TOOTH DISCOLORATION :-A NONTOXIC APPROACH TOWARDS THE TREATMENT

Thesis submitted to Department of Life Science for the partial fulfillment of M.Sc Degree

in

Life Science

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UNDER THE GUIDANCE OF

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Research is to see what everybody has seen but to think what nobody has thought. To go beyond the traditional way of thinking and utilizing age old techniques for creation of something new has been an amazing journey. I owe my gratitude to a number of people who have illuminated my path on this journey and helped me reach my destination.

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Last but not the least, I dedicate my work to **my parents** and **my brother** who are my greatest strength and always support me in every decision.

Date: 9.5.2015, **SUBHADARSHINI AGASTI**

Place: NIT,ROURKELA

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CERTIFICATE

This is to certify that the thesis entitled "***Tooth discolouration:-A nontoxic approach towards the treatment***" *by* ***Subhadarshini Agasti (413LS2023)*** submitted to the National Institute of Technology, Rourkela for the degree of Master of science is a record of bonafide research work, carried out by her in the Department of life science under my supervision .I believe that the thesis fulfill part of the requirements for the award of Master of Science. The result embodied in the thesis have not been submitted for the award of any other degree.

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Declaration

I hereby declare that the thesis entitled "**Tooth discolouration:-A nontoxic approach towards the treatment**", submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr.MONALISA MISHRA, Department of Life Science, NIT, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date: 9.5.2015

Place: Rourkela **Subhadarshini Agasti**

LIST OF FIGURES:-

LIST OF TABLES:-

LIST OF ABBREVIATION:-

gm	**Gram**
µl	**Microliter**
cm	**Centimeter**
NA	**Nutrient agar**
NB	**Nutrient broth**
C	**Centigrade**
%	**Percentage**
No.	**Number**

CONTENTS

ABSTRACT:-

Oral cavity is known as the house of all microbes. Bacteria mainly grow and starts colonize here. In human life structures, the mouth is the first pathway for which our body that gets nourishment and saliva. Many types of oral diseases can seen in our oral cavity e.g, dental caries, periodontal disease, black stained teeth, gingivitis, dental plaqe. Oral microbes include streptococci, lactobacilli, staphylococci, corynebacteria, and different anaerobes specifically bacteroides. The oral cavity of the new-born babies does not contain microorganisms but rather quickly gets to be colonized with microbes, for example, Streptococcus salivarius. Black stained teeth or teeth blackening mainly seen in case of childrens. It is a common problem now-a-days. Teeth blackening mainly occur due to several reasons. In case of children it may be causes due to taking of heavy amount of chocolate, cold drinks and in case of adults it occurs may be due to heavy smoking, taking heavy amount of tea or coffee (8-10)times per day, but in some cases it occurs due to certain types of bacteria. So far there is no proper treatment for this disease. The aim of the thesis is to treat teeth blackening by using some non-toxic approach.

INTRODUCTION:-

In human life structures, the mouth is the first divide of the nutritious waterway that gets nourishment and saliva.[1] The mouth incorporates the lips, cheeks, sense of taste (top of the mouth), floor of the mouth and the piece of the tongue in the mouth (oral tongue). A mucous layer lines and protects within the mouth. The structures in the oral depression assume an essential part in speech, taste and the first stage of digestion. The mouth, comprises of two districts, the vestibule and the oral cavity proper. The vestibule is the territory between the teeth, lips and cheeks.[2]The oral depression is limited along the edges and in front by the alveolar procedure (containing the teeth) and at the back by the isthmus of the fauces. Its rooftop is shaped by hard sense of taste and delicate sense of taste and the floor is framed by the mylohyoid muscles and is involved primarily by the tongue. Mucous film lines the sides and under surface of the tongue to the gum lining the internal part of the jaw mandible. It gets the emissions from the submaxillary and sublingual salivary organ. The oral cavity starts at the fringe between the skin and the lips (vermillion outskirt). The top of the mouth is shaped by the hard sense of taste. The oral depression leads into the oropharynx, which incorporates the delicate sense of taste, the back of the tongue and the tonsils. The internal surface of the cheeks frames the sides of the oral depression. The most minimal piece of the oral depression is the floor of the mouth, which is secured by the tongue.

The oral cavity can be divided into specific areas, including:

- lips
- labial mucosa (inner lining of the lips)
- commissure of lips (where the upper and lower lips meet at the corner of the mouth)
- vestibule (a space bounded by the teeth and gums on the inside and the mucosal surface of the lips and cheeks on the outside)
- oral tongue (the front two-thirds of the tongue)

- floor of the mouth
- buccal mucosa (the inner lining of cheeks)
- gingiva (gums)
- retromolar trigone (the area just behind the back molars in the lower jaw)
- hard palate (the bony part at the front of the roof of the mouth)
- teeth
- lower jaw (mandible)

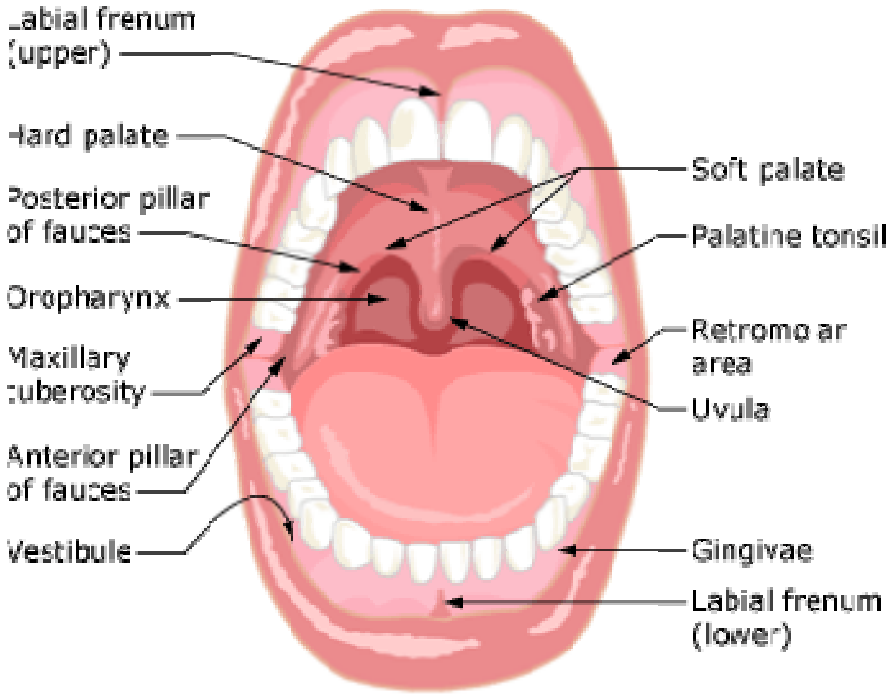

Figure 1(Structure of oral cavity)

IMPORTANT ORAL BACTERIA:-

Gram Positive organisms:-(3)

1. Bulk of oral bacteria

2. Rods (bacilli), cocci or irregular shape pleomorphic

3. Oxygen tolerance varies from aerobes to strict anaerobes

4. Most are fermentative

5. Cell wall has thick peptidoglycan layer (penicillin has effect by interfering production of this layer).

Gram Negative organisms:-(4)

1. Many Gram negative microorganisms found in the mouth, particularly settled in subgingival plaque.

2. They are Cocci bar shape, filamentous bars, shaft formed or winding molded.

3.Some fermentative , produce acids which different organic entities use acids as a vitality source, others deliver chemicals which separate tissue

4.Cell wall distinctive to Gram positive with a dainty peptidoglycan layer, has B-lactamase which separates penicillin, additionally has LPS or endotoxin

Oral micro organism causes two major dental diseases.

1. Dental caries

2. Periodontal diseases

DENTAL CARIES:-

Dental caries otherwise called tooth rot, holes, or caries, is breakdown of teeth because of the exercises of microbes. It is brought about by the microbes Streptococcus mutans. The cavities may be various distinctive colours from yellow to dark. symptoms may include pain and trouble with eating. Difficulties may include aggravation of the tissue around the tooth, tooth misfortune, and disease or canker formation.[5]The mouth contains a wide assortment of oral microscopic organisms, yet just a couple of particular types of microbes are accepted to bring about dental caries: Streptococcus mutans and Lactobacillus species among them. These creatures can create large amounts of lactic acid after maturation of dietary sugars, and are impervious to the unfriendly impacts of low pH, properties crucial for cariogenic bacteria.[6]

PERIODONTAL DISEASES:-

It is a biofilm, usually a pale yellow, that develops naturally on the teeth. Like any biofilm, It is formed by colonizing bacteria trying to attach themselves to the tooth's smooth surface. It has been speculated that forms part of the defense systems of the host by helping to prevent colonization of microorganisms that may be pathogenic. It is caused by Porphyromonas gingivalis.

Dental plaque:-

Dental plaque is a thin, tenacious microbial film that forms on the tooth surfaces. Microorganisms in the dental plaque ferment carbohydrate foodstuffs, especially the disaccharide sucrose, to produce acids that cause demineralization of inorganic substances and furnish various proteolytic enzymes to cause disintegration of the organic substances of the teeth, the processes involved in the initiation and
progression of dental caries. The dental plaque holds the acids produced in close contact with the tooth surfaces and prevents them from contact with the cleansing action of saliva.

