMARANTHUS CRUTENS

Kingdom: Plantae

Order : Caryophyllales

Family : Amarantheaceae

Genus : *Amaranthus*

Species : *crutens*

Useful Parts:both for their leaves and for their edible seeds.

Amaranthus cruentus is a tall annual herb topped with clusters of dark pink flowers. The plant can grow up to 2 m (6 ft) in height, and blooms in summer to fall. It is believed to have originated from *Amaranthu shybridus*, with which it shares many morphological features.

AMARANTHUS VIRIDIS

Kingdom: Plantae

Order : Caryophyllales

Family : Amarantheaceae

Genus : *Amaranthus*

Species : *viridis*

Useful parts :Leaves

Amaranthusviridis is an annual herb with an upright, light green stem that grows to about 60–80 cm in height. Numerous branches emerge from the base, and the leaves are ovate, 3–6 cm long, 2–4 cm wide, with long petioles of about 5 cm. The plant has terminal panicles with few branches, and small green flowers with 3 stamens.

AMARANTHUS CAMPESTRIS

Kingdom : Plantae

Order : Caryophyllales

Family : Amarantheaceae

Genus : *Amaranthus*

Species : *campestris*

Useful parts: Whole plant as leafy green vegetables and seed used directly or as flour.

Amaranth is a herbaceous plant or shrub that is either annual or perennial across the genus. Flowers vary interspecifically from the presence of 3 or 5 tepals and stamens, whereas a 7-porate pollen grain structure remains consistent across the family. Leaves are approximately 6.5–15 centimetres (2+½–6 inches) and of oval or elliptical shape that are either opposite or alternate across species, although most leaves are whole and simple with entire margins.

QUALITATIVE ANALYSIS

Phytochemical constituents were analyzed using aqueous and methanol extracts. Standard procedures were followed **(Horbone1984, Kokate *et al*., 1995, Harborne, 1998).**

Test for Alkaloid (Wagner's test):

About 1 ml of extract was taken and few drops of Wagner's reagent was added and the formation of a reddish brown precipitate indicate the presence of alkaloids.

Test for Flavanoid (Shinoda Test):

About 1 ml of extract was added to pinch of magnesium turnings and 1-2 drops of concentrated hydrochloric acid was added. Formation of pink color indicated the presence of Flavanoids.

Test for Phenol (Lead acetate test):

About 1 ml of extract was taken and 0.5 ml of 1% lead acetate solution was added and the formation of precipitate indicated the presence of tannins and phenolic compounds.

Test for Tannin (Ferric chloride test):

About 1 ml of extract was taken and 0.5 ml of 5% ferric chloride was added. The development of dark bluish black color indicated the presence of tannins.

Test for Steroid and Phytosteroid:

About 1 ml of extract was dissolved in 2 ml of chloroform and equal volume of concentrated sulphuric acid was added along the sides of the test tube. Appearance of brown ring indicated the presence of steroids. Appearance of blush brown colour indicated the presence of phytosteroids.

Test for Carbohydrate (Benedict's test):

About 5 ml of Benedict's solution was added to 1 ml of extract and boiled in water bath. The appearance of red or yellow or green precipitate indicated the presence of reducing sugars.

Test for Saponin (Foam test):

About 0.5 mg of extract was diluted with 20 ml distilled water and shaken well in a graduated cylinder for 15 min. The formation of foam to a length of 1cm indicated the presence of saponin.

Test for Glycoside:

About 0.5 ml of extract was dissolved in 1 ml of water and then aqueous NaOH solution was added. Formation of yellow color indicated the presence of glycosides.

Test for Protein & Amino Acid (Ninhydrin test):

About 0.5 ml of extract was taken and 2 drops of freshly prepared 0.2% ninhydrin reagent was added and heated. The appearance of pink or purple colour indicated the presence of proteins, peptides or amino acids.

Test for Terpenoid:

Five ml of the extract was mixed with 2 ml of chloroform and concentrated sulphuric acid to form a layer. A reddish brown coloration of the interface showed the presence of terpenoids.

Test for Phlobatannin

Sample was boiled with 1% aqueous hydrochloric acid to produce red precipitate indicated the presence of phlobatannins.

