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Effect of antimicrobial agents for the prevention of Streptococcus mutans biofilm formation

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Effect of antimicrobial agents for the prevention of *Streptococcus mutans* biofilm formation

THESIS SUBMITTED FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

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INSTITUTE OF BIOLOGICAL SCIENCES
UNIVERSITY OF RAJSHAHI
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By

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Session: 2014-2015

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Entomology and Biotechnology Laboratory
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University of Rajshahi, Bangladesh

DEDICATED
TO MY
BELOVED FATHER
DR HABIBUR RAHMAN &
MOTHER DR SHIRIN AKHTER
AND TWO CHILDREN
FARHAN AND ANIKA

DECLARATION

I do, hereby declare that, the thesis entitled **Effect of antimicrobial agents for the prevention of *Streptococcus mutans* biofilm formation** for the degree of **Doctor of Philosophy** in Medical science, contains no material, which has been submitted for the award of any other degree or diploma in any university. To the best of my knowledge and belief it contains no material previously published of written by any other person or any other forms except when due reference is made in the text of the thesis.

(**Tasnia Habib**)
Signature of the candidate

CERTIFICATION

This is to certify that the thesis entitled **Effect of antimicrobial agents for the prevention of *Streptococcus mutans* biofilm formation** has been prepared by **Tasnia Habib** under my guidance and supervision for submission to the Institute of Biological Sciences, University of Rajshahi, Bangladesh for the degree of **Doctor of Philosophy**. It is also certified that the materials included in this thesis are the original works of the researcher and have not been previously submitted for the award of any degree or diploma anywhere.

I have gone through the draft of the thesis and found it acceptable for submission.

Professor Dr Md Wahedul Islam
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CONTENTS

Page No.

Dedication	**i**
Declaration	**ii**
Certificate	**iii**
Acknowledgment	**iv**
Content	**v**
A checklist of the figures provided	**viii**
A checklist of the tables provided	**xi**
Abstract	**xiii**

CHAPTER 1: INTRODUCTION	**1-26**
Dental Plaque	3
Chemical Composition of Dental Plaque	5
Classification of Dental Plaque	6
Development of *Streptococcus mutans* Biofilm	6
Calculus or Tartar	9
Marginal gums	10
Attached gum	11
Interdental gum	11
Interdental Areas	12
Characteristics of healthy gums	12
The formation of plaque	13
Components of plaque	13
Dental plaque as a biofilm	15
Consequences of plaque formation	18
Detection of plaque formation	20
Plaque in dogs and cats	21
General notes on Medicinal plants	21
Medicinal Plants of Bangladesh	24
Field Survey	26

Page No.

CHAPTER 2: STUDY PROTOCOLS, AIMS AND OBJECTIVES	27-28

CHAPTER 3: LITERATURE REVIEW	29-48
Medicinal plants	32
History	34
Context	37
Phytochemical basis	38
Drug discovery	41
Cynodondactylon	43
Piper nigrum	44
Phytochemicals, folk medicine and research	48

CHAPTER 4: MATERIALS AND METHODS	49-92
Biological Investigation	49
In vitro Antimicrobial screening	50
Principle of Disc Diffusion Method	51
Microbiological Experimental work	52
Selection of Patient	53
Preparation of culture media	59
Composition of culture medium	62
Placement of the discs, diffusion and incubation	64
Antibacterial activity test	65
Phytochemical Investigations	66
General Methods	66
Others Methods and Instruments	76
PHYTOCHEMICAL STUDY ON *Cyndondactylon*	77
PHYTOCHEMICAL STUDY ON *Piper nigrum*	84

Page No.

Biofilm Production	88
General Methodology	88
Working Procedure for the Biofilm Formation	90
Protocol of biofilm production	91
Minimum biofilm inhibitory concentration (MBIC)	92

CHAPTER 5: RESULTS AND DISCUSSION	93-127
Microbiological Investigation	93
Phytochemical work on *Cyndondactylon*	99
Phytochemical work on *Piper nigrum*	107
Biofilm Production	117

SUMMARY AND CONCLUSION	128-130

REFERENCE	131-158

A CHECKLIST OF THE FIGURES PROVIDED

Sl. No.	Name of the figures	Page no.
Figure 1	Dental Plaque and Gingivitis formation	1
Figure 2	Dental Plaque formation	2
Figure 3	Dental Plaque formation	3
Figure 4	Decay equation	4
Figure 5	Flow chart of the Classification of Dental Plaque	6
Figure 6		7
Figure 7	Development of Dental Plaque. Confocal microscopy images of plaque growth showing the timescales involved (albeit in a laboratory model)	8
Figure 8	Properties of Bifilm	15
Figure 9		17
Figure 10	Distribution of respondents according to their sex. [n=480]	55
Figure 11	Distribution of respondents according to their Time of Oral Hygiene practice group	57
Figure 12		77
Figure 13	Schematic representation of solvent partitioning of the crude extract of *Cyndondactylon*	79
Figure 14	A (Materials used for TLC test of Cyndondactylon) B (TLC Plate showing number of visualized bands or spots) C (Cylindrical glass column containing silica gel with liquid solvent and extract sample)	82
Figure 15	Schematic representation of solvent partitioning of the crude extract of seed of *Piper nigrum*	85
Figure 16	Identifications of *S. mutans* bacteria on mitis salivarius bacitracin agar media	91
Figure 17	Flow chart for the biofilm production	92

Sl. No.	Name of the figures	Page no.
Figure 18	Effect of ethyl acetate and chloroform extract on Compound PN-1(200 and 400 µg/disc) on *Streptococcus mutans*	98
Figure 19	Inhibitory effect of chloroform, ethyl acetate and acetone extract on *Streptococcus mutans*	98
Figure 20	Characterization of *Cyndondactylon* on TLC spot	100
Figure 21	A, B (TLC plate visualized different types of compound bands which can be detected by using spray reagent)	101
Figure 22	Isolation and purification of *Cyndondactylon* using column-Chromatographic techniques	102
Figure 23	Phytol derivatives (3,7,11,15, Tetramethyl-hexadeca-2,4 dien-1-01)	103
Figure 24	Phytol derivatives (3,7,11,15, Tetramethyl-hexadeca-2-en-1-01)	104
Figure 25	Stigmasterol	105
Figure 26	^{1}H-NMR spectrum of the AS-1 in $CDCl_3$	106
Figure 27	Showed TLC spot	109
Figure 28	A (12-20), (21-2), (-56) No. test tubes are mixed with each other and dried, a single sport is detected after doing the TLC of 12 to 20 test tubes). B, C (TLC) was done of the dried sample of (21-32), (33-56), No. test tube in different solvent ratio and ($CHCl_3$ EtOAc-9 : 2) give the best result	110
Figure 29	Piperine	111
Figure 30	Spathulenol	112
Figure 31	Structure of the compound Piperine	114
Figure 32	Structure of the compound Spathulenol	115
Figure 33	^{1}H-NMR spectrum of the compound PG-1 in $CDCl_3$	116
Figure 34	Flow chart for the Protocol of biofilm production	117

Sl. No.	Name of the figures	Page no.
Figure 35	Process of biofilm formation A (Adding LB broth on 96 well plate) B (Adding 150 µL crystal violet) and C (Stain washed with deionized water)	118
Figure 36	Mean value of inbhibition of *P. aeruginosa, E. coli* and *S. mutans*	119
Figure 37	Mean value of inhibition of *P. aerginosa, E. coli* and *S. mutans*	120
Figure 38	Mean value of inbhibition of *P. aeruginosa, E. coli* and *S. mutans*	121
Figure 39	Mean value of inbhibition of *P. aeruoginosa, E. coli* and *S. mutans*	122
Figure 40	Mean value of inbhibition of *P. aeruginosa, E. coli* and *S. mutans*	123

A CHECKLIST OF THE TABLES PROVIDED

Sl. No.	Name of the tables	Page no.
Table 1		3
Table 2	Shows the distribution of respondents according to their age group. [n=480]	54
Table 3	Shows the distribution of respondents according to their Marital Status. [n=542]	56
Table 4	Shows the distribution of respondents according to their educational status group. [n=480]	56
Table 5	Shows the distribution of respondents according to their occupational status group [n=480]	57
Table 6	Shows the distribution of respondents according to their Oral Hygiene practice group [n=480]	58
Table 7	Shows the distribution of respondents according to their Material use for oral hygiene practice group. [n=480]	58
Table 8	Shows the distribution of the frequency of visit of the respondents to their Dentist for regular care [n=480]	59
Table 9	Composition	60
Table 10	Composition of Nutrient agar medium	63
Table 11	Selected plant specimen for dental plaques prevention	65
Table 12	Antibacterial activity assay-initial/ primary anti bacterial sensitivity test Result (1 mg/disc): zone of inhibition (mm)	65
Table 13	Solvent system used in column analysis of chloroform fraction	81
Table 14	Developing solvent system and Rf value of the compounds	83
Table 15	Solvent systems used in column chromatography analysis of chloroform extract	87
Table 16	Developing solvent system and Rf value of the compounds	88
Table 17	Selected plants specimen showing antibacterial sensitivity	93

Sl. No.	Name of the tables	Page no.
Table 18	Anti bacterial activity test result for selected specimen (2mg/disc) zone of inhibition (mm)	94
Table 19	Anti bacterial activity test result for selected specimen (3mg/disc) zone of inhibition (mm)	94
Table 20	*In vitro* antibacterial activity of two fractions of the *Piper nigrum* extract	95
Table 21	*In vitro* antibacterial activity of compound PN-1	96
Table 22	*In vitro* antibacterial activity of three fractions of the *C. dactylon* extract	97
Table 23	*In vitro* antibacterial activity of compound CD-1	97
Table 24	The compound PN-1 was found to be identical with the chemdraw structure	113
Table 25	Effect of 3,7,11,15-tretramethyl-hexadeca-2,4dien-1-01 of Cynodondactylon on the biofilm of *P. aeruginosa*, *E. coli* and *S. mutans*	119
Table 26	Effect of , 7,11,15-tretramethyl- hexadeca-2 en 1-01 of *Cynodondactylon* on the biofilm of *P. aeruginosa, E. coli* and *S. mutans*	120
Table 27	Effect of Stigmasterol of *Cynodondactylon* on the biofilm of *P. aeruginosa, E. coli* and *S. mutans*	121
Table 28	Effect of Spathulenol of *Piper nigrum* on the biofilm of *P. aeruginosa, E. coli* and *S. mutans*	122
Table 29	Effect of Piperine of *Piper nigrum* on the biofilm of *P. aeruginosa, E. coli* and *S. mutans*	123
Table 30	Effect of growth of inhibition different compound of *Cynodondactylon* on the biofilm of *P. aeruginosa, E. coli* and *S. mutans*	124
Table 31	Effect of growth of inhibition of different compound of *Piper nigrum* on the biofilm of *Pseudomonas aeruginosa, Escherichia coli* and *Streptococcus mutans* bacteria	124