CAUSES OF DENTAL DISEASES:-

The two principle etiologies of oral diseases have been credited to Porphyromonas gingivalis which causes periodontal illnesses (like periodontitis) and Streptococcus mutans which causes dental caries . Both these microscopic organisms are not universally found in the mouth in light

of the fact that the ordinary greenery in the oral depression is commensal and cooperate to keep pathogenic microbes from influencing the host. Thus caries and periodontal diseases is an after effect of a mix poor oral cleanliness and high sugar based eating diet which causes steady recolonization by pathogenic microscopic organisms. Emperical studies by Kigure et al. have observed that there is higher fixation P. gingivalis and Treponema denticola are found in the periodontal pockets of grown-ups experiencing periodontitis .The proposed component for periodontitis is that both these living beings have indicated hemolytic action (by conceivable destructiveness element) and this influences the connection of the gums with the teeth. If there should be an occurrence of dental caries, Sucrose has been indicated to help S. mutans colonize by helping it follow better. S. mutans separate sucrose to create glucans (extracellular polymer) which can then be utilized for building a biofilm which is not the same as biofilms created by ordinary florae. This biofilm has expanded porosity and permits acids to uninhibitedly diffuse to the enamel and reason a pH drop, which can lead at last prompt dental caries .

CURE AVAILABLE FOR TEETH DISEASE:-

Increase the resistance of the teeth[7–11].

Systemic use of fluoride:-

(i) Fluoridation of water, milkand salt;
(ii) fluoride supplementation in the form of tabletsand lozenges;

(iii) consuming a fluoride-rich diet such as tea, fish, etc.

(iv)Use of fluoridated toothpaste and mouthwash;

(v) use of fluoride varnishes (in-office application,longer duration of action, high fluoride content);

The use of various interdental cleaning aids such as dentalfloss, interdental brush, water pik, etc. supplements the cleansing effect of a toothbrush. Use of an electronic
toothbrush in children and persons with decreased manual dexterity is recommended.

Modify the diet.[12-15]:-

Reduce the intake and frequency of refined carbo-hydrates. Avoid sticky foods and replace refined with unrefined natural food. Increase the intake of fibrous food
to stimulate salivary flow, which is protective against caries.Consume caries-protective foods such as cheese, nuts, rawvegetables, fruits, etc. Stimulate salivary flow with sugar-free chewing gum.
Using sugar substitutes such as saccharine, xylitol,mannitol, aspartame, etc. in paediatric medicinal syrups,bakery products, jams, marmalade, etc. Making toothbrushes and fluoridated toothpaste available to the masses at low cost. Regular use of fluoridated toothpaste is proven to reduce the incidence of dental caries by 30%.Salt mixed with pepper can be of great use when a tooth becomes extremely sensitive as both the ingredients have antibacterial, anti-inflammatory and analgesic properties. Mustard oil and charcoal played a great role for increase the strength of the teeth and teeth whitening simultaneously. It also gives the relaxation from various type of tooth pain.

REVIEW OF LITERATURE:-

ORAL CAVITY:-

The oral cavity includes the oral tongue (the foremost 66% of the tongue); hard sense of taste floor of the mouth; retromolar trigone; lips; buccalmucosa; alveolar edges; and gingiva . The mandible and maxilla are the characterizing hard edges of the oral depression. mouth, additionally called Oral Cavity, or Buccal Cavity, in human life structures, orifice through which sustenance and air enter the body. The mouth opens to the outside at the lips and purges into the throat at the back; its limits are characterized by the lips, cheeks, hard and delicate palates, and glottis. It is separated into two areas: the vestibule, the zone between the cheeks and the teeth, and the oral depression fitting. The last area is generally filled by the tongue, a vast muscle immovably tied down to the floor of the mouth by the frenulum linguae. Not with standing its essential part in the admission and starting assimilation of sustenance, the mouth and its structures are key in people to the development of discourse.

The boss structures of the mouth are the teeth, which tear and toil ingested sustenance into little pieces that are suitable for assimilation; the tongue, which positions and blends nourishment furthermore conveys tactile receptors for taste; and the sense of taste, which divides the mouth from the nasal depression, permitting separate entries for air and for nourishment. Every one of these structures, alongside the lips, are included in the arrangement of discourse sounds by adjusting the entry of air through the mouth.

The oral depression and vestibule are altogether lined by mucous layers containing various little organs that, alongside the three sets of salivary organs, bathe the mouth in liquid, keeping it wet and clear of nourishment and different garbage. Particular layers structure both the gums (gingivae), which encompass and support the teeth, and the surface of the tongue, on which the film is rougher in composition, containing numerous little papillae that hold the taste buds. The mouth's sodden surroundings and the compounds inside its emissions help to diminish sustenance, encouraging gulping and starting the methodology of absorption.

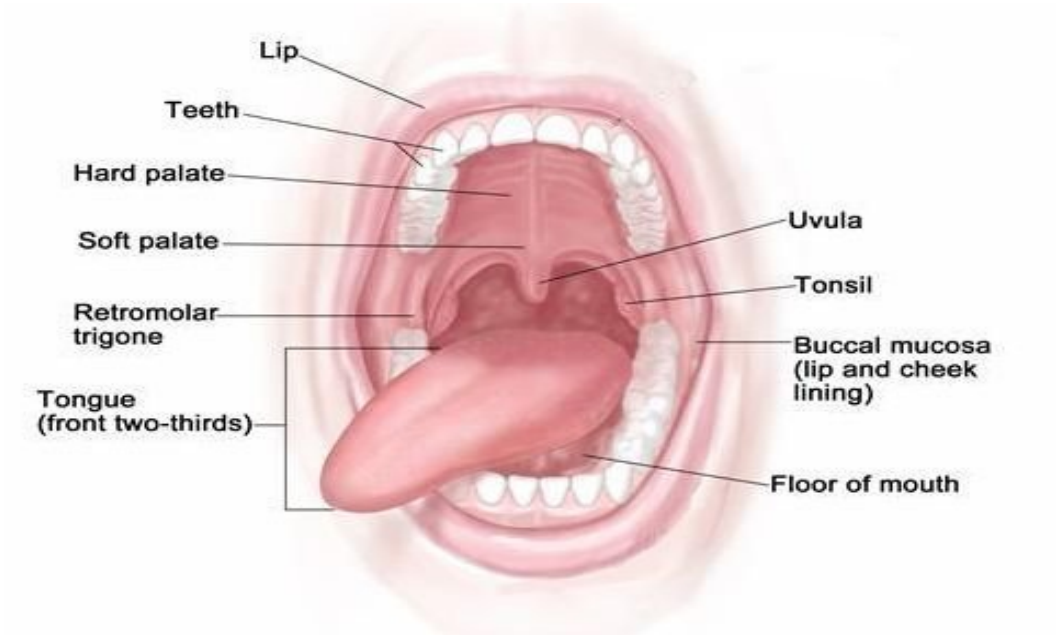

Figure 2:- [ANATOMY OF MOUTH "HARD PALLETE ROOF OF MOUTH"]

The teeth are the hardest substances in the human body. Besides being essential for chewing, the teeth play an important role in speech. Parts of the teeth include:

• Enamel: The hardest, white outer part of the tooth. Enamel is mostly made of calcium phosphate, a rock-hard mineral.

• Dentin: A layer underlying the enamel. Dentin is made of living cells, which secrete a hard mineral substance.

• Pulp: The softer, living inner structure of teeth. Blood vessels and nerves run through the pulp of the teeth.

• Cementum: A layer of connective tissue that binds the roots of the teeth firmly to the gums and jawbone.

• Periodontal ligament: Tissue that helps hold the teeth tightly against the jaw.

A normal adult mouth has 32 teeth, which (except for wisdom teeth) have erupted by about age 13.

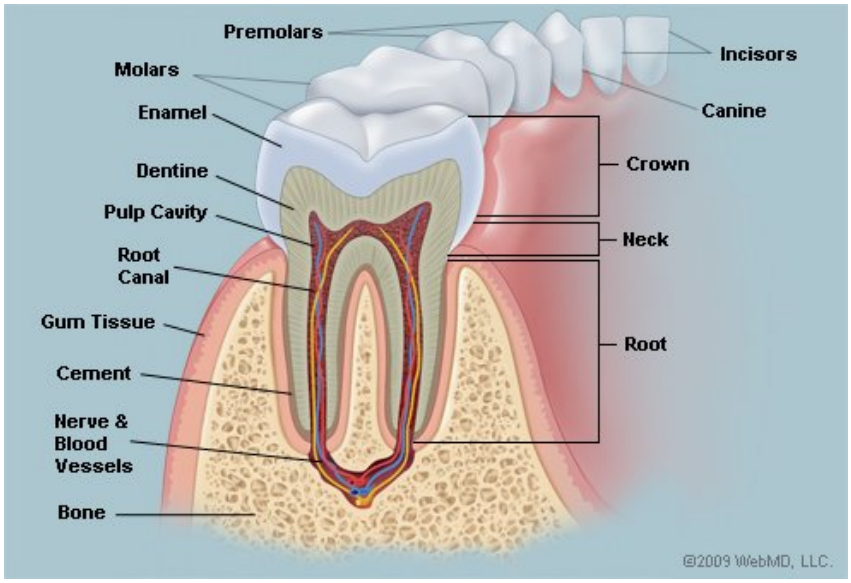

Figure 3 [Structural anatomy of teeth]

The mouth, consists of two regions, the vestibule and the oral cavity proper. The vestibule is the area between the teeth, lips and cheeks.[16] The oral cavity is bounded at the sides and in front by the alveolar process (containing the teeth) and at the back by the isthmus of the fauces. Its roof is formed by hard palate and soft palate and the floor is formed by the mylohyoid muscles and is occupied mainly by the tongue. Mucous membrane lines the sides and under surface of the tongue to the gum lining the inner aspect of the jaw mandible. It receives the secretions from the submaxillary and sublingual salivary glands.