Coumarin:

About 3 ml of 10% NaOH were added to 2 ml of plant extracts. The formation of a yellow colour was an indication for the presence of coumarins.

Cardiacglycoside (Keller-Killani Test):

Two ml of plant extract were treated with 2 ml glacial acetic acid containing a drop of $FeCl_3$. A brown coloured ring or brown-violet under a brown greenish layer indicated the presence of cardiac glycosides.

QUANTITATIVE ESTIMATION OF NUTRIENT COMPOSITION

Total Soluble Protein (Lowry *et al*., 1951)

Requirements:

- Alkaline copper reagent
- Solution A- 20% Sodium carbonate in 0.1 N sodium hydroxide
- Solution B- 1% Sodium potassium tartarate
- Solution C- 0.5% copper sulphate

To prepare 100 ml alkaline copper reagent, 98 ml of solution A, 1ml of solution B and 1 ml of solution C were mixed together freshly.

Folin-ciacalteau reagent (commercial reagent was diluted in the ratio of 1:1 with distilled water at the time of use).

Procedure:

100 mg of sample was homogenized in 10 ml of distilled water and filtered through a muslin cloth and centrifuged at 3000 rpm for 10 minutes. To the supernatant, 10% trichloro acetic acid (TCA) was added in 1:1 ratio and left in an ice bath for 30 minutes to precipitate protein. Then centrifuged at 3000 rpm for 5 minutes and discarded the supernatant. The precipitate was dissolved in 0.1N sodium hydroxide and diluted to a known volume.

To 0.5 ml of protein extract, 5 ml of alkaline copper reagent was added. After thorough mixing, 0.5 ml of folinciocalteau reagent was added and allowed to stand for 30 minutes; the blue colour appeared and absorbance was measured at 650 nm using UV visible spectrophotometer (Model No: UV 2371). The analysis was performed in triplicates and the amount of protein was calculated and expressed as mg/g DW. Bovine serum albumin (BSA) was used as standard.

Estimation of Carbohydrates: Phenol-Sulpuric Acid Method (Dubois *et al*., 1956)

Requirements:

- 5% phenol (5 ml phenol + 95 ml distilled water)
- 96% Sulphuric acid (96% sulpuric acid + 4 ml distilled water)

Procedure:

100 mg of sample was grounded with 10 ml distilled water. It was then filtered and centrifuged. The filtrate was collected. To 0.1 ml of the filtrate, 0.9 ml of distilled water, 1 ml of 5% phenol and 5 ml of 96% H_2SO_4 were added. After 30 minutes absorbance was measured at 490 nm using UV visible spectrophotometer (Model No: UV 2371). The analysis was performed in triplicates and the results were expressed as mg/g sample. Glucose was used as standard.

Estimation of Free Amino Acids (Moore and Stein, 1948)

Total free amino acids (ninhydrin method) were determined according to the procedure given by Moore and Stein.

Requirements:

- Ninhydrin reagent
- n-propanol

Procedure:

1 ml of the sample was mixed with 1 ml of Ninhydrin reagent in a test tube. Tubes were kept in boiling water bath for 20 minutes and then added 5 ml of diluent (equal volume of water and n-propanol) incubated at room temperature for 15 minutes and absorbance was read at 570 nm against a reagent blank. The analysis was performed in triplicates, and the results were expressed as mg/g sample.

Determination of Total Lipid (Folch *et al*., 1957)

Requirements:

- $CHCL_3$
- CH_3OH
- H_2SO_4
- Phosphovanillin reagent

Procedure:

500 mg of dried sample was taken in a screw capped test tube, 10 ml of 2:1 $CHCL_3$:CH_3OH solvent mixture was added. The tube was loosely capped and heated in a water bath at 60ºC for 30 minutes. After cooling the solution, the volume was made up to 10 ml with the solvent mixture. 0.4 ml of the extract was pipetted in a separate test tube, allowed to dry completely and digested with 0.4 ml of concentrated H_2SO_4 by boiling in a water bath for 10 minutes. After cooling the tube, 5 ml of phosphovanillin reagent was added and allowed to stand for 30 minutes for colour development. The absorbance was then measured at 520nm against a reagent blank using spectrophotometer (Model No: UV 2371). Cholesterol was used as standard.