ABSTRACT

Oral diseases especially *Streptococcus mutans* bacterial biofilm are the most significant issue among all the infectious diseases of the world. The present study is attempt to generate interest among the society regarding the potential natural alternatives of two medicinal plants such as-*Cyndon dactylon* or *Pipper nigrum* which are used for the prevention of *Streptococcus mutans* bacterial biofilm, detection of adhesion strength of *S. mutans* bacterial biofilm and structure determination of the isolated compounds using phytochemicals (Mass, IR, NMR) of analysis. Two types of medicinal plants such as *Cyndon dactylon* and *Pipper nigrum* were drying, washing and pulverization and added with ethyl acetate and methanol, filtration of the crude extracts, isolation and identifications of *S. mutans* biofilm sphectrophotometrically. The effect of different plant extracts on biofilm through antibacterial disc diffusion methods and biofilm formation methods were conducted. The extracts were isolated and purified using column chromatographic techniques and structure determination were using (NMR, IR, MASS) analysis. Finally the results of the crude ethyl acetate extract of *Cyndon dactylon* showed highest antimicrobial activities. The present results found three specific compounds from *Cyndon dactylon*- 3,7,11,15 tetramethyl hexadeca 2-4di en 1- 01, 3,7,11,15 tetramethyl hexadeca 2en 1-01 from phytol derivatives and Stigmasterol. Among the compounds 3,7,11,15 tetramethyl hexadeca 2en 1-01 showed the highest antimicrobial activities. The results further found that black pipper have two compounds - Piperine and Spathulenol and among the two compounds piperine showed highest antimicrobial activities.

CHAPTER 1
INTRODUCTION

Periodontal diseases with the dental caries are the most serious or significant issue among all the infectious diseases of these whole world. Oral health can affect our normal life style and poor oral hygiene is connected with the chronic infection and systemic disease. The relationship between the oral diseases and the oral microorganisms are well established. Lack of oral hygiene and excess of fleshy foods and sweets can harm our teeth by causing pain in the teeth, bleeding from gums and dental caries (Palombo 2011). The development of dental caries usually connects with acidogenic and aciduric gram positive bacteria (*Mutans streptococci, Lactobacilli* and *Actinomycetes*) and periodontal diseases are associated with the gram-negative microorganisms such as *Porphyromonasgingivalis, Actinobacillus, Provotella* and *Fusobacterium*.

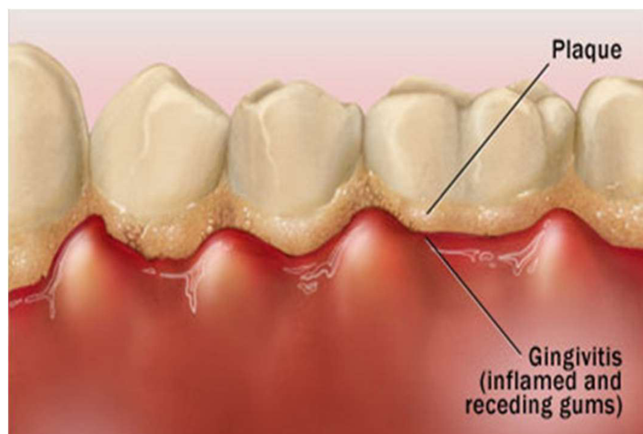


Figure 1. Dental Plaque and Gingivitis formation

Recently, a human oral mcrobioe project indicates that more than 764 kinds of bacteria and 102 kinds of fungi specially among 132 kinds of bacteria can causes the development of diseases in oral cavity. The indicated pathogenic bacteria were closely related with the opportunistic pathogens involved in bacterial endocarditis,

aspiration pneumonia, osteomyelitis, preterm low birth weight, or any heart disease, cerebral infarction etc.

These reports indicate that the dental microorganism not only responsible for various types of disease in the oral cavity but also could be implicated in systemic disease. In case of these situations, it would be impossible to protect pathogenic bacteria of dental plaque biofilm from antibiotics. Although chlorohexidine gluconate and essential oil is widely used to prevent dental biofilm formation as an antimicrobial agents but it has some disadvantages like dental pigmentation, burning sensation in oral cavity and changes in the sensation of the taste (Choi *et al*. 2017).

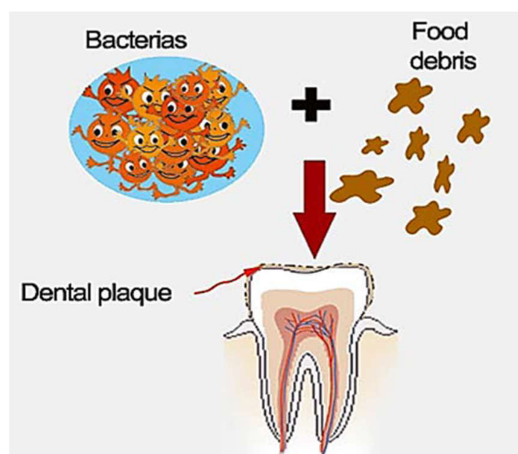


Figure 2. Dental Plaque formation

So, there is a necessary requirement for discovering the drug molecule which will have both antibiotic and antimicrobial properties against all the cariogenic bacteria specially *Streptococcus* species isolated from dental plaque biofilm (Sharma and Singh 2017).

Dental Plaque

The plaque is a colorless sticky film that forms by the bacteria and deposited mainly at the base of human teeth. But when the tarter was formed, it converted into brown or pale yellowish color. It is commonly observed between the teeth, in the front portion and behind the chewing surfaces of the teeth along with the gum line or below the gum line of the cervical margins. This dental plaque is commonly referred to as microbial plaque biofilm or bacterial plaque biofilm.

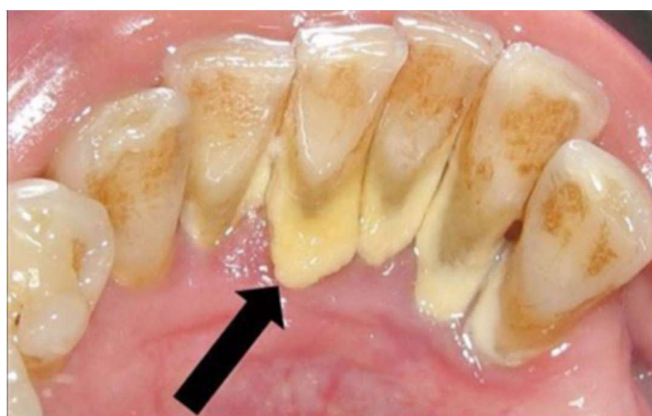


Figure 3. Dental Plaque formation

Scientifically the plaque was termed as "biofilm" because it's actually a community of living microbes surrounded by gluey polymer layer. The sticky coating helps the microbes attach to surface in our mouth so they can grow in to living microbes (Christine Franck 2019).

Table 1.

Steps of Plaque Formation	Description
Association	Dental pellicle forms on the tooth (normally on tooth), and provides bacteria surface to attach
Adhesion	Within hours, bacteria loosely binds to the pellicle.
Proliferation	Bacteria spreads throughout the mouth and begins to multiply.
Microcolonies	Microcolonies are formed. Streptococci secrete protective layer (slime layer).
Biofilm formation	Microcolonies form complex groups with metabolic advantages.
Growth or maturation	The biofilm develops a primitive circulatory system

The dental plaque decays the tooth and destructs the tooth local tissues by the acid produced from the degradation of bacteria as well as fermentation of the sugar and periodontal problems like periodontitis and gingivitis; (Wolf and Hassell 2006*)*. Therefore, it is perquisite to remove the bacterial mass (Verkaik *et al*. 2011). The control and removal of the plaque can be accomplished by regular and correct brushing of the tooth once or twice a day and some interdental aids like interdental brushes and dental floss should be recommended to use (Darby and Walsh 2010*)*.

The dental biofilm formation disrupts the oral health as it is acidic and caused demineralization in the teeth which is called dental caries or sometimes it become harden into dental calculus (tartar) (Summitt *et al*. 2006). Calculus is a hard dental tartar that cannot be treated by tooth brushing or with any other interdental aids, it can be only removed by the professional cleaning of the tooth (*Wolf and Hassell 2006).*

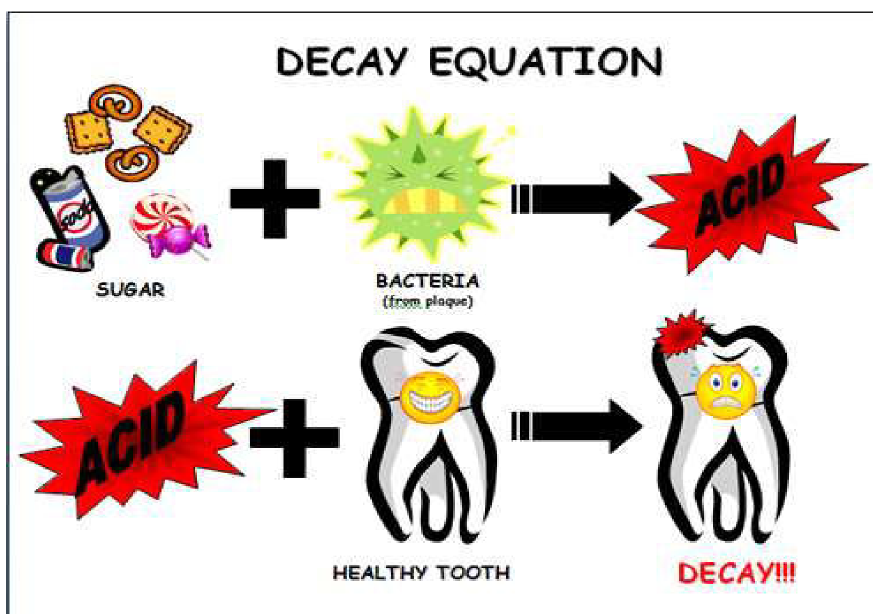


Figure 4. Decay equation

Chemical Composition of Dental Plaque

- 80% water
- 20% solids including bacterial cells (35% dry weight) and extracellular components (65% the dry weight).
- Other Microorganisms including-
  - Mycoplasma
  - Yeast
  - Protozoa
  - Viruses
- Host cells in Dental plaque.
  - ✓ Epithelial cells
  - ✓ Macrophages
  - ✓ Leukocytes

Intracellular Matrix in Dental Plaque

- ✓ Organic components
- ✓ Inorganic components
- ✓ Material from Saliva, GCF and bacteria

Organic Constituents

- ❖ Polysaccharides - dextran 95% (adhesion), levan 5%, Sialic acid and fructose
- ❖ Proteins - Albumin
- ❖ Glycoproteins - saliva
- ❖ Lipid materials - Membrane remnants of bacteria and host cells.