The teeth and the periodontium (i.e. the tissues that backing the teeth) are innervated by the maxillary and mandibular divisions of the trigeminal nerve. Maxillary (upper) teeth and their related periodontal ligament are innervated by the unrivaled alveolar nerves, branches of the maxillary division, termed the back predominant alveolar nerve, foremost prevalent alveolar nerve, and the variably present center prevalent alveolar nerve. These nerves shape the predominant dental plexus over the maxillary teeth. The mandibular (lower) teeth and their related periodontal ligament are innervated by the substandard alveolar nerve, a branch of the mandibular division. This nerve runs inside the mandible, inside the sub-par alveolar trench

underneath the mandibular teeth, giving off branches to all the lower teeth (second rate dental plexus).[17][18] The oral mucosa of the gingiva (gums) on the facial (labial) part of the maxillary incisors, canines and premolar teeth is innervated by the predominant labial branches of the infraorbital nerve. The back prevalent alveolar nerve supplies the gingiva on the facial part of the maxillary molar teeth. The gingiva on the palatal part of the maxillary teeth is innervated by the more prominent palatine nerve separated from in the incisor locale, where it is the nasopalatine nerve (long sphenopalatine nerve). The gingiva of the lingual part of the mandibular teeth is innervated by the sublingual nerve, a branch of the lingual nerve. The gingiva on the facial part of the mandibular incisors and canines is innervated by the mental nerve, the continuation of the substandard alveolar nerve rising up out of the mental foramen. The gingiva of the buccal (cheek) part of the mandibular molar teeth is innervated by the buccal nerve (long buccal nerve).[19]

The mouth plays an important role in eating, drinking, breathing and speaking. Infants are born with a sucking reflex, by which they instinctively know to suck for nourishment using their lips and jaw. The mouth also helps in chewing and biting food.

Oral microbiology is the study of the microorganisms (microbiota) of the oral depression and their cooperations between oral microorganisms or with the host. Of particular interest is the role of oral oral microorganisms in the two noteworthy dental sicknesses: dental caries and periodontal disease.[20]

Oral microbes include streptococci, lactobacilli, staphylococci, corynebacteria, and different anaerobes specifically bacteroides. The oral cavity of the new-born babiesdoes not contain microorganisms but rather quickly gets to be colonized with microbes, for example, Streptococcus salivarius. With the presence of the teeth during the first year colonization by Streptococcus mutans and Streptococcus sanguinis happens as these organic entities colonize the dental surface and gingiva. Different strains of streptococci stick unequivocally to the gums and cheeks yet not to the teeth. The gingival hole region (supporting structures of the teeth) gives a living space to a mixed bag of anaerobic species. Bacteroides and spirochetes colonize the mouth around puberty.

Dental plaque :-

Dental plaque is the material that holds fast to the teeth and comprises of bacterial cells (fundamentally S. mutans and S. sanguis), salivary polymers and bacterial extracellular items. Plaque is a biofilm on the surfaces of the teeth. This gathering of microorganisms subject the teeth and gingival tissues to high concentration of bacterial metabolites which brings about dental diseases. If not taken care off, by means of brushing or flossing, the plaque can transform into tartar (its solidified shape) and lead to gingivitis or periodontal infection.

Gum diseases:- Gum disease is one of the most common dental problems adults face, but gum disease can begin at just about any age.

Gingivitis — Gingivitis is a term used to describe inflammation of the gums.

Cavities — A cavity is a very small hole that forms on the surface of a tooth.

Oral Cancer — Oral cancer is a disease resulting from abnormal cell growth in the mouth, lips, tongue or throat.

Salt water has been utilized by multiple cultures over countless generations to clean injuries and wash out mouths. Salt has antibacterial and safeguarding properties when applied in abundance to foods, yet its influence on microorganisms when disintegrated and weakened in water is less clear. Salt water changes the pH of the mouth, which deters the proliferation of numerous microorganisms, however it doesn't through and through slaughter numerous structures. In any event, salt water is alleviating to the mucous layers of your mouth, yet it ought not be gulped in amount. Converse with your dental specialist about the advantages of washing your mouth with salt water.

Brief History :-

The sympathy toward oral cleanliness goes back no less than 5,000 years to old China and India. Numerous references are made to mouth flushing and cleaning in customary Chinese and Indian

Ayurvedic therapeutic archives and works on going back to those right on time times, as per the book "The Way of Ayurvedic Herbs." Ayurvedic solution is like conventional Chinese home grown prescription, yet construct more with respect to identity attributes called doshas. Teeth brushing and mouth washing got to be generally normal in Greek and Roman periods, and Hippocrates is said to have prescribed a mixture of well water, ocean salt and vinegar. Different seeds and herbs were additionally noted for the capacity to refresh breath. These days, numerous drug store mouth washes are liquor based and contain an assortment of antimicrobial mixes.

Uses of some unconventional methods:-

1. Salt:-

Raw salt, which is essentially sodium chloride, restricts bacterial development in numerous nourishments and preserves them because it absorbs water molecules. Microscopic organisms need moisture in order to thrive , so without enough water they can't develop well. Salt water is not viewed as an anti-infection in light of the fact that it furnishes microbes with water and does not execute them upon prompt contact. salt water rinses are beneficial because they temporarily alkalinize or increase the pH in the mouth, which deters bacterial proliferation.

2. Charcoal:-

Charcoal helps in teeth whitening. Rural people mainly use it to maintain the oral health.

3. Mustard oil:-

Mustard oil is thought to be an oil that has low soaked fat when compared with other cooking oils. Stimulates digestion, circulation and excretory system. Helps preventing fungal growth, thus it can be used as anti-fungal. Protects teeth from germs if rubbed on gums and make gum strong.

4.Calcium chloride:-

Calcium is a mineral that is an essential part of bones and teeth. The heart, nerves, and blood-coagulating frameworks additionally require calcium to work. Taking calcium by mouth, together with vitamin C and vitamin D supplements, appears to decrease fluoride levels in childrens and enhance indications of fluoride harming

***5.Alternanthera sesillis*:-**

Alternanthera sessilis is an aquatic plant known by several common names, including sessile joyweed and dwarf copperleaf. It is used as an aquarium plant. The plant occurs throughout the tropical and subtropical regions of the Old World. It has been introduced to the southern United States, and its origins in Central and South America are uncertain.

The leaves are used as a vegetable.[21] Young shoots and leaves are eaten as a vegetable in Southeast Asia. Occasionally it is cultivated for food or for use in herbal medicines.

This is a perennial herb with prostrate stems, rarely ascending, often rooting at the nodes. Leaves obovate to broadly elliptic, occasionally linear-lanceolate, 1-15 cm long, 0.3-3 cm wide, glabrous to sparsely villous, petioles 1-5 mm long. Flowers in sessile spikes, bract and bracteoles shiny white, 0.7-1.5 mm long, glabrous; sepals equal, 2.5-3 mm long, outer ones 1-nerved or indistinctly 3-nerved toward base; stamens 5, 2 sterile. In the wild it flowers from December till March.

Taxonomic classification:-

Kingdom: Plantae

(unranked): Angiosperms

(unranked): Eudicots

(unranked): Core eudicots

Order: Caryophyllales

Family: Amaranthaceae

Subfamily: Gomphrenoideae

Genus: *Alternanthera*

Species: ***A. sessilis***

Alternanthera sessilis is an annual or perennial prostate weed, found throughout the hotter part of India, ascending to an altitude of 1200 m. [22]The plant has been scientifically proven to consist of chemical constituents like α-and β- spinasterols,[23] lupeal isolated from roots. [24]Apart from the above, plant also contains β- sitosterol, stigmasterol etc.[25] The herb has been reported as galactagogue, chologague, abortificient, and febrifuge and is also said to be used in indigestion. [26]The leaves are used in eye diseases; in cuts and wounds; antidote to snake bite and scorpion sting; in skin diseases. [27]

6. Achyranthes aspera:-

Achyranthes aspera (common name: prickly chaff flower,[28] devil's horsewhip,[29] Sanskrit: *apamarga*) is a species of plant in the Amaranthaceae family. It is distributed throughout the tropical world.[30] It can be found in many places growing as an introduced species and a common weed.[31] It is an invasive species in some areas, including many Pacific Islands environments.[32]

It is one of the 21 leaves used in the Ganesh Patra Pooja done regularly on Ganesh Chaturthi day. In Uttar Pradesh the plant is used for a great many medicinal purposes, especially in obstetrics and gynecology, including abortion, induction of labor, and cessation of postpartum bleeding.[33] The Maasai people of Kenya use the plant medicinally to ease the symptoms of malaria.[34]

Taxonomic classification:-

Kingdom – Plantae

Subkingdom - Tracheobinota

Super Division - Spermatophyta

Division - Mangoliophyta

Class - Mangoliophsida

Subclass - Caryophyllidae

Order - Caryophyllales

Family - Amaranthaceae

Genus - *Achyranthes*

Species – ***A.aspera***

Traditionally, the plant is used in asthma and cough. It is pungent, antiphlegmatic, antiperiodic, diuretic, purgative and laxative, useful in oedema, dropsy and piles, boils and eruptions of skin etc. Crushed plant is boiled in water and is used in pneumonia. Infusion of the root is a mild astringent in bowel complaints. The flowering spikes or seeds, ground and made into a paste with water, are used as external application for bites of poisonous snakes and reptiles, used in night blindness and cutaneous diseases [35]

AIM AND OBJECTIVE:

- Isolation of microbes from oral cavity from the dental swab of the patient.
- Study of effect of salt, sugar, calcium, chlorine, $KMnO_4$, charcoal and mustard oil on the growth of the dental isolates.
- Study of the effect of stem and leaf extract of *Alternanthera sessilis* and *Achyranthes Aspera on the growth of the isolates.*
- Evaluation of biofilm forming potential of the isolated bacteria and the effect of the above mentioned agent on biofilm formation.