DETERMINATION OF ANTIOXIDANTS

Estimation of Total Phenolic Content (Duan *et al*., 2006)

Requirements:

- 50% Folin – ciocalteau reagent (Folin – phenol)
- 20% Sodium carbonate

Procedure:

100 mg of plant material was homogenized with 10ml of distilled water and filtered through a muslin cloth. 1 ml of the filtrate was added to 1.5ml of de-ionized water and 0.5ml of 50% Folin – ciocalteau reagent and the content were mixed

thoroughly. After one minute, 1 ml of 20% sodium carbonate solution was added and mixed. The blank contains all the reagents and solution except the sample. After 30 minutes of incubation at 37ºC, the absorbance was measured at 750nm. Galic acid was used as standard.

Estimation of Total Flavonoid Content (Zhinshen *et al*., 1999)

Requirements:

- 5% Sodium nitrate
- 10% Aluminium chloride
- 1M Sodium hydroxide

Procedure:

100mg of sample was homogenized with 10ml of distilled water and filtered through a muslin cloth. 1ml of extract was added with 4ml of distilled water and mixed. After 5 minutes, 0.3ml of 5% sodium nitrate was added and again after 5 minutes, 0.3ml of aluminium chloride was added. After 5 minutes, 2ml of 1M sodium hydroxide was added and final volume was made up to 10ml with distilled water. A blank was prepared by adding all the reagents except the sample. The absorbance was read at 510nm against blank. Quercetin was used as standard.

Estimation of Total Tannin Content (Julkunen – Titto, 1985)

Requirements:

- 4% Vanillin (prepare with methanol)
- Concentrated hydrochloric acid

Procedure:

100 mg of sample was homogenized with 10 ml of distilled water and filtered through a muslin cloth. 1ml of aliquot of aqueous extract was mixed with 3ml of 4% vanillin (prepare with methanol) and 1.5ml of concentrated HCL was added. The solution was shaken vigorously and left to stand at room temperature for 20 minutes in darkness. A blank was prepared by adding all the reagents except the sample. The absorbance was read at 500 nm. Tannic acid was used as a standard.

SALT STRESS:

Certified seeds of uniform size were surface sterilized with 0.1% Mercuric chloride for two minutes and rinsed thoroughly with distilled water. Various concentration of Sodium chloride solutions (50mM, 100mM, 150mM) were prepared. The selected germination trays were filled with soil and organic manures. About 10 seeds were sown in each tray. The seed trays with seeds were treated with NaCl salt using the following concentrations 50mM (Treatment I), 100mM (Treatment II) and 150Mm (Treatment III). The control seed tray was watered with distilled water.

MORPHOLOGICAL PARAMETERS:

Seed germination percentage

Seed germination study was carried out on 10^{th} day and 20^{th} day after sowing. From all the treatments, the number of seeds germinated were counted and recorded. Germination percentage was worked out by the following method.

$$\textbf{Germination percentage} = \frac{\textbf{Number of seeds germinated}}{\textbf{Total number of seeds sown}} \textbf{ X 100}$$

Length of the root and shoot

The root length and shoot was taken on 20th day after sowing by selecting 3 seedlings at random from all the treatments. The length was measured randomly by selecting three seedlings and the average value was recorded.

Fresh and dry weight of the seedlings

Three seedlings were taken from all the treatments and the fresh weight was calculated. The average value for each treatment was recorded in g/seedling. The seedlings were then kept in hot air oven at 80˚C for 48 hours to obtain a constant weight. The dry weight was found out and the average value was recorded in g/seedling.

Vigour index

Observations for the germination, root length, shoot length and vigour index were made on 20th day after sowing. Vigour index was calculated using the following formula.