Inorganic Constituents

- ❖ Primarily - Calcium & Phosphate
- ❖ Traces - Sodium, Potassium and Fluoride
- ❖ Fluoride - From external sources like is derived tooth paste, mouth washes

Classification of Dental Plaque

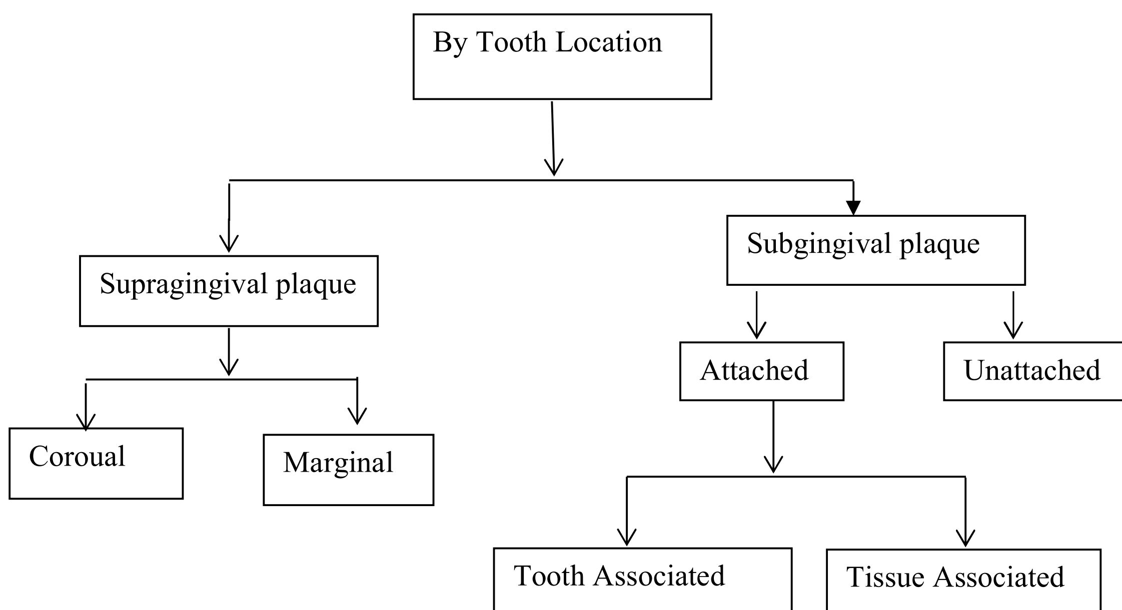


Figure 5. Flow chart of the Classification of Dental Plaque

Development of *Streptococcus mutans* Biofilm

Six stages for the development of plaque biofilm formation are mentioned below-

Stage 1: Formation of an acellular layer

The layer consists of salivary glycoproteins, phosphoproteins and lipids called the acquired pellicle but bacteria are unable to grow immediately on naked enamel surfaces.

Stage 2: Initial attachment

Streptococcus sanguinis is found to float freely on the teeth. It is common inhabitants on the human mouth and forms primary attachment to the pellicle by the process of weak and reversible Vander Waals forces. This bacterium multiplies and forms an adhesive structures by the help of pili.

Stage 3: Irreversible attachment:

Organisms, which are not able to attach with the pellicle, they initiate to adhere to the first colonizer layer with the irreversible attachments by forming specific adhesion-receptor interactions. The bacteria multiply and form micro colonies that embedded to the extracellular matrix.

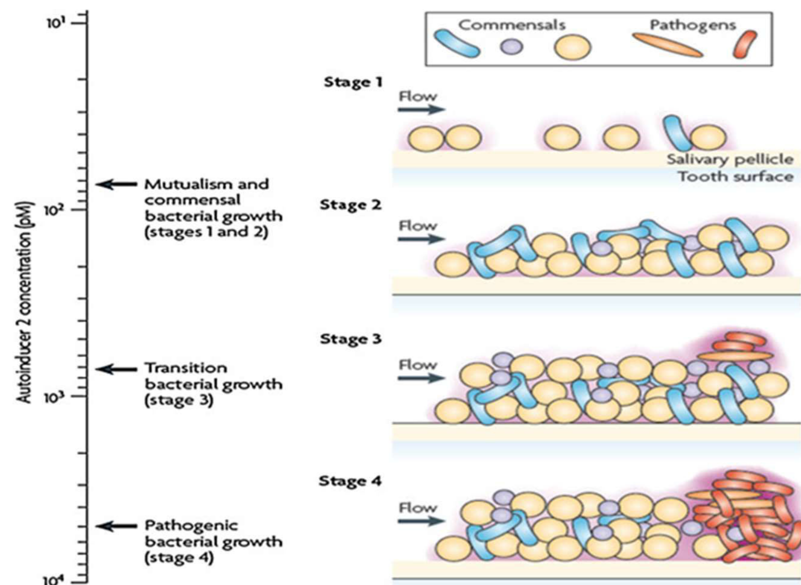


Figure 6.

Stage 4. Early Maturation (Maturation I)

When bacteria develop attachments, early colonizers established their existence. This process accelerate to increase the dental plaque complexity as a result of allogenic factors like anaerobic zones due to oxygen consumption, food chain formation and creation of receptor sites for bacterial attachments. Cell division was occurred and new bacteria were formed due to the bacterial multiplication.

Stage 5: Late Maturation (Maturation II)

In this maturation stage, microbial diversity has the negative correlation with cell division rate. The plaque heterogeneous nature was formed due to development of the mosaic microenvironments when the various concentrations of pH, oxygen and secondary metabolite are accumulated in the micro colonies. The microbial ecology creates a pseudo-steady-state climax community where the cell was constantly turned over, but the overall cellular state remains unchanged. Therefore, three-dimensional thick layer of dental plaque biofilm was generated.

Stage 6: Dispersion

The biofilm degradating Enzymes (dispersion B) allow several bacteria to disperse themselves from the biofilm. Sometimes this process happens in the deleterious environmental conditions the new surfaces are spread and colonized on the oral cavity.

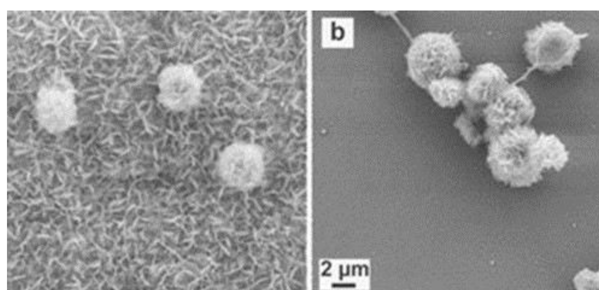


Figure 8. Development of Dental Plaque. Confocal microscopy images of plaque growth showing the timescales involved (albeit in a laboratory model).

24h: Cells and microcolonies with monolayer (stages i-iv).

48h: The three dimensional growth and development of the microcolonies in the cross-section at the bottom of the image (stages iii and iv).

96h: The coverage of entire surface (stages iv and v) (Hope and Wilson 2006).

Calculus or **Tartar**

In dentistry, **tartar** or **calculus** is a hardened form of dental plaque. It is formed on the teeth due to the precipitation of the minerals secreted from saliva and gingival crevicular fluid (GCF). This precipitation kills the bacteria in dental plaque, but the formation of the rough and hardened surface provides an ideal surface for further plaque formation. This process leads to calculus formation that forms both along with the gum line referred to as supra gingival and within the narrow sulcus that found between the teeth and the gingiva referred to as sub gingival.

Calculus formation is associated with the clinical manifestations that include bad breath, receding gums and chronically inflamed gingiva. The regular brushing and flossing of the teeth can remove the plaque. Calculus in the teeth can also be removed by the help of ultrasonic tools or dental hand instruments (popularly referred to as a periodontal scaler).

Structure

The gums are the part of the soft tissue lining in the mouth. It surrounds the mouth and provides basement and support to the teeth. Unlike the soft tissue linings of the lips and cheeks, the gums are tightly bound to the underlying bone which helps resistant and the friction of food passing over them. Thus healthy gum provides an effective barrier to the barrage of periodontal insults to deeper tissue. Healthy gums are usually coral pink in light skinned people, but may be naturally darker with melanin pigmentation.

The color changes in the teeth particularly redness with swelling and bleeding tendency as well as inflammation might be due to the accumulation of bacterial plaque. Overall, the clinical appearance of the tissue reflects the underlying histology in health and disease. The unhealthy gum tissue can provide support for periodontal disease into the deeper tissue of the periodontium that lead to a weaker prognosis for long time retention of the teeth health. Both the periodontal therapy and homecare instructions delivered to the patients by dental professionals and restorative on the basis of the clinical conditions of the tissue (Bath-Balogh and Fehrenbach 2011).

Anatomically, human gums are divided into the followings:

Marginal gums

The marginal type of the gum is the edge of the gums that surrounds the teeth in a collar-like fashion. Nearly in half of the individuals, it is free from gingival groove that is demarcated from the adjacent and attached gums by a shallow linear depression. This slight depression was formed on the outer surface of the gum which does not connected to the gingival sulcus depth instead of the apical border of the junctional epithelium. This outer groove differs in the depth that is based on oral cavity's area, which is very prominent on mandibular anteriors and premolars.

The marginal gum has different sizes that ranges from 0.5 to 2.0 mm from the undulating gingival crest to the attached gingiva. The marginal gingiva follows the scalloped pattern that is established by the contour of the cement enamel junction (CEJ) of the teeth. The marginal gingiva has more translucent appearance than the attached gingiva, although it showed the similarity in clinical appearance of pinkness, dullness and firmness. On the contrary, the marginal gingiva does not have the stippling appearance where the tissue is not attached to the underlying tooth surface which can be demonstrated by the help of periodontal probe. The

marginal gingiva is fleshy appearance and is not supported by bone. It is only stabilized by the gingival fibers. The gingival margin is clinically also observed and its location could be recorded on the chart (Bath-Balogh and Fehrenbach 2011).