MATERIALS AND METHODS:-

1. ISOLATION OF CULTURE:-

- Dental swab was collected from patient.
- Nutrient Broth (NB) Media was prepared.
- Swabs were put in different test tubes containing NB and incubated at 37ºC for 24 hours.
- After detection of growth in the test tubes, the cultures from each tube were plated onto Nutrient Agar plates for isolation of pure colonies.
- The isolated microbes were observed for their colony morphology, given isolate code and the pure cultures were stored in 4°C for further use.

2. GRAM STAINING:-

- A loopful of bacterial sample was smeared onto a clean glass slide and air dried. Three slides were prepared for three different bacteria.
- For staining the smear was flooded with crystal violet solution for 1 minute.
- Crystal violet was rinsed with distilled or tap water.
- Then the slide was flooded with iodine solution for one minute and then washed with distilled or taps water.
- Then decolorizer was added onto slides for one to five seconds and rinsed off with distilled or tap water.
- Then the slide was flooded with safranin for 30 seconds and finally washed off with distilled or tap water.
- The slides were air dried and placed in an upright position and microscopically under a 100X objective to study the morphology as well as whether the bacterial samples are Gram positive or Gram negative.

3. CATALASE TEST:-

- Three clean glass slides were taken for the three isolates.

- With the help of sterilized and cooled inoculation loop small amount of culture was taken from the nutrient agar, emulsified to make a smooth suspension and smeared onto the slides.
- With a pasture pipette one drop of hydrogen peroxide was placed on the test smear and observed for effervescence.

4. HAEMOLYTIC TEST:-

- Nutrient agar was prepared and autoclaved. On cooling to 45ºC 5% blood was added to it and mixed properly and poured it to the 3 different petriplates.
- After plates solidified the bacterial samples were smeared (smear size is 1cm diameter) onto the plates and the plates incubated at 37ºC for 24 hours.
- Next day, the zone formation was observed.

5. EFFECT OF SALT AND SUGAR ON DENTAL ISOLATES:-

- Nutrient broth was prepared.
- Salt solutions of different concentrations ranging from 1M-5M were prepared. In each test tube, 100 cfu of bacteria along with Nutrient broth and different concentration of salt were added and the tubes were incubated for 18-24 hrs at 37°C.
- Next day the bacterial growth in the form of turbidity was measured by spectrophotometer at OD 600 nm. The zeta potential was also measured.
- To determine whether the concentrations are bactericidal or bacteriostatic, the cultures from each tube were sector streaked onto Nutrient Agar plates.
- The same procedure was repeated to study the effect of sugar concentration.

5. STUDY OF EFFECT OF CALCIUM CHLORIDE, $KMnO_4$, CHAROAL AND MUSTARD OIL ON THE GROWTH OF DENTAL ISOLATES:-

- For this well diffusion method was used.
- Nutrient agar plates were prepared. Onto it the bacterial isolates were swabbed.
- Using the reverse of 1ml tip holes of 9 mm diameter were punched into it and the samples to be tested were added.
- The plates were incubated at 37°C for 24 hrs. The next day the zone of bacterial growth inhibition was checked.

6. PREPARATION OF PLANT EXTRACT AND EVALUATION FOR ANTIMICROBIAL ACTIVITY:-

- Fresh leaves of Alternanthera sessilis and Achyranthes Aspera were collected from NIT RKL campus and washed several times to remove the dust particles and then sundried to remove and grinded to form powder. Then the samples were dissolved in 1 ml distil water.

Fig 4: Picture of *Alternanthera sessilis*.

Fig 5: Picture of *Achyranthes Aspera*.

- For the antimicrobial activity well diffusion method was used.
- Nutrient agar plates were prepared. Onto it the bacterial isolates were swabbed.
- Using the reverse of 1ml tip holes of 9 mm diameter were punched into it and the samples to be tested were added.
- The plates were incubated at 37°C for 24 hrs.
- The next day the zone of bacterial growth inhibition was checked.

7. EVALUATION OF BIOFILM:-

- Organisms was inoculated in 10 mL of nutrient broth in test tubes and incubated at 37ºC for 24 h.
- Tubes were washed with phosphate buffer saline (pH 7.3) and dried.
- Tubes were then stained with crystal violet (0.1%) and excess stain was washed with distilled water.
- Tubes were dried in inverted position.
- Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube.

8. EVALUATION OF DIFFERENT AGENTS ON BIOFILM FORMING POTENTIAL OF THE ISOLATED BACTERIA:-

- Overnight cultures of dental isolates were diluted. 1:100 with fresh medium and 100 µL added to individual wells of sterile 96 well-flat bottom plate followed by 100 µL of different agents mentioned in previous experiments.
- After incubation at 37ºC for 24 h, contents of each well were removed by gentle tapping and washed with phosphate buffer saline (pH 7.2) to remove free floating bacteria.
- Biofilm formed by bacteria stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying.
- Optical density (OD) of stained adherent biofilm was obtained by using ELISA reader at wavelength 570 nm.

9. FTIR:-

- Prepared plant samples were dissolved H_2O.

- 20ul was placed in the probe and scanned between 4000-400cm^{-1}
- Data obtained was analyzed for functional groups.

RESULT AND DISCUSSION:-

1. CHARACTERIZATION OF BACTERIAL ISOLATES:-

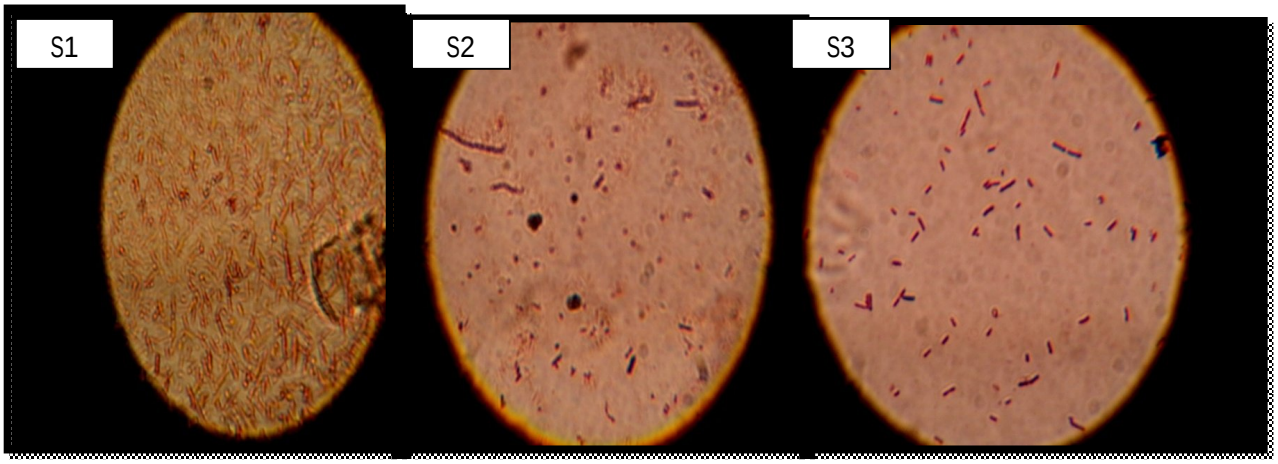

Figure 6(Gram staining pictures)

Serial no.	Isolate code	Morphology	Gram staining	Blood agar haemolysis	Catalase test
1	S1	cocci	+	β haemolytic	-
2	S2	rod	+	No zone	-
3	S3	rod	-	α haemolytic	-

Table 1(Morphological characteristics)

2. EFFECT OF SALT:-

If we add salt to foods it can also cause microbial cells to undergo osmotic shock, resulting in the loss of water from the cell and thereby causing cell death or retarded growth[36]. It has also been suggested that for some microorganisms, salt may limit oxygen solubility, interfere with cellular enzymes, or force cells to expend energy to exclude sodium ions from the cell, all of which can reduce the rate of growth [37].salt remains a commonly used component for creating an environment resistant to spoilage and inhospitable for the survival of pathogenic organisms in foods. Salt commonly plays a central role in the fermentation of foods.