Vigour Index = (Root length + Shoot length) x **Percentage of germination**

RESULTS AND DISCUSSION

The human life and culture has directly or indirectly been influenced by their surrounding environment. The primitive people were well acquainted with the properties and uses of plants of their surroundings. They have inherited rich traditional knowledge of surrounding plants used as food, fodder, fibers, woods, fuel, medicine, beverage, tannin, dye, gum, resin, cosmetics, crafts and religious ceremonies. The listings of plants and animals of ethno biological value are important for knowing and evaluating human plant relationship to their environment. *Amaranthus* is a common plant which is regularly used by few of the people in the world.

PRELIMINARY PHYTOCHEMICAL ANALYSIS

Table 1 show the preliminary phytochemical analysis of selected *Amaranthus* species. Aqueous extract of all the selected leafy vegetables showed the presence of four phytoconstituents such as carbohydrates, proteins, aminoacids and alkaloids. Ethanolic extract revealed the presence of ten metabolites namely alkaloid, flavanoid, phenol, tannin, carbohydrate, saponin, glycoside, protein, amino acid and terpenoid. In plants, these secondary metabolites function to attract beneficial and repel harmful organisms, serve as phytoprotectants and respond to environmental changes. In humans, however the compounds have beneficial effects including antioxidant, anti-inflammatory effects, modulation of detoxification enzymes, stimulation of the immune system, modulation of steroid metabolism and antibacterial and antiviral effects **(Johanna, 2003).**

QUANTITATIVE ESTIMATION OF PRIMARY METABOLITES

The nutritional value of leafy green vegetables is due to their protein, fibre, vitamin, mineral contents and low - fat levels. They are very useful for vegetarian diets because they provide the essential nutrients. Besides, it contains many different bioactive compounds with various human health benefits. Several nutritional parameters were measured for the selected *Amaranthus* species.

Carbohydrates act as an associate energy supplier and they are found in several foodstuffs. In the present study carbohydrate content was ranged between 4.3 mg/g to 8.9 mg/g dry weight. Samples I, II, III, IV and V showed 4.3 mg/g, 7.8 mg/g, 6.5 mg/g , 7.1 mg/g, 8.9 mg/g carbohydrate content respectively. Protein content was found to be higher in sample II (2.94 mg/g DW) followed by sample III, I, V and lesser in sample V (1.4 mg/g DW). Sample III contain higher amount of protein (12.4 mg/g DW) than other samples. Sample to has lesser amount of protein in the selected samples. As like other primary metabolites lipid content of all the five investigated Amaranthus species showed variation. Highest lipid content was observed in sample V (0.22 mg/g DW) followed by sample II and IV. Lowest amount of lipid was found in sample III and I. **(Table 2, Figure 1)**

QUANTITATIVE ESTIMATION OF SECONDARY METABOLITIES

Secondary metabolites are a large group of secondary plant metabolites which play a major role in the protection of oxidation processes. These compounds have antioxidant properties and can act as free radical scavengers, hydrogen donators and singlet oxygen quenchers. Numerous studies have conclusively demonstrated that *Amaranthus* species also contain many secondary phytoconstituents which are

important plant constituents because of their scavenging ability. In addition, phenolics exhibit a wide range of biological effects including antibacterial, anti-inflammatory and anti-hyperglycemic activities.

The present study revealed highest phenolic content in sample II (4.54 ± 0.12 mg /g DW) followed by sample III (4.09 ± 0.17 mg/g DW) and it was lower in sample I (1.57 ± 0.2 mg/g DW). As like phenolic content flavonoid is also higher in sample II (4.4 ± 0.11 mg/g DW) followed by sample V, I, IV and III. Highest tannin content was noticed in sample II (2.99 ± 0.09 mg/g DW) **(Table 3, figure 2)**

NACL STRESS:

Amaranthus has been rediscovered as a promising food crop mainly due to its resistance to heat, drought, diseases and pests, and the high nutritional value of both seeds and leaves. Leaves are rich in proteins and micronutrients such as iron, calcium, zinc, vitamin C and vitamin A **(Enoch, 2014).** In the present study selected *Amaranthus* seeds were exposed to salt stress. The effect of salt stress in the morphological parameters of 20 days old *Amaranthus* species was represented in **Table 4.** In all the selected seeds germination potential was higher in control series except in sample II and IV. In Sample II and IV treatment I induced the germination capacity than control and other treatment. Except than sample III all the other samples displayed inhibition in root length with response to salt stress. Root length also considerably affected proportional to higher the salt concentration **(Figure 3 and 4)**. Results depicted in the table clearly explain that weight of the 20 days old seedling also affected by salt stress by showing the less weight than control series. Reduction in dry weight depended upon the decrease in the lengths of shoot and root **(Salim, 1991)**. This findings were corroborated with the findings of **Jenifer and Natarajan,**

(2018) who reported suppressed growth in *Vigna radiata* with response to NaCl stress.