Attached gum

The attached gum is the continuation of the marginal gum. It is firmly attached to the underlying periosteum of alveolar bone. The facial portion of this gum extends to the relatively loose and movable alveolar mucosa where it is delimited by the mucogingival junction. Attached gum is found to be present with the surface stippling. The dried tissue is dull, tightly bounded and immobile with various amounts of stippling. The attached gum has various width based on its location. It's width in the facial portion varies in different parts of the mouth. The greatest width is found in the incisor zones of maxilla (3.5 to 4.5 mm) and the mandible (3.3 to 3.9 mm) and less one is observed in the posterior portions as well as the least one in the first premolar area (1.9 mm in the maxilla and 1.8 mm in the mandible). However, several portions of attached gum might be indispensible for the stability of the underlying root of the tooth (Bath-Balogh and Fehrenbach 2011)

Interdental gum

The interdental gum exists between the teeth which occupies the gingival embrasure where the interproximal space is observed beneath the tooth contact area. The interdental papilla is found as pyramided or "col" shaped. Attached gums are covered with keratin that have resistant to the chewing forces.

On the basis of the contacting tooth surface expansion, the col shape varies in both depth and width. The col is non keratinized except epithelium covered col, which consists of the marginal gum of the adjacent teeth. It is generally found in the broad interdental gingiva of the posterior teeth but absence in the interproximal tissue of anterior teeth as the adjacent tissue is narrower. Due to the lack of contact

with adjacent teeth, the attached gum extensions are uninterrupted from the facial to the lingual parts. The col may be played a pivotal role in the periodontal disease formation but it is clinically identified only when teeth are extracted properly (Bath-Balogh and Fehrenbach 2011).

Interdental Areas

It is the part of gum which extends in between two teeth up to the contact point. There are facial and lingual portions in interdental papilla. Interdental papilla has a summit and margins which are concave. The tip and the margins are not attached and the central part is found to be attached. In the inflammations, the interdental papilla loses its concavity.

Characteristics of healthy gums

Color

The coral pink is the symptom of the healthy gum. Red, white and blue colours can be signified as inflammatory (gingivitis) or pathogenic gum. Variation in the colour of gum might be due to the factors of thickness and degree of epithelial keratinization, blood flow, natural pigmentation, disease and medications (Mosby's Medical Dictionary, 8th edition, 2009).

The gum colour is a key factor for gum health. Melanin deposits at the base of the interdental papillae can cause dark spots or patches on the gums. Gum depigmentation (aka gum bleaching) is the procedure that is used to remove these discolorations in cosmetic dentistry.

Contour

Healthy gums bear a smooth and curved edge around each tooth. In healthy gums, the space between the teeth are filled and fitted properly, unlike the swollen gum papilla that is found in gingivitis or the empty interdental embrasure in periodontal

disease. Healthy gums are always firmly attached to each tooth where the gum surface becomes narrower to knife-edge at the free gingival margin. On the other hand, inflamed gums have a puffy margin.

Texture

Healthy gum is always found as a strong attachment and firm texture that is resistant and protective to the movement and the texture often exhibit surface stippling. In contrast, the unhealthy gums is often swollen and less firmly attached. Healthy gums have an orange-peel like structure to it due to the stippling.

Reaction to disturbance

Healthy gums do not show any reaction to normal disturbance like brushing and periodontal probing. The probing (BOP) and purulent exudates are found to bleed in the unhealthy gums. Tooth cervical margin is the surface above the junction of the crown and the root of the tooth.

The formation of plaque

Dental plaque is a biofilm which is attached to tooth surfaces, restorations and prosthetic appliances (including dentures and bridges) if it is left undisturbed. The proper knowledge in plaque formation, composition and characteristics is very much helpful to control it (Chetrus and Ion 2013). The acquired pellicle is a salivary layer which is consisted of glycoproteins and forms new teeth shortly (Kreth, Merritt and Qi 2009). When bacteria affected the pellicle layer and multiply on teeth, which results in oral diseases.

Components of plaque

Different bacteria are generally existed in the mouth. These bacteria, leukocytes, neutrophils, macrophages and lymphocytes are the normal compositions in oral cavity and keep contribution to dental health (Darby and Walsh 2010).

Approximately 80–90% of water is found in the plaque. Seventy percent bacteria and remaining 30% of polysaccharides and glycol proteins occupy the dry weight of plaque (Marsh and Bradshaw 1995).

Bacteria

Streptococcus mutans and other anaerobes (*Fusobacterium* and *Actinobacteria*) are the biofilm forming microorganisms. The composition of the biofilm varies on the basis of the location of these microorganisms in the mouth (Darby and Walsh 2010). *Streptococcus mutans*, *Fusobacterium* and *Actinobacteria* are primarily colonized on the tooth surface and they play a vital role for in the establishment of the existence of early biofilm formation (Kolenbrander 2000). These microorganisms are normally existed in the human oral cavity and are found to exhibit harmless in dental health. However, the regular brushing of tooth may be failed to remove the dental plaque and allows the microorganisms to proliferate and thus form a thick layer, which can lead to various kinds of dental diseases for the host. These microorganisms absorb energy from the nearest tooth surface by the process of fermenting dietary sucrose and produce acids.

The equilibrium position of the bacteria differs at the various stages of the plaque formation. The following is a summary of the bacteria which may be existed during the plaque maturation phases:

Early biofilm: early gram positive cocci

Older biofilm (3–4 days): increased numbers of filaments and fusiforms

4–9 days undisturbed: more complex flora with rods and filamentous forms

7–14 days: Vibrio species, spirochetes, more gram negative organisms (Wilkins 2009)

Dental plaque as a biofilm

Dental plaque is a biofilm and adhered to the tooth surface. It creates microbial community that is particularly organized to the functional structure (Marsh 2006). Plaque is enriched with around 1000 different bacterial species that is estimated by using modern techniques (ten Cate 2006).

The salivary pellicles immediately colonize the clean tooth surface that acts as an adhesive. This recognizes and allows the early colonizing bacteria to adhere and attach to the tooth, then finally multiply and grow. After the growth of these early colonizers, the biofilm becomes more compliant to other bacterial species and commonly referred to as late colonizers (ten Cate 2006).

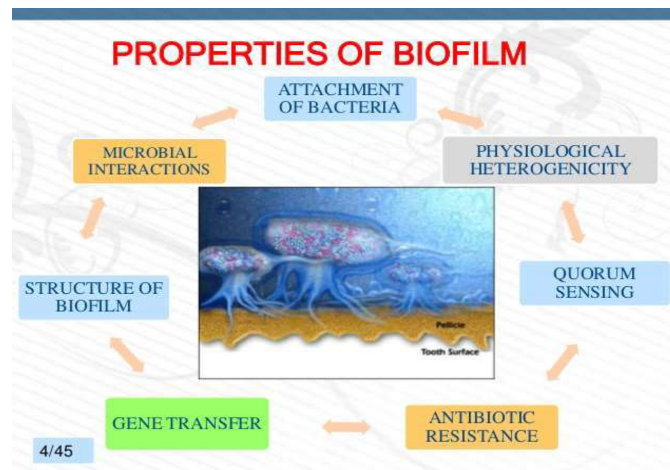


Figure 8. Properties of Bifilm

Early colonizers

(ten Cate 2006)

Mainly *Streptococcus* species (60-90%)

Eikenella spp.

Haemophilus spp.

Prevotella spp.

Priopionibacterium spp.

Capnocytophaga spp.

Veil- lonella spp.

Late colonisers

(ten Cate 2006)

Aactinomycetemcomitans

Prevotellaintermedia

Eubacterium spp.

Treponema spp.

Porphyromonasgingivalis

Fusobacterium nucleatum is observed between the early and late colonizers and the both are linked together. The plaques ecosystem is enhanced by some salivary components. The salivary alpha-amylase plays a vital role as a key component for binding and adhesion (*Scannapieco et al*. 1993). Proline rich proteins (PRP) and statherins are also the key factors for the plaque formation (ten Cate 2006).

Supra gingival biofilm

Supra gingival biofilm is a dental plaque which forms and found above the gums. It is one kind of plaque that is formed after the teeth brushing. It is commonly found in between the teeth and in the pits and grooves of the teeth along gum lines, which is consisted of mostly aerobic bacteria. If the plaque persists on the tooth for long time, anaerobic bacteria start to multiply on this plaque (Chetrus and Ion 2013*)*.

Environment

The environment of mouth is the most crucial factor for the tooth surfaces that are uniquely hard as well as non-shedding components like any other part of the body. The warm and moist conditions of the mouth are convenient factors for the growth and multiplication of the microorganisms for dental plaque development (ten Cate 2006).

The temperature, pH, redox reactions and saliva are the responsible components for the dental plaque formation in the human mouth (Marsh 2003, Marsh and

Devine 2011). The range of normal pH in the saliva is about 6 to 7 but the plaque biofilm is observed to develop in the pH of 6.7 and 8.3 (Mc Dermid *et al*. 1988; Humphrey and Williamson 2001). This may indicates that the natural atmosphere of the mouth is conducive for the growth and development of bacteria in the dental plaque formation. Saliva acts as a buffer for the maintenance of the normal pH in the mouth (Marsh 2003). In addition, saliva and other gingival crevicular fluid pertain primary nutrient factors of amino acids, proteins and glycoproteins that are responsible for the bacterial growth in plaque formation. The host dietary factor does not play major role in supplying the nutrients for the microflora existing in the mouth (Marsh *et al.* 2011). 35 and 36 °C are normal ranges of the temperature in the mouth but only two-degree (°C) change in the temperature enhances the growth of the dominant bacterial species in the mouth for the plaque formation (Marsh and Devine 2011). Redox reactions by aerobic bacteria keep the oxygen levels at semi-stable homeostatic condition in the mouth that allows the bacteria to survive (Marsh and Devine 2011).

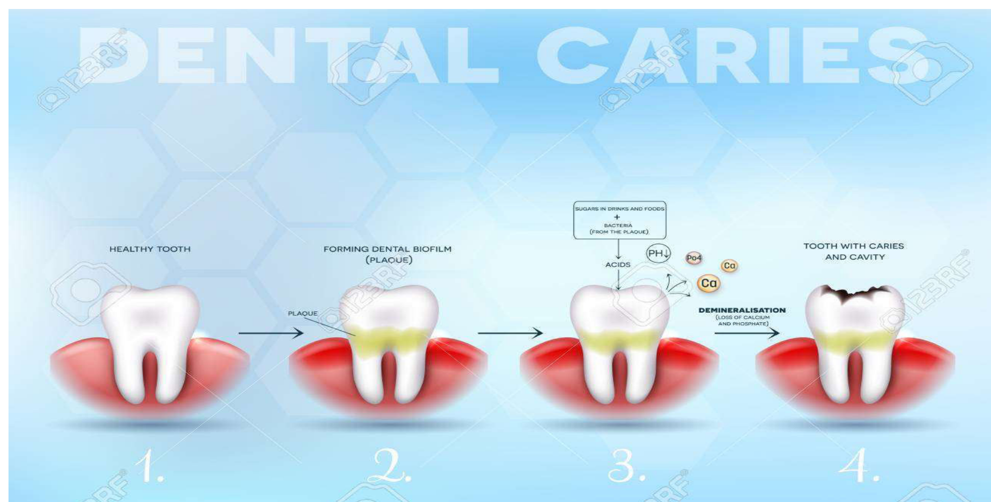


Figure 9.