Fermentation is a common process for preserving foods, in which fresh foods are transformed to desirable foods that can be preserved for longer periods of time than their fresh counterparts due to the actions of particular types of microbes [38].Products such as pickles, sauerkraut, cheeses, and fermented sausages owe many of their characteristics to the action of lactic acid bacteria. Salt favors the growth of these more salt-tolerant, beneficial organisms while inhibiting the growth of undesirable spoilage bacteria and fungi naturally present in these foods [39].

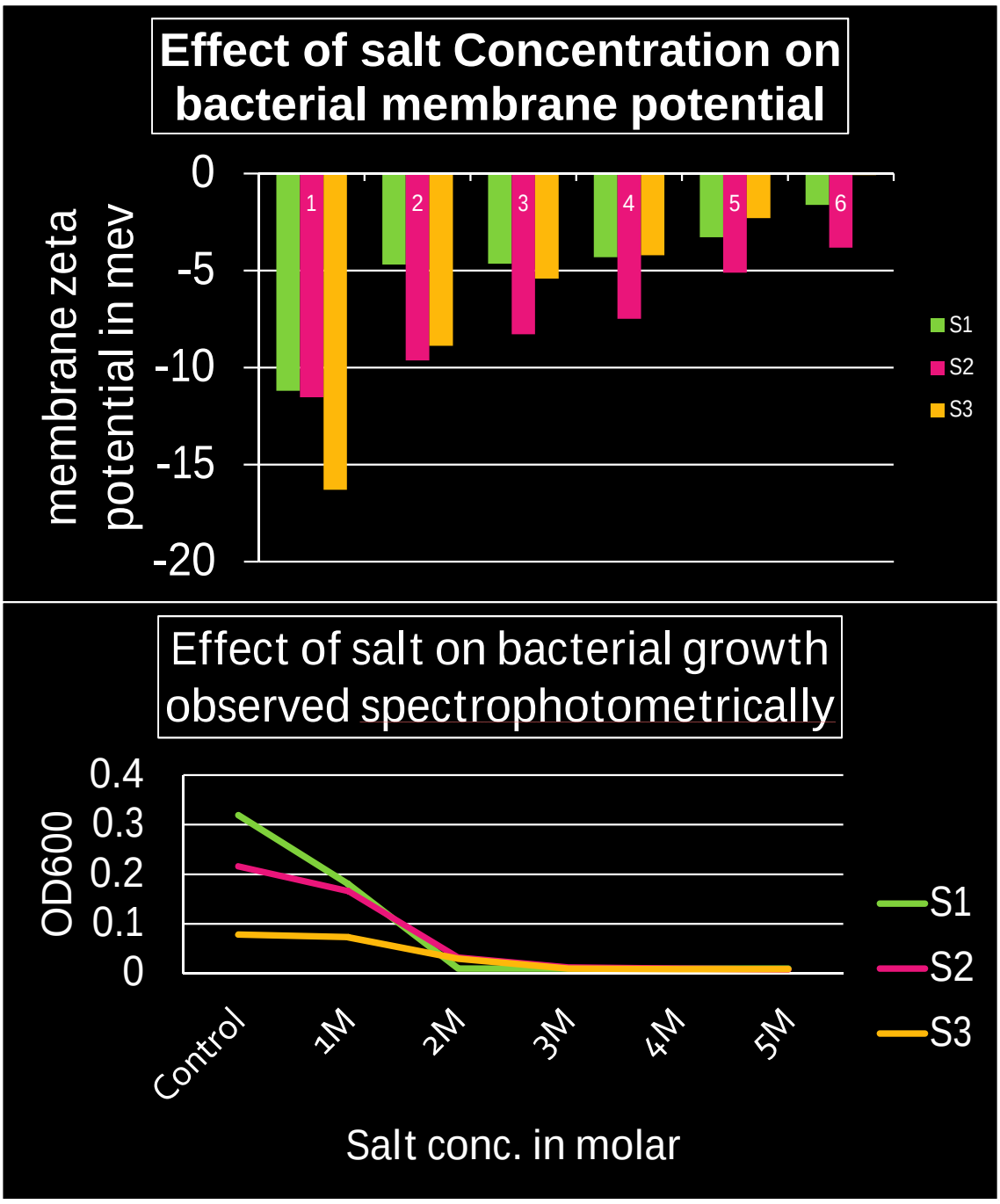

Figure 7:-(Effect of salt concentration on bacterial growth measured by spectrophotometer and zeta analyser)

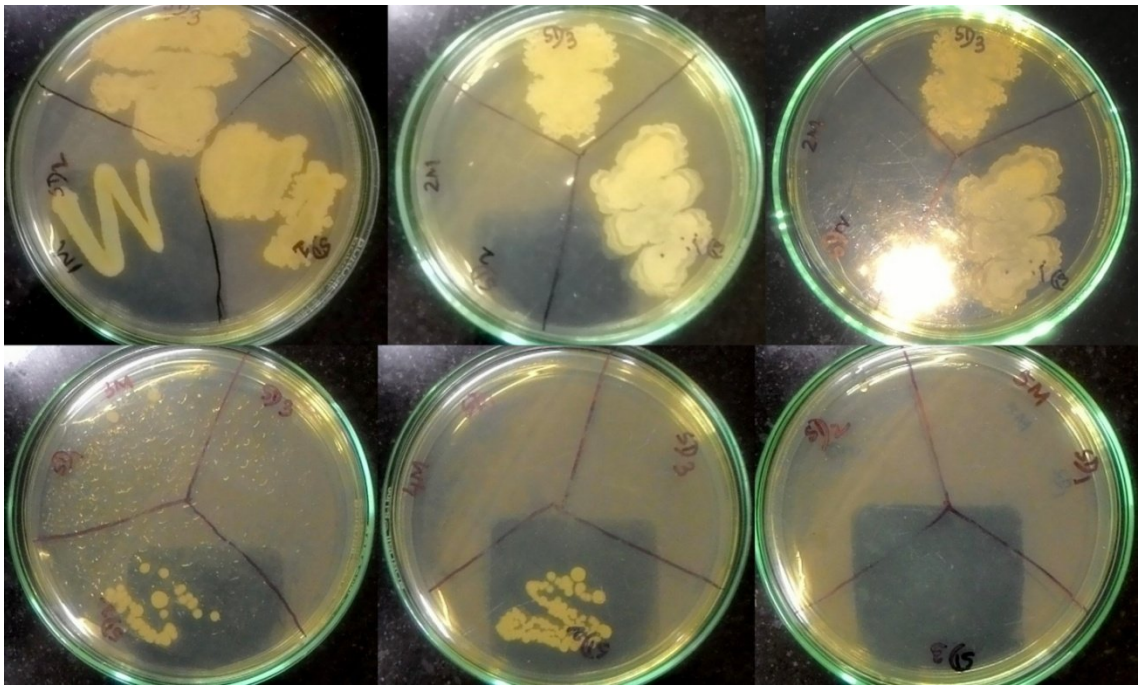

Figure 8:-(Photographs of effect of salt concentration on petriplates)

EFFECT OF GLUCOSE:-

Glucose is one of the primary molecules which serve as energy sources for almost all organisms, including bacteria. When we add glucose to the nutrient broth it may increase the overall growth rates [40].In some cases, glucose may inhibits the cell growth if the concentration is too high. Increased concentrations of glucose have been shown to limit bacterial growth by inhibiting proteinaceous enzymes; this strain on protein degradation may limit growth of bacterial populations in sugar solutions by reducing a cell's ability to breakdown and reincorporate proteinacious resources[41],[42]. In the absence of glucose, growing bacteria populations were found to exhibit higher initial growth rates, implying that glucose has an inhibitory effect on the early stages of growth(even at low glucose concentrations). In the presence of glucose growth rates were initially hampered. Bacteria grown in the 1% glucose solution attained highest growth rates and overall biomass.