SUMMARY AND CONCLUSION

Regular intake of sufficient amounts of certain dietary phytochemicals was proven to reduce the incidence of noncommunicable chronic diseases and certain infectious diseases. In addition, dietary phytochemicals were also reported to reduce the incidence of metabolic disorders such as obesity in children and adults. Human consumption of wild plants has been documented from antiquity into the common Era. Dietary use of wild fruits, nuts, seeds, and leaves appear in numerous records of history. Today, most human plant food is based on rather limited number of crops, but it is clear that in many parts of the world the use of wild plants is not negligible. Many publications have emphasized on the diversity and value of wild edible plants **(Dhyani *et al.*, 2007).** The nutritional value of traditional wild plants is higher than several known common vegetables and fruits. Approximately 75,000 species of plants world-wide are believed to be edible **(Walters and Hamilton, 1993)**.

The present study confirmed the presence of alkaloid, flavanoid, phenol, tannin, carbohydrate, saponin, glycoside, protein, amino acid and terpenoid. Metabolites like carbohydrates, proteins, aminoacids, lipids, phenols, flavonoids and tannins were estimated. In addition to that response of *Amaranthus* species against NaCl stress was studied which shows considerable changes in its growth performance.

The selected species of *Amaranthus* leaves contain various pharmacologically active compounds. Results from the current study indicate that these plants are of ethno-pharmacological importance further confirming the pharmacological basis in the use of the said plant in traditional medicine for the treatment of infections and consumption. It is also hoped therefore that this study will contribute to the

improvement of food habits and public health. Developing salt resistance *Amaranthus* variety may help to grow *Amaranthus* against salt stress.

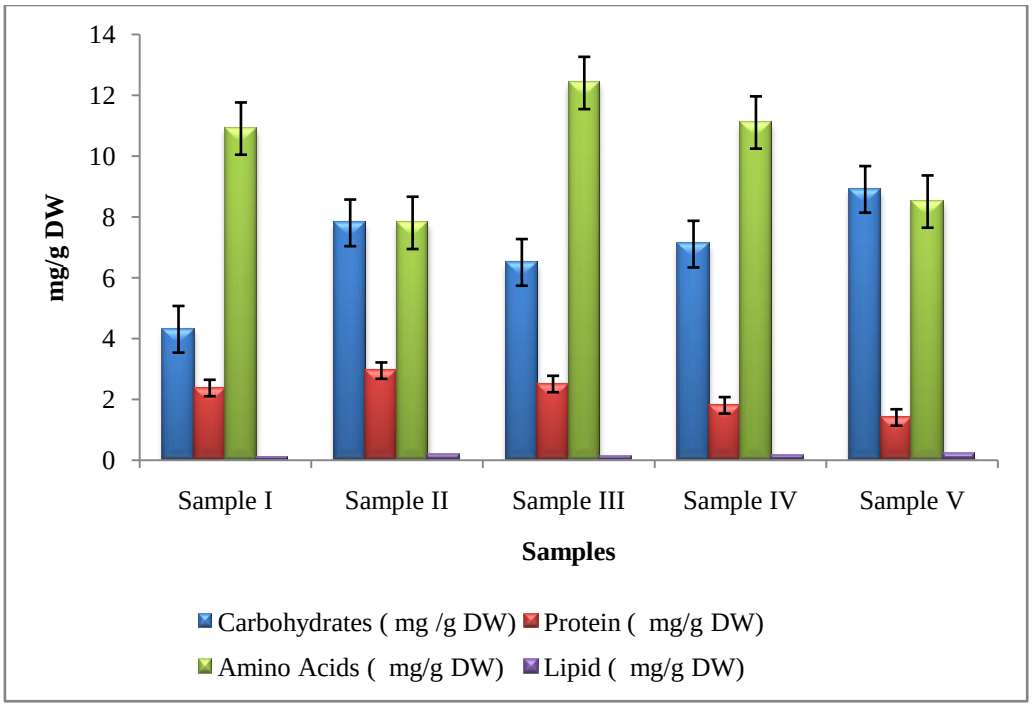

Figure 1: Quantitative estimation of Primary Metabolites of Selected *Amaranthus* Species