Consequences of plaque formation

Gingivitis

Gingivitis is an inflammatory wound of the gingival tissue which is affected by the plaque formation. This process is mediated by host-microbe interactions. The bacteria responsible for biofilm formation elicit the host response resulting in the inflammation of localized tissue (Armitage 1999). This characterization is considered as the cardinal inflammatory signs of red, puffy appearance of the gums and bleeding during the brushing or flossing (Chandrasoma and Taylor 2005). Gingivitis is medicated by elimination of the plaque from teeth. However, if the condition is allowed to continue for long time, the inflammation may affect the supporting and adjacent tissues in a progression, which is referred to as periodontitis (Noble 2012).

Periodontitis

Periodontitis is an infection of the gums which leads to bone destruction around the teeth in the jaw. Periodontitis is the next step of the gingivitis has been affected. But it does not mean that all individuals who have gingivitis will be affected by periodontitis (Noble 2012, Rateitschak *et al*. 1985). Plaque infection is vital factor for the progression of periodontitis as the bacteria in plaque formation release enzymes which affect and break down the bone and thus lead to periodontitis. This disease can be medicated by strict and proper oral health hygiene including tooth brushing and cleaning as well as surgical debridement by a dental professional (Tonetti *et al*. 2015).

Periodontitis and its relation to systemic disease development

The bacteria responsible for to the periodontists from dental plaque may be circulated to distant parts of the body through the respiratory and circulatory system and effectively develop different kinds of systematic diseases. The infectious bacteria existed in the oral cavity can spread within the systems of the

human body and may cause adverse health effects (Gulati *et al*. 2013). Bacteria originated from the ulcerated epithelium of the periodontal pocket that may cause infection within the gingiva. Conditions and diseases can be as follows:

Atheromas (Han 2015)

Cardiovascular disease (CDE World 2019)

Respiratory disease (Scannapieco 1999)

Diabetes mellitus (Loe 1993)

Dental caries

Dental caries is an infectious disease which is primarily caused by *Streptococcus mutans*. This disease is characterized by the demineralization of acid in the enamel that can progress and ultimately lead to further breakdown of the more organic matters and inner dental tissue (dentin) (Darby and Walsh 2010). The acidogenic, acid-tolerant bacteria (*Mutans streptococci* and *lactobacilli*) and other species are susceptible to the dental caries. But the intensity of development of this disease depends on the capacity of the patient's immune system. The factors that are considered as high-risk for developing carious lesions on the teeth as follows:

- Low fluoride exposure
- The frequency, length and time of sugar consumption
- Tooth cleaning quality
- Fluctuations in salivary flow rates and composition
- The individual behavior
- Biofilms' quality and composition (Darby and Walsh 2010)

Organic acids exuded from the dental plaque can cause the demineralization of the adjacent tooth surface and consequently lead to dental caries. Saliva can not enhance the plaque formation and thus cannot neutralize the acid produced by the bacteria.

Detection of plaque formation

The application of medicine and visual observation are popular methods of detecting dental plaque in the oral cavity. Plaque detection is clinically estimated by plaque disclosing agents. Disclosing agents pertain the dye which turns bright to red that indicates the plaque formation (Darby and Walsh 2010). It is very important for everybody to be aware of dental plaque (Darby and Walsh 2010).

Plaque disclosing gel

Plaque disclosing products referred to as disclosants make plaque clinically visible. It is observed that cleaned teeth surfaces can not absorb the disclosant but only rough surfaces can do it. Plaque disclosing gels can cure this disease that can either be used in home or in the dental clinic. Before using this gel, precautions should be checked out and being aware of any allergic condition to iodine, food colouring or any other ingredients that may be existed in this product. The gel is used to visualize in assessing existence of plaque biofilm formation that can also exhibit the dental plaque maturity.

Disclosing tablets

Disclosing tablet is similar to that of disclosing gels except that it should be placed in the mouth and chewed for approximately one minute where the saliva spit it out. Disclosing gels will elicit the existence of the plaque, but it will often not express the maturity level of the plaque. Disclosing tablets are prescribed to the patients for the application with orthodontic appliances before and after the brushing of tooth to ensure optimal cleaning. These are also appropriate tools for young children or patients who want to eliminate the dental plaque in certain areas of the mouth. For the patients of all ages, disclosing tablets are effectively useful tools in the elimination of dental plaque.

Visual or tactile detection

Dental biofilm starts to create on the tooth within the minutes after brushing. It is difficult to observe dental plaque on the hard tissue surface; however it can be visible in the rough surface. It is found as a thick and fur-like structure that may exhibit the yellow or brown stain. The dental plaque is assessed through several dental plaque assessment tools in the dental clinic where these tools are able to remove the plaque. The patients can easily find out the most common areas of the dental plaque in the teeth along with cervical margins.

Plaque in dogs and cats

Dental plaque is also commonly found in domestic animals like cats and dogs. However, the canine and feline plaques associated with the bacteria are found to appear different from that of human plaque (Dewhirst *et al*. 2012, Dewhirst *et al*. 2015). If this plaque remains untreated it may lead to more acute gum disease of periodontitis where veterinarians often recommend oral healthcare products for affected pet animals.

The dental caries and periodontal diseases are the major health problems among the most global infectious diseases. Oral health affects the general quality of life where the oral health disease influenced systemic diseases in the other parts of the body. The excess fleshy food and sweets affect the oral health by causing pyorrhea, tooth ache, bleeding from the gums and dental caries.

General notes on Medicinal plants

Human being has been using plants as therapeutic agents since the prehistoric period of times and depending on them for health care. WHO estimated that 5.86 billion of world people used traditional medicines for their primary dental health care, majority of these inhabitants used medicinal plants. The biotic and abiotic components of the nature are always interdependent. The plants are indispensable

for human life. Plant is the store house of remedies to cure health complexities for mankind and traditional medicine systems are formed from these plants.

Medicinal plants are rich sources of bio-active compounds that serve as important raw materials for medicine production.

World Health Organization (WHO) estimated that 80% of the population in developing countries of the world depends on traditional medicinal plants for the health care system even today (Fransworth *et al*. 1985). Realizing the great role, WHO in its 30th Assembly in 1977 gave formal recognition to traditional medicine and emphasized the global researches into medicinal herbs and to associate traditional healers in the National Health Care Program. However, the inadequacy of the modern drugs to combat degenerative diseases, the undesirable side effects, the time, effort and high cost of production of clinically acceptable synthetic drugs, efficacy of a number of phytopharmaceuticals and herbal drugs and the need for naturally occurring drug intermediates and more so the recent concept of antioxidative prophylaxis and therapy focused renewed global attention to herbal remedies and neutraceuticals or better phytoneutrient for the last three decades or so. In fact WHO has not only been advocating the use of traditional medicines but also sponsored multicentered international R&D projects.

Bangladesh has the rich source of herbal medicine throughout the countries in South-East Asia. Moderate weather and existence of fertile soil all over the country have made this region an ideal place of the growth of various important medicinal plants which are utilized for the health care of the millions of people of the country. In rural Bangladesh and among a considerable number of populations including the tribal and peripheral people, which has been included the mainstay of the therapeutic arsenal till now. Microbial infection is a very common health problem in Bangladesh that are caused by various microorganisms like pathogenic

bacteria and fungi. Peoples of Bangladesh especially the rural people use various portions of plants for the treatment of different bacterial and fungal infections. Medicinal plants play a significant role for treating and cure of various infections against the microbes where over doses of these medications have produced long-term negative effects on human health is increasing at alarming range. Ayurvedic, Unani and Humdard are well known practices of folk medicine holding century old tradition. In the present years, the scientific interest for research is increasing day by day in the application of medicinal plants for the development of new medicinal patterns. The usages of herbal medicines are being popular for the various disease treatments from simple diseases to very complex incurable diseases like cancer. The medicinal plants are mostly used as antimicrobial agents for the effectivity and inexpensive sources which appear now as alternative and potential a agents for controlling and treating microbial infections.

Searching for new and potent drugs as an antimicrobial agent is an important measure to solve the world most lethal problem "bacterial resistance and its associated health hazards". Scientists are now engaged to develop new and effective chemotherapeutic agents, which can fight against the resistance organisms. Plants, microbes and animals are important source of modern drugs and a lot of researches' are till now continuing based on plants to investigate the biological property as well as to explore potent and biologically effective principles.

Antimicrobial studies of the plants are going on throughout the world in full swim. In this regards various plants and their isolated compounds have been found to be very effective for the treatment of various kinds of microorganisms (Machado *et al*. 2003, Palombo and Semple 2002, Candan *et al*. 2003, Karaman *et al.* 2003, Iwalokun *et al*. 2001, Srinivasan *et al*. 2001, Perumal Samy *et al*. 1998).

Medicinal Plants of Bangladesh

Since time immemorial herbal medicine is in use all over Bangladesh. With the introduction of synthetic medicines and establishment of pharmaceuticals industries, herbal medicine remained neglected. Proper exploration of varies medicinal plants in the country and their stock assessment were not thoroughly carried out. Many valuable plants have become endangered or threatened with extinction.

Considering safe use of herbal medicines, they are gaining importance all over the world and Bangladesh is no exception. In recent years, the people are inclined to the herbal medicines which are popularly used in the Ayurvedic, Unani and other system of the treatment.

In general, practice of administering has been to combine a number of medicinal plants or their products into formulae. The huge number of medicinal plants is now widely used in Bangladesh for the development and production of both traditional and modern medicine. There are about 5000 species of plants like phanerogams and Pteridophytes growing in different lands that have both medicinal and poisonous properties. Among which 546 plants have recently found to have the medicinal properties and therapeutic uses (Yusuf *et al.* 1994). It is recorded that the present annul demand for the medicinal plants in Bangladesh are more than one thousand metric tons for only traditional medicine production (Mia and Ghani 1990). For that region they are now commercially cultivated in many countries and traded as valuable items of commerce in the world market. Bangladesh can export this natural resources and earn huge amount of foreign currency.