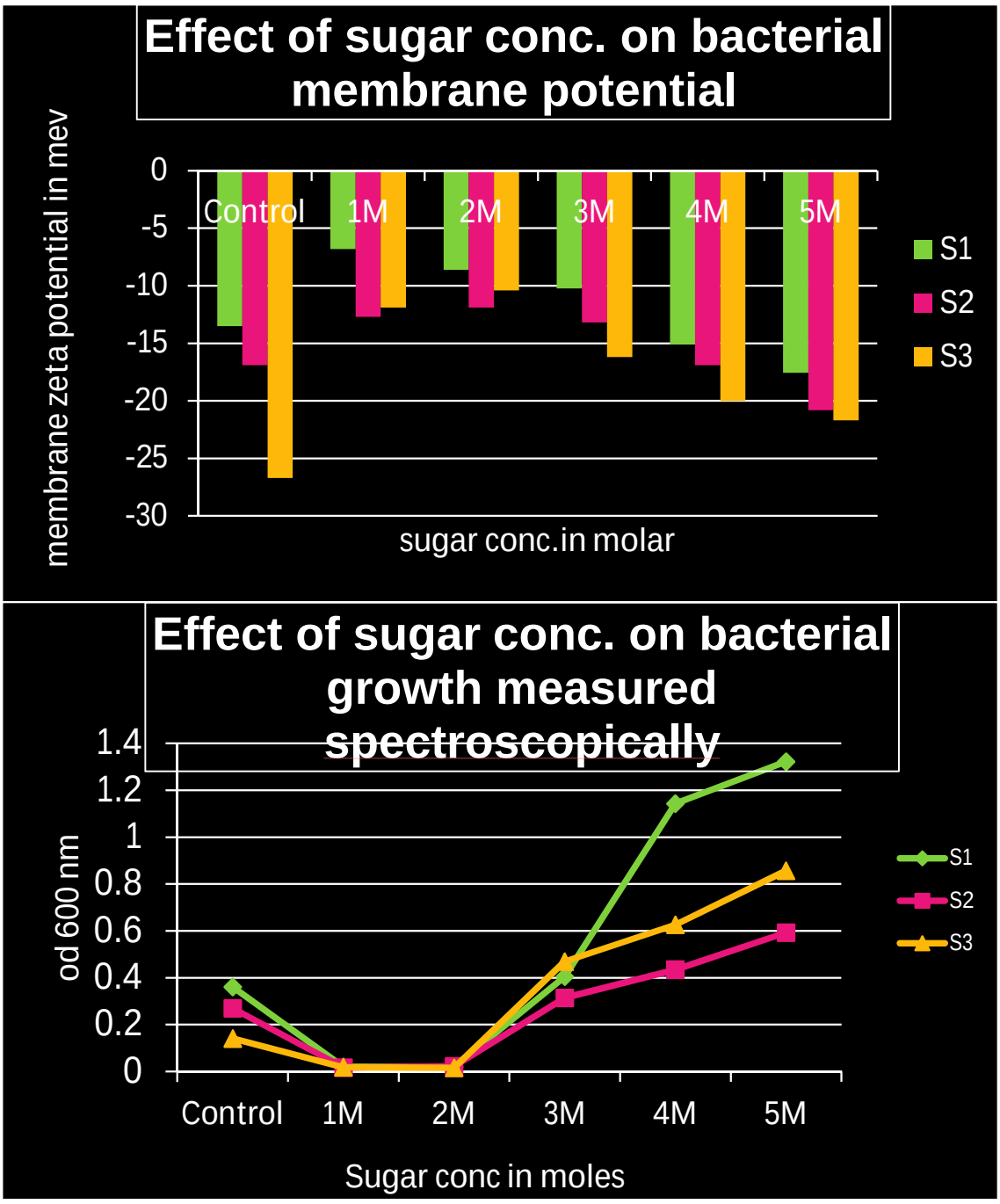

Figure 9 (effect of sugar concentration measured by spectrophotometer and by zeta analyser)

Figure 10 (Effect of sugar concentration result shown in petriplates in different concentrations).

3. EFFECT OF $KMNO_4$, $CACl_2$, CHARCOAL AND MUSTARD OIL ON THE GROWTH OF DENTAL ISOLATES:-

It controls the biological growth and remove iron and manganese. high dosage rates were required to accomplish complete inactivation of bacteria . Early research showed that a dose of 2.5 mg/L was required for complete inactivation of coli form bacteria[43]. [44] investigated the disinfectant ability of potassium permanganate on several water borne pathogenic microorganisms. The investigation studied Vibrio cholerae, Salm. typhi, and Bact. flexner.

The effects of NaCl reduction and its substitution with KCl on cell membranes of a cheese starter bacterium (Lactococcus lactis ssp. lactis), probiotic bacteria (Bifidobacterium longum, Lactobacillus acidophilus, and Lactobacillus casei), and a pathogenic bacterium (Escherichia coli) were investigated using Fourier-transform infrared (FTIR) spectroscopy. In some studies, It was found that *L. monocytogenes* can grow in the presence of 1-9% NaCl and 1-11% KCl. The higher the concentration of salt used, the longer the lag phase induced. In addition, it was observed that *L. monocytogenes* tolerate KCl better than NaCl when using the same percents in broth[45]Calcium chloride also proved to have a marked effect on the inhibitory behavior of a series of imidazole compounds.

Charcoal is “activated” because it is made to have a very small particle size. This increases its overall surface area and absorptive capacity. This activated charcoal absorbs the toxins from your body and then, since it is not absorbed by the body, the toxins along with the charcoal are expelled, leaving you cleaner and more toxin-free. Hippocrates, the Greek physician considered the father of natural medicine, used activated carbon to cure many patients. Activated charcoal (also referred to simply as *carbon*) has been called black magic because of it’s color and healing abilities. Carbon has a negative ionic charge that attracts the positive ionic charges of toxins and poisons causing them to bind to the charcoal. Removes parasites and bacteria from the body. This includes bugs that cause everything from a stuffy nose to more invasive parasites. It removes fungal toxins. It can be used as a tooth paste to whiten teeth and eliminate foul breath since it kills bacteria.

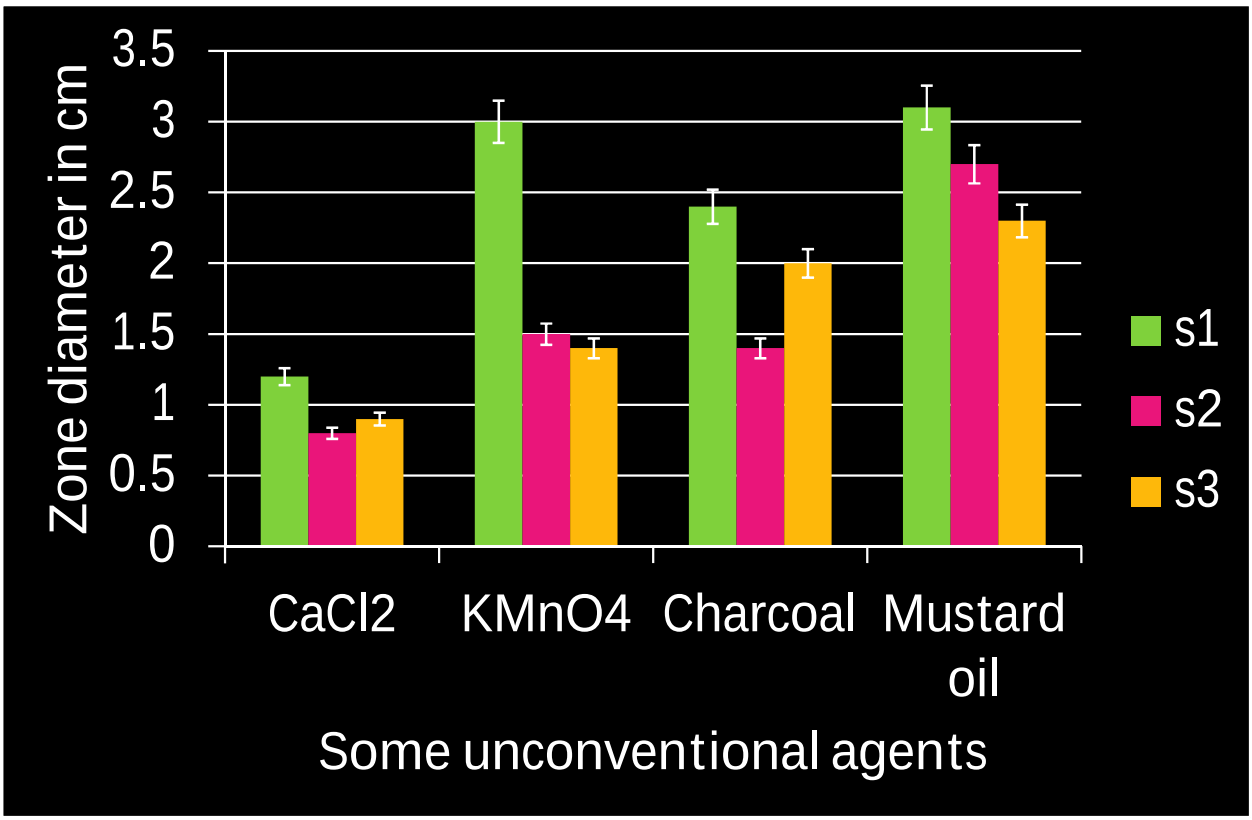

Figure 11 (Figure showing anti microbial property of some unconventional agents)

Figure 12 (Petriplate containing charcoal)

4. EFFECT OF PLANT EXTRACT ON BACTERIA:-

Plants from the genus Alternanthera are thought to possess antimicrobial and antiviral properties. *Alternanthera sesillis* has also the wound healing property,[46]. [47] revealed degenerative and necrotic changes in the liver and kidney in Swiss mice, caused by oral administration of water extract of A. sessilis in high doses through histopathological test. Alternanthera sessilis (L.) DC. (Sessile joy weed; Amaranthaceae is a popular leafy vegetable in Sri Lanka and also used as traditional medicine in China, Taiwan, India and Sri Lanka. The antibacterial effectof the leaves, inter-nodal, leaves and inter-nodal segments derived from A. Sessilis was evaluated against Proteus vulgaris, Streptococcus pyogenes, Bacillus subtilisand Salmonella typhii. The ethanolic extracts of leaves and leaves were more effective against the selected bacteria than other and solvents.