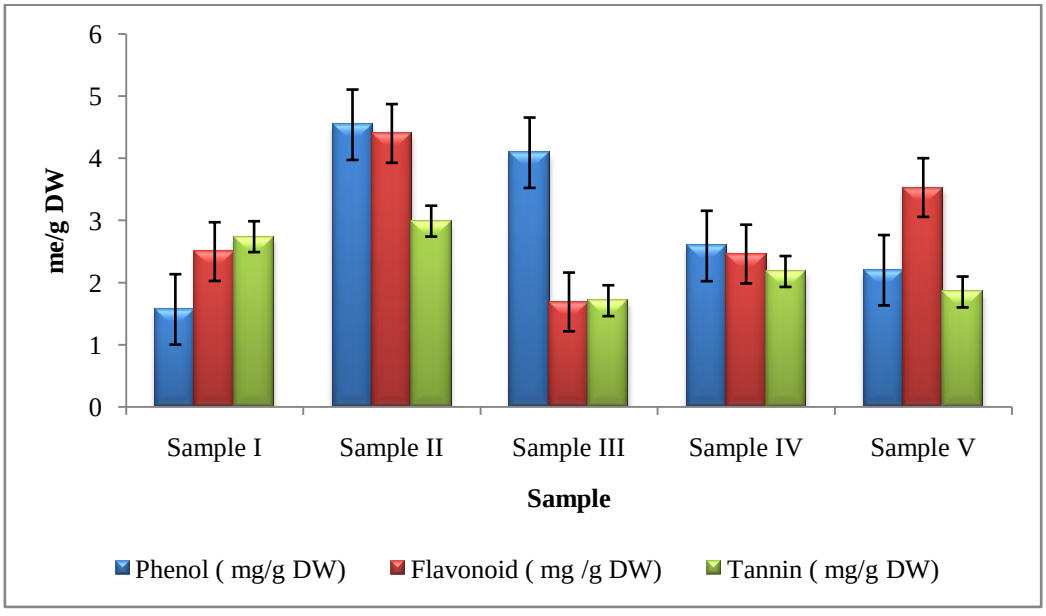

Figure 2: Quantitative Estimation of Secondary Metabolites of Selected *Amaranthus* Species