A large number of plant materials and extracts are imported for the Ayurvedic, Unani and Homeopathic medicine productions. Nowadays, ensuring availability of health care and medical facilities to the people is the main concern throughout the

world. Due to adverse toxic effects of synthetic and chemical medicines, the herbal medicine has been getting huge popularity for human medication system all over the world.

In the ancient time most of the people were depended on plants as remedy of disease. Still now some of the patients in Bangladesh take medicine in the form of Aurvedic and Unani formulation which are derived from plants. In many cases plants or their anatomical parts are taken directly as extract, sections etc. In Bangladesh most of medicinal plants for Ayurveda cannot meet the demand. The situation is compounded by the depletion of local resources due to habitat degradation, unsustainable harvesting in an optimal way. Establishment of correct identity of the medicinal plants used in traditional medicine is a serious problem. In addition to these, there are other difficulties which have to be surmounted. No standard methods of identification and proper scientific descriptions of the recommended medicinal plants have been in the older publications. Modern technologies for describing the different plant parts have not been develop rightly in early age. Many of the plant mention in the old record with recognition and identification and the description recorded in them are so scanty and vague that one cannot be certain these descriptions whether the specimen used currently are the particular plants recommended earlier. In Bangladesh about 546 plant species are so far reported with medicinal properties deserve current identification and right use for specific disease. More over liberal destruction of forest sites and Jungles endowed with vast resources of important medicinal plants are going to be extinct without proper identification and nourishment. Some of the important medicinal plants, serving as the valuable resources of local remedies have already been assumed extent. Thus it is our national concern to intensify our efforts towards immediately identifying the important medicinal plants running threatened in the country and looking in to the potential biotechnological measures for their conservation.

Field Survey

Bangladesh possesses high resources of medicinal plants which grow in widely distributed forest, jungles wastelands and roadsides. Although more than 546 medicinal plants have been reported to grow in the wild environment of Bangladesh and used for medicinal purpose. These medicinal plants are collected from the natural habitats for the production of Ayurvedic, Unani, and Homeopathic drugs. Many medicinal plants are used widely for the treatment of toothache and daily oral hygiene practice. *Vitex negundo*, *Mimusopselengi*, *Meliaazedarach/ spervirens*, *Azadirachta indica*, *Croton tiglium*, *Eucalyptus globulus*, *Zingiber officinale*, *Allium sativum*, *Syzygium aromaticum*, *Morus alba* Linn, *Eritrina variegata* Linn *Paederia foetida*, *Andrographis paniculata*, *Psidium guajava*, *Swertia chirayita* , *Terminalia chebula*, *Paedera foetica*, *Adhato davasica*, *Cynodomdactylon*, *Terminalia arjun*, *Zingiber officinale*, are now under the threat of extinction. In Bangladesh, the majority of the people can not live wealthy life, so, the low cost and safe health care is very essential. Traditional medication systems should be improved. But unfortunately, these traditional medication systems are largely eradicated due to the lack of support and recognition as well as the rapid destruction of natural habitats of medicinal plants. Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as important therapeutic agents as well as important raw materials for the materials for the manufacture of traditional and modern medicines. Substantial amount of foreign exchange can be earned by exporting medicinal plants.

CHAPTER 2
STUDY PROTOCOLS, AIMS AND OBJECTIVES

Study Protocol

Streptococcus biofilm or dental plaque is a yellow pale biofilm that develops naturally on the tooth surface and is formed by colonizing bacteria such as S*treptococcus mutans* which is facultatively anaerobic, Gram positive organism and other anaerobes such as fusobacterium and actinobacteria are trying to attach themselves on the tooth surfaces.

Streptococcus mutants biofilm or dental plaque is composed of a thousand species of bacteria that take part in the complex ecosystems of the mouth. It has been estimated that about 1,000 species can exist as part of the dental biofilm ecosystem.

Bioflim may be defined as aggregation of microorganism in which cells are embeded within self- produced matrix of extra cellular polymeric substance such as – DNA, Protein and long chain polysaccharides and the cells are stick to each other on a surfaces.

The aim and objectives this research work is to prevention of Dental Plaque formation by isolation and identification of specifics bacteria such as- *Streptococcus mutans* by preventing their growth and adhesion by using phytochemicals against the isolated bacteria from the medicinal plant of Bangladesh.

Aims

No serious attempts have been made to study the medicinal plant extracts for preventive dental plaque formation. Keeping this in mind the present investigations were undertaken.

Objectives

- To collect the dental plaque from the affected teeth, Dental unit, RMCH,
- isolation and identification of *Streptococcus mutans* bacteria which are responsible for dental plaque formation,
- to culture the bacterial strain,
- to find out the adhesion strength of biofilm developed by *S. mutans*,
- to find out the effect of different medicinal plant extracts on these biofilm and
- structure determination of the isolated bioactive compounds using physical methods (Mass, IR, NMR) of analysis.

CHAPTER 3
LITERATURE REVIEW

Oral disease is one of the major health problems in the world (Petersen *et al*. 2005). Among which, the dental caries and periodontal problems are the most significant global oral health diseases after the oral and pharyngeal cancers and oral tissue lesions (Petersen 2003). In case of oral and dental health, the dental caries prevalence was about 90% in school going children where the majority of adults were also found to be affected (Petersen *et al*. 2005). Oral health is a determining factor for general well-being of the people and relates to the quality of life that extends beyond the functions of the craniofacial complex. The poor oral health was related to chronic conditions of the diseases such as there is a potential evidence of the association between severe periodontal diseases and diabetes (Petersen *et al*. 2005, Petersen 2003, 2005). The poor oral health is also linked to systemic diseases like cardiovascular diseases, osteoporosis and rheumatoid arthritis (Rautemaa *et al*. 2007). Periodontal disease was studied to increase the pregnancy complications risking the preterm low birth weight (Yeo *et al*. 2005). Tooth loss due to poor periodontal health led to significant morbidity and premature death (Petersen 2003, 2005). The oral diseases affected the economic condition increasing 10% public health expenditure in the most of developed countries (Petersen *et al*. 2005). Jenkinson and Lamont (20050) established linkage between oral diseases and the microbial activities that formed micro biota part in the oral cavity where more than 750 bacterial species were observed to inhabit in the oral cavity. The dental caries development inhabits acidogenic and aciduric gram-positive bacteria like *Mutans streptococci* (*Streptococcus mutans* and *S. sobrinus*), lactobacilli and actinomycetes metabolizing sucrose to organic acids that dissolve the calcium phosphate in teeth, causing decalcification and eventual decay. Among the bacteria responsible for dental plaque, *Streptococcus mutans* is

one of harmful microorganisms for early colonization on a tooth surface during the dental plaque formation (Loshche 1986). The ability of acidogenic properties of *S. mutans* for synthesizing extracellular glucan are the major factors for the cariogenic biofilms development and establishment (Gamboa *et al*. 2004). Biofilm related infectious disease cause major constrains in the society from both economical and health perspectives. Biofilms can be defined as an accumulation of microorganisms where the related cells are frequently embedded in self formed matrix of extracellular polymeric substances that are closely adhered to each other and or a surface (Jiao Franklin *et al*. 2019). The EPS matrix not only gives the microorganisms with a multilayered scaffold where the most of the cells contact cell to cell communication either in flocks or surface attached biofilm but also forms the microenvironment that is found to be different from other portions in terms of main environmental inputs that is known to affect microbial behaviors including pH, redox and nutrient availability. Comparing the planktonic microorganisms, the mature biofilm microorganisms exhibited increased rate of the tolerances to antimicrobial agents (Jiao Franklin *et al.* 2019). The lifestyle of classical biofilm might be described as the multi stage procedure that involved microbial attachment, biofilm maturation and biofilm dispersal. The strategies can disrupt biofilm formation step which are considered as potential and valuable aspects in controlling measures of biofilm related infections (Jiao Franklin *et al*. 2019). The nutritious, warm and moist environment of oral cavity facilitates the growth and proliferation of microbes in the forms of complex dynamic interactions among the microorganisms. The microbial colonization and pathogenic biofilm formations are enhanced subsequently by host and diet. Biofilms that are formed on the tooth or dental surfaces referred to as oral biofilms (Jiao Franklin *et al*. 2019) have been conspicuously defined as a virulence factor in many oral infectious cases involving dental caries, endodontic infections and periodontitis (Jiao Franklin *et al*. 2019). The dental caries is a supra gingival condition whereas

periodontal diseases are sub gingival conditions that are closely related to gram-negative anaerobic bacteria such as *Porphyromonas gingivalis*, *Actinobacillus* sp. *Prevotella* sp. and *Fusobacteriums* (Jenkinson and Lamont 2005, Tichy and Novak 1998). In accordance with the recent report of WHO, dental caries were more frequently persist in both children and adults (Choi Cheong *et al*. 2017). Diet habits, dentine mineralization, bacterial adhesion and biofilm formations are the key factors for the dental caries formations. In the primary stages of dental caries, cariogenic plaque biofilms are formed by combined enzymatic actions in the tooth surfaces (glucosyl transferase and fructosyl transferase) that is secreted by *Candida albicans*, *S. mutans* and the adhesive enhancing agents (glucans), which facilitate the growth and proliferation of etiologic bacteria (Choi Cheong *et al.* 2017). On the contrary, in case of the periodontal diseases, the gingival crevice is massively infected by causing the cellular inflammatory response in the gingival and its connective tissue. These inflammatory responses are manifested and known to as gingivitis or periodontitis (Loesche 2007). The dental plaque is specialized bacterial biofilm that is developed in the teeth surfaces, dental restorations and prosthetics (Bernimoulin 2003). The colony forming bacteria produce the biofilm consortium and lactic acids by carbohydrate metabolism resulting the dental caries formation (Park 2003). Chung *et al*. in 2006 and Steinberg *et al*. 2004 studied that some of methods were applied to evaluate the effectivity of natural product to eliminate the complexity of oral biofilms. On the contrary, the methods were used to isolate antimicrobial properties where the methods were found to be effective to assess the ability to prevent the bacterial absorption or adhesion to the surfaces (Chung *et al.* 2006 and Steinberg *et al.* 2004).

and economical comes from the rise in disease incidence (particularly in developing countries), Due to the development of the resistance by pathogenic bacteria to the antibiotics and chemotherapeutics, the global demand of such

alternative prevention and treatment measures for oral diseases were increasing in developing countries (Tichy and Novak 1998, Badria and Zidan 2004).