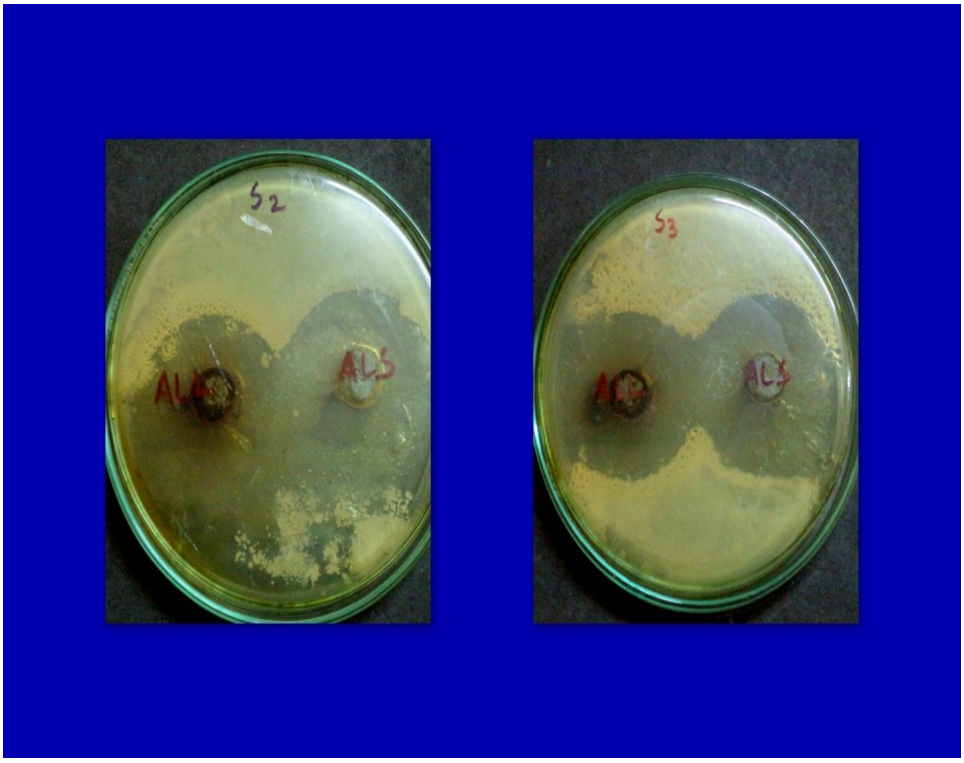

Figure 13 (Two petriplates showing the zone of diameter of the Alternanthera leaf and stem)

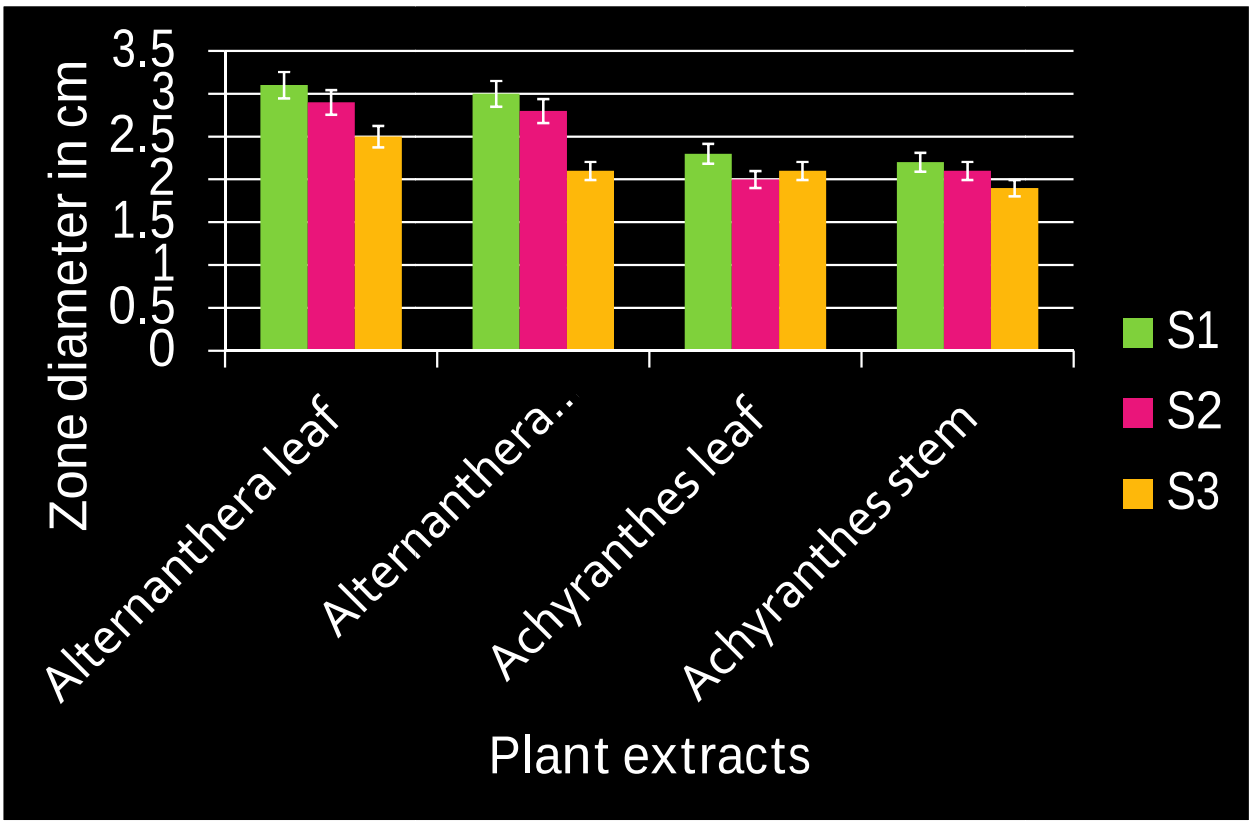

Figure 14 (Figure showing anti microbial property of two plant extract).

5. EFFECT ON SHELL:-

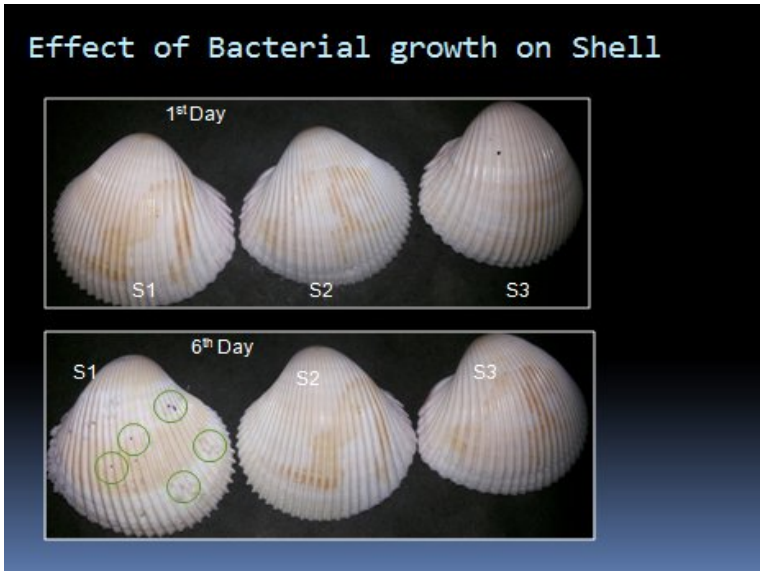

Figure 14 (The photograph showing the bacterial growth on the shell surface).6. BIOFILM FORMATION:-

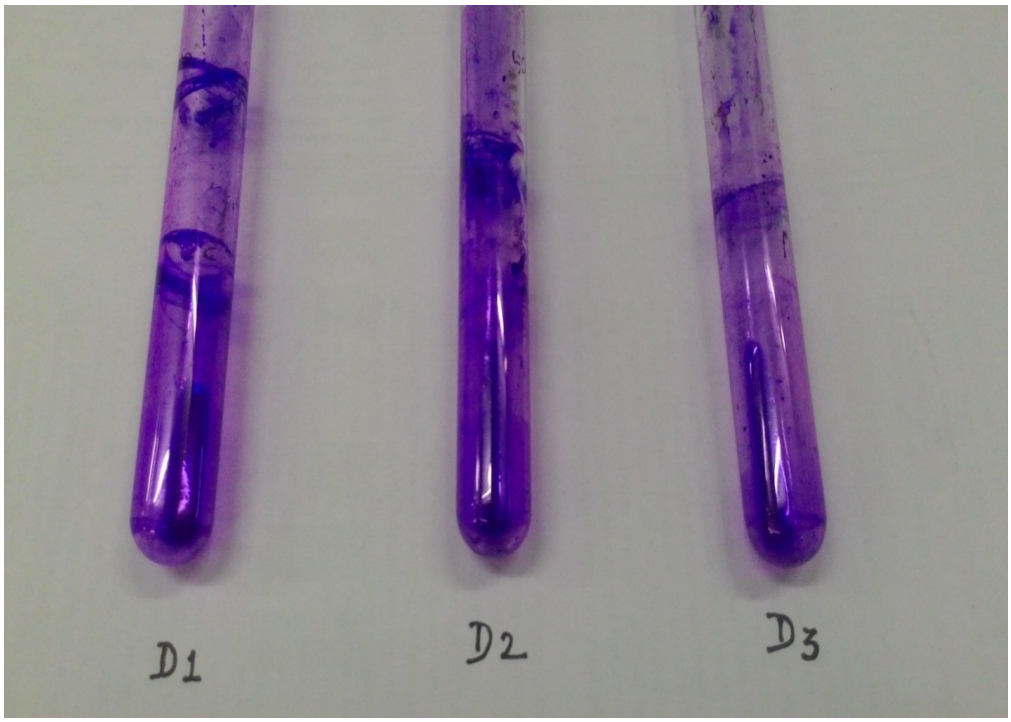

Figure 15 (Biofilm formation in test tube method).