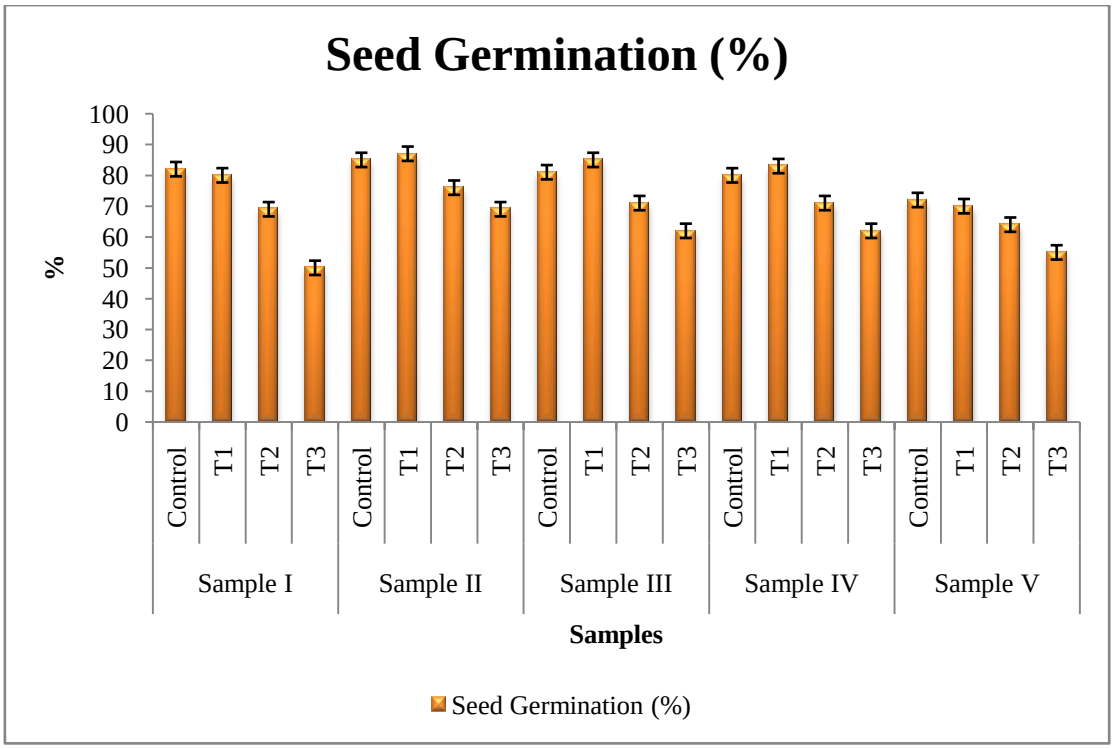

Figure 3: Effect of NaCl stress on Seed Germination of Selected *Amaranthus* Species

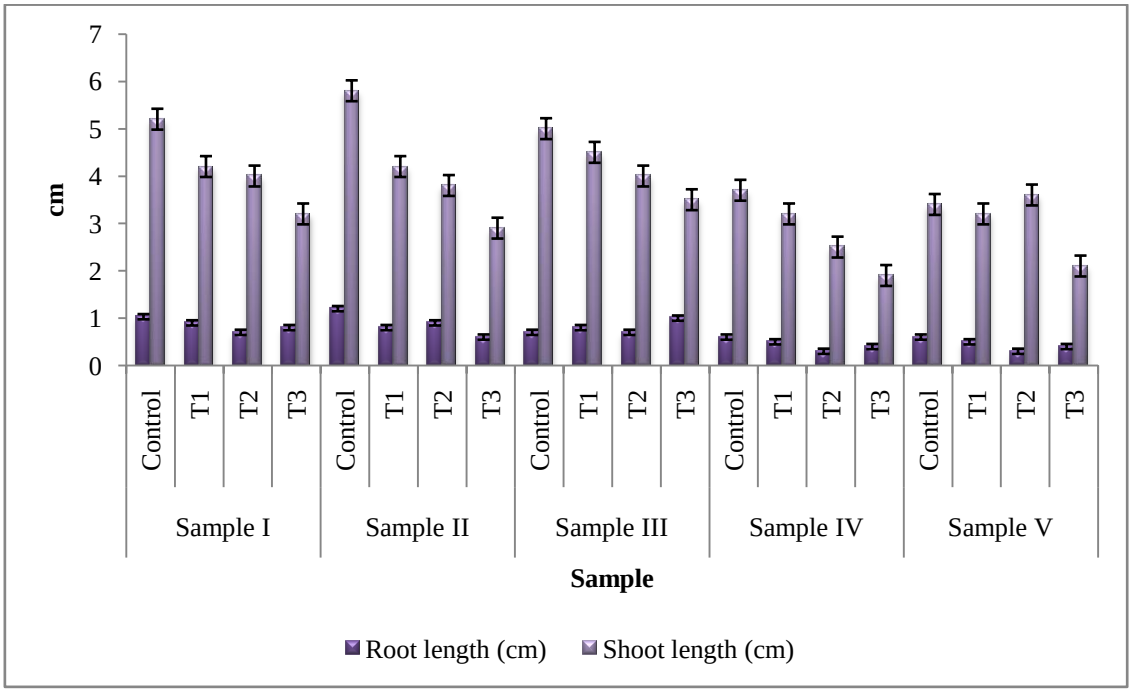

Figure 4: Effect of NaCl stress on Root Length and Shoot Length of Selected *Amaranthus* Species