Some chemicals can alter oral microbiota and produce some undesirable side-effects such as diarrhea, vomiting and tooth staining (Park *et al*. 2003, Chung *et al*. 2006). The bacterial resistance to the most of the antibiotics like penicillins and cephalos porins, erythromycin, tetracycline and derivatives and metronidazole that are commonly used to cure the oral diseases and cetylpyridinium chloride, chlorhexidine, amine fluorides were reported to show the toxicity causing teeth staining where ethanol (commonly found in mouthwashes) was linked to oral cancer (Knoll-Köhler Stiebel 2002, Lachenmeier 2008, McCullough and Farah 2008, Neumegen *et al*. 2005, Rodrigues *et al*. 2007). Hence, the exploration of alternative natural phyto chemicals isolated from plants were observed effective in traditional medicine considering good alternative measures to synthetic chemicals (Prabu *et al*. 2006).

Medicinal plants

Plants are the significant sources of active ingredients for medication. Since the long time, medicinal plants are well known to have bioactive compounds and were used in different forms for the treatment of various diseases (Rahmatullah *et al.* 2010). These compounds are generally concentrated as secondary metabolites in the plant cells and play active role in the modern pharmacology or as the main compounds for new drug discovery (Vijayalakshmi and Ravindhran 2012). The antimicrobial efficiency was found in different plants that were used as the secondary metabolites such as phenols, tannins, flavonoids (Karuppiah and Mustaffa 2013, Koo *et al.* 2003). Hence, there is a strong quest for bio-screening plant extracts for anti-biofilm and quorum sensing inhibitory activity, which is followed by the isolation of the compounds that is responsible for this activity (Zaki *et al*. 2013). The plant materials from *Piper nigram* and *Cynodondactylon*

were extracted for natural biofilm inhibition activity. These medicinal plants were considered to have bioactive compounds that are used for the treatments of various ailments. *Cynodondactylon* is under the family of Poaceae, commonly referred to Bermuda grass and is traditionally used for the treatments of diabetes, jaundice, kidney and urinary diseases etc (Jarald *et al.* 2008). Burmuda grass is locally known as doob grass or durva grass or dogs tooth grass. It grows in the tropical climates of latitude *30°* N. The antidiabetic, diuretic activity, antioxidant, anticancer potentials, anti-ulcer activity and anti allergic effects were studied in *Cynodondactylon*. Traditionally whole plant of *C. dactylon* is directly applied as a rejuvenator and for wound healing (Das Mukhes Chandra *et al.* 2013).

The study found that the microorganisms showed resistant to several antimicrobial agents. Due to advancement in technology, the plant derived bioactive potential compounds were utilized against antibiotic resistant microorganisms (Al-Hussaini and Mahasneh 2009). *Piper nigrum* (common name: peppercorn) is widely used in the Ayurvedic medicine as a natural component. It is applied for the treatment of asthma, cough, diabetes and heart diseases (Rao *et al*. 2006). On the other hand, *Piper betle* (common name: betle leaves) was found to show the compounds that have anti-diabetic and anti-allergic properties (Arambewela *et al.* 2005, Mali *et al*. 2008).

Plant derived materials have new therapeutic and anti-pathogenic agents (Hentzer and Givskov 2003). The phytochemical properties might be used and analyzed against microbial growth patterns having strong antioxidant activity. So the study was conducted to find out the anti quorum sensing capability of three plants extracts. Subsequently, the antibiofilm potential of these plants was also studied. The antioxidant properties of the potential extracts were studied for the phytochemical analysis.

Hundreds of chemical compounds were synthesized from plants for the various functions including fungus contro, diseases and herbivorous mammals. The biological activity if numerous phytochemicals was identified. However, a single plant has widely diverse phytochemicals, the effects of using a whole plant for medicinal purposes are quite uncertain (Ahn 2017).

From the ancient period of time, plants are using as very rich sources of medicines. Various types of chemical compounds are found in plants that are of many kinds including four major biochemical classes like alkaloids, glycosides, polyphenols and terpenes.

Medicinal plants are widely used in non-industrialized forms as they are readily available and cheaper than any other kinds of modern medicines. The thousands of plants with suspected medicinal properties was exported who's cost was estimated about US$2.2 billion in the year of 2012 ("Medicinal and aromatic plants trade programme" .Traffic.org.2017). Several hundred billion of dollars was estimated for the export of botanical extracts and medicines in 2017 (Ahn 2017).

History

Prehistoric times

Plants, including culinary herbs and spices were used as medicines from prehistoric period of the times. Spices are used partly to counter food spoilage bacteria, especially in hot climatic situation (Tapsell *et al*. 2006, Billing, Jennifer and Sherman 1988). Angiosperms (plants) were originally the high source of the medicines (Angiosperms: Division Magnoliophyta: General Features. Encyclopedia Britannica 1993). Human habitats are surrounded by weeds that are used as herbal medicines, such as nettle, dandelion and chickweed (Stepp John 2004, Stepp John, Moerman Daniel 2001). Not only humans but also some

animals like primates, monarch, butterflies and sheep ingest medicinal plants spontaneously when grazing (Sumner Judith 2000).

Plant samples from prehistoric burial sites are among the lines of evidence that Paleolithic peoples had knowledge of herbal medicine. For instance, a 60 000-year-old Neanderthal burial site, "Shanidar IV", in northern Iraq has yielded large amounts of pollen from eight plant species, seven of which are used now as herbal remedies (Solecki Ralph 1975). A mushroom was found in the personal effects of Ötzi the Iceman, whose body was frozen in the Ötztal Alps for more than 5,000 years. The mushroom was probably used against whipworm (Capasso 1998).

Ancient times

In ancient Sumeria, hundreds of medicinal plants with myrrh and opium are included in clay tablets. The ancient Egyptian Ebers Papyrus listed more than 800 medicinal plants like aloe, cannabis, castor bean, garlic, juniper and mandrake (Sumner Judith 2000). From ancient period, Ayurvedic medicine is documented in the Atharva Veda, the Rig Veda and the Sushruta Samhita has applied hundreds of chemically active herbs and spices as turmeric, which pertains curcumin substance (Aggarwal 2007, Girish Dwivedi and Shridhar Dwivedi 2007). The Chinese pharmacopoeia, the Shennong Ben Cao Jing recorded plant derived medicines such as chaulmoogra for leprosy, ephedra and hemp (Sumner Judith 2000). This type of medicines was expanded in the Tang Dynasty Yaoxing Lun (Wu Jing-Nuan 2005). In the 4th century BC, Aristotle's pupil Theophrastus wrote the first systematic botany text, Historia plantarum (Grene Marjorie 2004). In around 60 AD, the Greek physician Pedanius Dioscorides, worked for the Roman army and documented more than 1000 items for medicines using over 600 medicinal plants in De material medica (Collins Minta 2000).

Middle Ages

In the Early Middle Ages, Benedictine monasteries reserved medical knowledge in Europe by translating and copying the classical texts and maintained herb gardens (Arsdall Anne 2002, Mills Frank 2000). Hildegard of Bingen wrote Causae et Curae ("Causes and Cures") on medicine (Ramos-e-Silva Marcia 1999). In the Islamic Golden Age, scholars translated many classical Greek documents with Dioscorides into Arabic, adding including their own commentaries (Castleman Michael 2001). Herbalism was flourished in the Islamic world, particularly in Baghdad and in Al-Andalus. Among the works in medicinal plants, Abulcasis (936–1013) of Cordoba wrote the Book of Simples, and Ibn al-Baitar (1197–1248) recorded hundreds of medicinal herbs such as Aconitum, nux vomica, and tamarind in his Corpus of Simples (Castleman Michael 2001). Avicenna included many plants in 1025 The Canon of Medicine (Jacquart Danielle 2008). Abu-Rayhan Biruni (Kujundzić and Masić 1999), Ibn Zuhr (Krek 1979) Peter of Spain and John of St Amand wrote further pharmacopoeias (Brater *et al*. 2000).

Early Modern

The Early Modern period showed flourishing illustrated herbals across Europe starting 1526 Grete Herball. John Gerard wrote his famous The Herball or General History of Plants in 1597, on the basis of Rembert Dodoens, and Nicholas Culpeper that published "The English Physician Enlarged (Singer Charles 1923)". Many new plant medicines arrived in Europe as the products of early modern exploration resulting Columbian Exchange, in which livestock, crops and technologies were transferred between the Old World and the Americas in the 15th and 16th centuries. Medicinal herbs in the Americas included garlic, ginger, and turmeric; coffee, tobacco and coca travelled in the other direction (Nunn, Nathan and Qian Nancy 2010, Heywood Vernon 2012). In Mexico, the sixteenth century Badianus Manuscript described medicinal plants available in Central America (Gimmel Millie 2008).

19th and 20th centuries

The place of medicinal plants was basically altered in the 19th century by the usage of chemical analysis. Alkaloids were isolated from medicinal plant that was started with morphine isolation from the poppy in the year of 1806 and was followed by ipecacuanha and strychnos in 1817 with quinine from the cinchona tree and then many others. Due to the progress of chemistry, chemically active substances were discovered in medicinal plants (Petrovska 2012, Atanasov, Atanas G *et al*. 2015). Commercially purified alkaloid extraction included morphine from medicinal plants that began at Merck in 1826. Salicylic acid was first synthesized in the medicinal plant in the year of 1853 (Atanasov Atanas *et al*. 2015). At the end of the 19th century, the trend of pharmacy turned against medicinal plants due to the modification of active ingredients by the enzymes as the whole plants were dried where alkaloids and glycosides were purified from plant material that started to be preferred (Petrovska 2012). Drug was discovered from the plants that was found to be important since the 20th century and at the 21st, significant anti-cancer drugs from yew and Madagascar periwinkle was isolated (Atanasov Atanas *et al*. 2015).

Context

The plant that is applied for the purposes of people's health maintenance and was administered for the specific heath condition in case of modern medicine and traditional medicine (Ahn 2017, Smith-Hall *et al*. 2012). The Food and Agriculture Organization estimated in the year, 2002 that more than 50,000 medicinal plants are used all over the world (Schippmann Uwe *et al*. 2002).