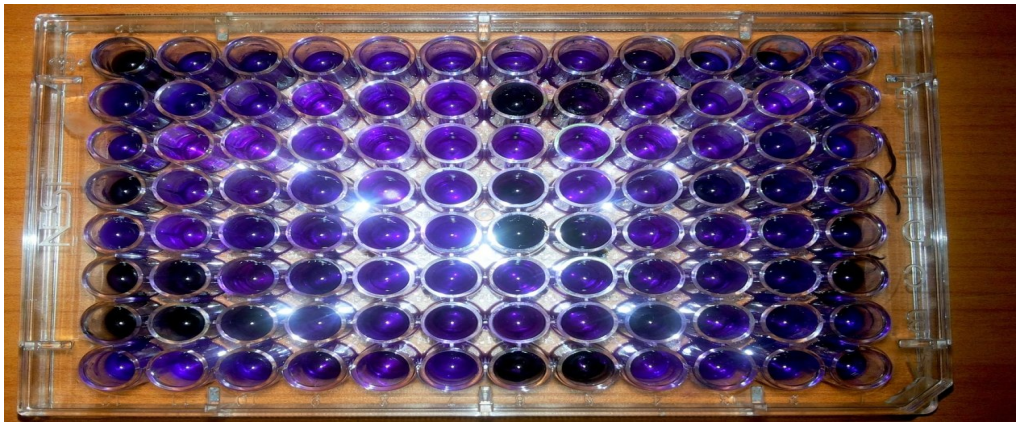

Figure 16 (Biofilm formation on 96 well plate method).

EFFECT OF VARIOUS PARAMETERS ON BIOFILM FORMATION:-

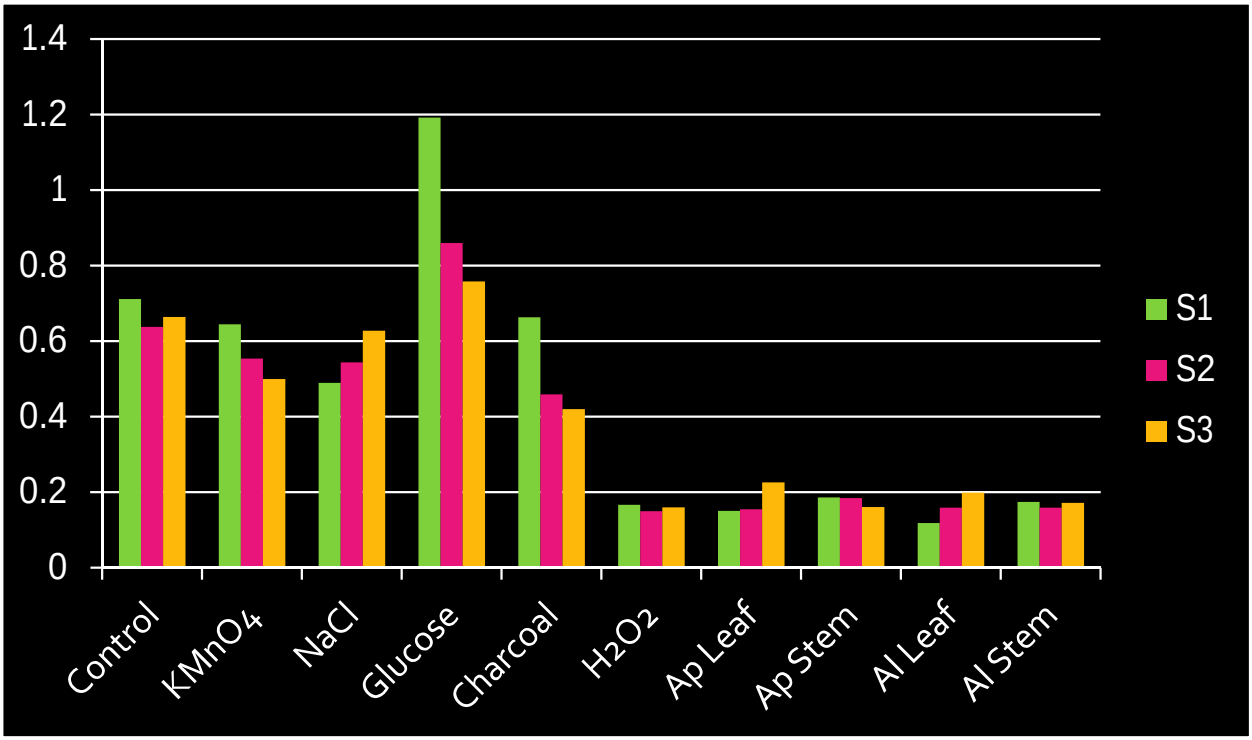

Figure 15 (Biofilm formation is showing the result in various parameters).

7. FTIR (FOURIER TRANMISSION OF INFRA RED SPECTROSCOPY):-

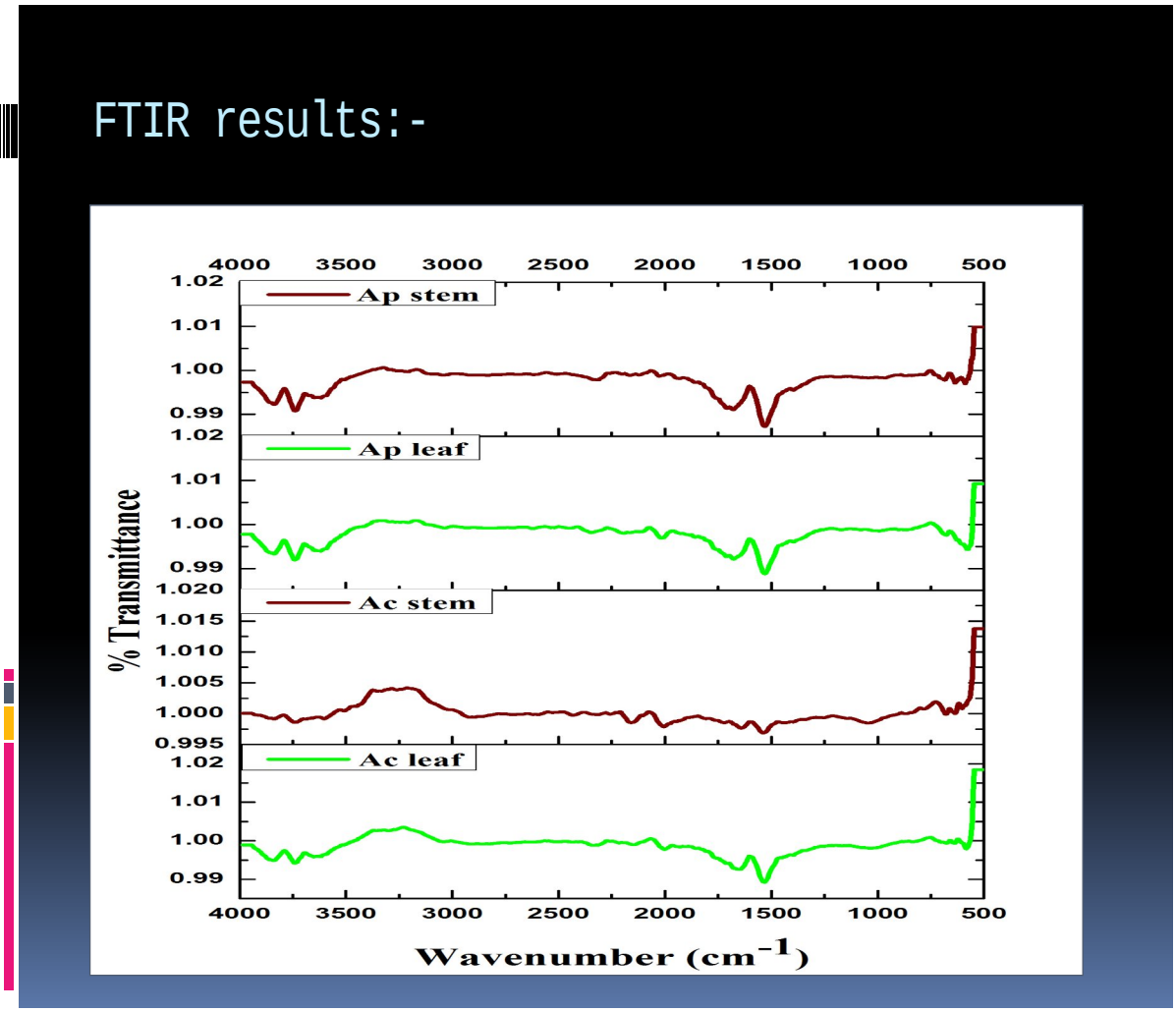

Figure 18 (The result of FTIR on the two plant extract *Alternanthera sesillis* and *Achyranthes aspera*)

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