TABLE 1: PRELIMINARY PHYTOCHEMICAL ANALYSIS OF SELECTED AMARANTHUS SPECIES

S. No.	Test	Sample I		Sample II		Sample III		Sample IV		Sample V	
		AE	EE	AE	EE	AE	EE	AE	EE	AE	EE
1.	Alkaloid	+	+	+	+	+	+	+	+	+	+
2.	Flavanoid	-	+	-	+	-	+	-	+	-	+
3.	Phenol	-	+	-	+	-	+	-	+	-	+
4.	Tannin	-	+	-	+	-	+	-	+	-	+
5.	Steroid	-	-	-	-	-	-	-	-	-	-
6.	Carbohydrate	+	+	+	+	+	+	+	+	+	+
7.	Saponin	-	+	-	+	-	+	-	+	-	+
8.	Glycoside	-	+	-	+	-	+	-	+	-	+
9.	Protein and Amino Acid	+	+	+	+	+	+	+	+	+	+
10.	Terpenoid	-	+	-	+	-	+	-	+	-	+
11.	Phlobatannin	-	-	-	-	-	-	-	-	-	-
12.	Coumarin	-	-	-	-	-	-	-	-	-	-
13.	Cardiacglycoside	-	-	-	-	-	-	-	-	-	-

'+' indicates presence '**-**' indicates absence AE – Aqueous Extract EE – Ethanol Extract

TABLE 2: QUANTITATIVE ESTIMATION OF PRIMARY METABOLITES OF SELECTED AMARANTHUS SPECIES

S. No.	Sample	Carbohydrates (mg /g DW)	Protein (mg/g DW)	Amino Acids (mg/g DW)	Lipid (mg/g DW)
1.	Sample I	4.3	2.37	10.9	0.11
2.	Sample II	7.8	2.94	7.8	0.18
3.	Sample III	6.5	2.5	12.4	0.13
4.	Sample IV	7.1	1.8	11.1	0.15
5.	Sample V	8.9	1.4	8.5	0.22

TABLE 3: QUANTITATIVE ESTIMATION OF SECONDARY METABOLITES OF SELECTED AMARANTHUS SPECIES

S. No.	Sample	Phenol (mg/g DW)	Flavonoid (mg /g DW)	Tannin (mg/g DW)
1.	Sample I	1.57 ± 0.2	2.50 ± 0.17	2.74 ± 0.13
2.	Sample II	4.54 ± 0.12	4.4 ± 0.11	2.99 ± 0.09
3.	Sample III	4.09 ± 0.17	1.69 ± 0.18	1.71 ± 0.46
4.	Sample IV	2.59 ± 0.19	2.46 ± 0.24	2.18 ± 0.3
5.	Sample V	2.20 ± 0.15	3.53 ± 0.42	1.85 ± 0.29

TABLE 4: EFFECT OF SALT STRESS ON THE GROWTH

PERFORMANCE OF SELECTED AMARANTHUS SPECIES

S. No.	Sample	Treatment	Seed Germination (%)	Root length (cm)	Shoot length (cm)	Weight (g)
1.	Sample I	Control	82	1.03	5.2	0.015
		T1	80	0.9	4.2	0.007
		T2	69	0.7	4.0	0.004
		T3	50	0.8	3.2	0.001
2.	Sample II	Control	85	1.2	5.8	0.012
		T1	87	0.8	4.2	0.006
		T2	76	0.9	3.8	0.005
		T3	69	0.6	2.9	0.009
3.	Sample III	Control	81	0.7	5	0.016
		T1	85	0.8	4.5	0.010
		T2	71	0.7	4	0.009
		T3	62	1	3.5	0.004
4.	Sample IV	Control	80	0.6	3.7	0.013
		T1	83	0.5	3.2	0.005
		T2	71	0.3	2.5	0.004
		T3	62	0.4	1.9	0.005
5.	Sample V	Control	72	0.6	3.4	0.011
		T1	70	0.5	3.2	0.006
		T2	64	0.3	3.6	0.005
		T3	55	0.4	2.1	0.004

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