In this modern period, about a quarter[a] of the drugs prescribed to the patients which are derived from medicinal plants and these are rigorously tested (Smith-Hall *et al*. 2012, Farnsworth, Norman *et al*. 1985). In other medicine systems, medicinal plants are treated as informal attempted that was not tested scientifically (Tilburt Jon and Kaptchuk Ted 2008). The World Health Organization estimated

that about 80 percent of the world's population is depended basically on the traditional medicine; perhaps around two billion of people are largely relied on medicinal plants (Smith-Hall *et al*. 2012, Farnsworth, Norman *et al*. 1985). The usage of plant derived substances included herbal or natural medicinal products with assumed health benefits that are increasing day by day in developed countries (Ekor Martins 2013). This may increase the toxicity risks and other health effects despite the safe usage of herbal remedies (Ekor Martins 2013). Herbal medicines have been using since long period of time before modern medicine inoculated. The World Health Organization speculated the policy for traditional medicine in 1991, and published the guidelines with a monograph series on widely aapplied herbal medicines (Singh Amritpal 2016, Cravotto *et al*. 2010).

Medicinal plants may give three basic types of benefit: health benefits to the consumers who taken them as medicines; financial benefits to people who harvest, process and distribute them for sale and society-wide benefits like job opportunities, taxation income and a healthier labour force (Smith-Hall *et al*. 2012). However, plant extract and medicine synthesis are developed by weak scientific evidence, poor practices of drug development and insufficient financing (Ahn 2017).

Phytochemical basis

Plants produce chemical components which provide evolutionary advantage like defending against herbivores by salicylic acid used as a hormone for plant defenses (Hayat and Ahmad 2007). These types of plant chemicals have showed potentiality for usage as drugs content and known as pharmacologically active substances in medicinal plants on the scientific basis for their use in modern medicine, when it’s scientifical proof was confirmed (Ahn 2017). For example, daffodils (Narcissus) have nine groups of alkaloids including galantamine which was licensed for the usage against Alzheimer's disease.

Alkaloids

The alkaloids are toxic with bitter taste which concentrated in the different parts of the plant like the stem that is most likely to be consumed by herbivore animals where these substances might protect the parasites (BastidaJaume *et al*. 2006, Aniszewski Tadeusz 2007, Galantamine. Drugs.com. 2017, Birks 2006).

There are several classes of drugs on the basis of different modes of action. Medicines have the different classes including atropine, scopolamine and hyoscyamine (all from nightshade), (The European Agency for the Evaluation of Medicinal Products 2017) berberine (from plants such as Berberis and Mahonia), caffeine (Coffee), cocaine (Coca), ephedrine (Ephedra), morphine (opium poppy), nicotine (tobacco), reserpine (Rauwolfiaserpentina), quinidine and quinine (Cinchona), vincamine (Vinca minor) and vincristine (Catharanthusroseus) (Elumalai, Eswariah Chinna 2012, Gremigni *et al*. 2003).

Glycosides

Anthra quinone glycosides are reported in medicinal plants such as rhubarb, cascara, and Alexandrian senna (Wang Zhe *et al*. 2013, Chan and Lin 2009). Plant-based laxatives made from such plants include senna (Hietala *et al.* 1987) rhubarb (Akolkar Praful 2012) and Aloe (Elumalai, Eswariah Chinna 2012).

The powerful cardiac glycoside drugs are isolated from medicinal plants including foxglove and lily of the valley. These drugs pertain digoxin and digitoxin which are used in the support of the heart beating and also act as diuretic drugs ("Active Plant Ingredients Used for Medicinal Purposes". United States Department of Agriculture 2017).

Polyphenols

Polyphenols are found in plants that play effective roles in the plant defenses against the diseases and predators ("Active Plant Ingredients Used for Medicinal Purposes". United States Department of Agriculture 2017). Polyphenols are hormones that mimick phytoestrogens and astringent tannins (Elumalai, Eswariah Chinna 2012, Da Silva Cecilia *et al*. 2013). Plant phytoestrogens are administered for gynecological disorders such as fertility, menstrual and menopausal problems (Muller-Schwarze Dietland 2006).

Polyphenolic extracts are used as dietary supplements and cosmetics that are popularly accepted without proof or legal health claims due to the positive health outcome (European Food Safety Authority 2010). In Ayurveda, the astringent rind of the pomegranate, punicalagins consisting of polyphenols is widely used as the medicine (Jindal and Sharma 2004).

Terpenes

Wiart Christopher (2014) reported the existence of various kinds of terpenes and terpenoids in medicinal plants and in resinous plants like conifers. These plants are the rich source of aromatic compounds and act as herbivore repellants. These aromatic compounds are used in essential oils for perfumes such as rose and lavender, or for aromatherapy (Elumalai , Eswariah Chinna 2012, Tchen, 1965, Singsaas Eric 2000). Some aromatic compounds have medicinal applications like thymol that is an antiseptic and was once used as a vermifuge (anti-worm medicine) ("Thymol (CID=6989)" NIH 2017].

Usage

Plant medicines are widely used all over the world (WHO 2017)., local traditional herbal medicine (herbalism) is the only one source of health care system for the people of rural areas in the most of the developing countries, while in the

developed world, alternative medicine are used. As of 2015, forThe safety and efficacy of the most of medicinal plant products are not tested properly but these products are marketed and provided in the undeveloped countries with low quality containing dangerous contaminants (Chan Margaret 2015). A wide variety of plant materials are used in traditional Chinese medicine among other materials and techniques ("Traditional Chinese Medicine: In Depth (D428)" 2017).

Effectiveness

Plant medicines are mostly used randomly without proper systematic and scientific tests all over the world. Around 16% herbal medicines are found to observe potentially effective in 2007. Very limited in vitro or in vivo evidence was studied for roughly half of the medicines where only phytochemical evidence traced out for about 20% and 0.5% medicines showed allergenic and toxic effects and nearly12% was basically never studied scientifically (Cravotto *et al*. 2010). Cancer Research Center of the UK did not find out any reliable evidence of herbal remedies for cancer treatment ("Herbal medicine". Cancer Research UK 2019). A phylogenetic family tree was drown upto to genus level with 20,000 species to compare the medicinal plants of Nepal, New Zealand and the South African Cape in 2012. It showed very strong phylogenetic relationship in this regards (Saslis-Lagoudakis *et al*. 2012). Many plants produced pharmaceutical drugs that belonged to the groups and were independently studied in three different regions where the results showed that these plant groups have the strong potentiality for medicinal efficacy (Saslis-Lagoudakis *et al*. 2012).

Drug discovery

The pharmaceutical industry had strong roots in the apothecary shops of Europe in the 1800s, where pharmacists delivered local traditional plant medicines to the consumers that included the extracts such as morphine, quinine and strychnine ("Emergence of Pharmaceutical Science and Industry: 1870-1930" 2005).

Therapeutically significant drugs like camptothecin and taxol which were extracted from medicinal plants (Heinrich and Bremner 2006, Atanasov Atanas *et al*. 2015) The anti-cancer efficacy of the drugs like Vinca alkaloids vincristine and vinblastine were identified in the year of 1950s from *Madagascar periwinkle* and *Catharanthusroseus* (Moudi Maryam *et al*. 2013).

Hundreds of the compounds were isolated from plants using ethnobotany by indigenous peoples for possible applications of medicinal entity (Fabricant and Farnsworth 2001). The efficacy of curcumin, epigallocatechingallate, genistein and resveratrol are studied that showed unreliable data. As a result, the phytochemicals were frequently proved unsuitable data as leading compounds in the discovery of the drug (Baell Jonathan and Walters Michael 2014, Dahlin Jayme and Walters Michael 2014). Food and Drug Administration was only able to show the sufficient evidence for medicinal values of two botanical drugs among several hundred applications for new drug status in the United States over the period of 1999 to 2012 (Ahn 2017).

The pharmaceutical industry had enough interest in traditional applications of medicinal plants in its drug discovery efforts (Atanasov, Atanas *et al*. 2015). Among 1073 small- drug molecules that were approved in the years of 1981 to 2010, more than half of which were directly derived from or inspired by natural substances (Atanasov Atanas *et al*. 2015,Newman David and Cragg Gordon 2012).

Safety

Plant medicines can produce harmful effects that may lead to the death due to the side-effects, adulteration, contamination, overdose and inappropriate prescription. Many of such adverse effects are well known but others are remained to be explored scientifically. As the product originates from the nature it might be safe but the existence of natural poisons such as atropine and nicotine shows these to be untrue. Further, the high standards are used to the conventional medicines but plant

medicines are not always been prescribed where the dose can vary widely on the basis of the growth conditions (Ernst 1998, Talalay 2001, Elvin-Lewis 2001, Vickers 2007, Ernst 2007, Pinn 2001).

Pharmacologically active plant compounds can react with traditional drugs due to increased dose of same compounds and interference of some phytochemicals with the body which metabolize and accumulate the drugs in the liver including the cytochrome P450 system where the drugs exist long time in the body and have produced more powerful cumulative effects (Nekvindová and Anzenbacher 2007). Plant medicines showed the dangerous effects during the pregnancy (Born and Barron 2005). Since the various chemical substances are existed in the plant that impart complex effects to human body (Tapsell *et al*. 2006).

Cynodondactylon

Overview Taxonomy

Kingdom: Plantae
- Phylum: Magnoliophyta
  - Class: Liliopsida
    - Order: Cyperales
      - Family: Poaceae
        - Genus: Cynodon
          - Species: C. dactylon
            - Subspecies: *C. dactylon*
              - Scientific Name
                - *Cynodondactylon* (L.) Pers
                  - Scientific Name Synonyms
                    - *Panicumdactylon* (L.) Pers
                      - *Cynodonaristiglumis* (L.) Pers
                        - *Caprioladactylon* (L.) Pers
                          - Common Names
                            - bermudagrass, common
                            - bermudagrass, devil grass

Appearance

Cynodondactylon is a prostrate and perennial grass that grows on all types of soil in warm weather.

Foliage

Leaves are gray-green in color and length ranges about 4-15 cm. The ligume has a ring like structure with white hairs which is one of salient features for its identification.

Flowers

Flowering is found to occur in late summer where the spikes are about 3-7 cm in length.

Fruit

The scaly rhizomes are observed in this grass and flat stolons are allowed to foliage a dense resilient turf.

Ecological Threat

Cynodondactylon is native plant to eastern Africa. It prefers to grow in the moist and warm climates with high illumination. It was introduced to North America in the year of 1800s as a pasture grass. *Cynodondactylon* is widely used as a turf grass.

Diagnostic Characteristics:

The distinguishing features are the peculiar ring with white hairs in the legume. The fringed hairs are observed in the keel of the lemma with the gray-green foliage.

Piper nigrum

Black pepper (*Piper nigrum*) is found as a flowering vine in Piperaceae family and it is cultivated for the fruits and commonly known as a peppercorn. This vine is

generally dried and taken as a spice. The fresh and fully matured one is around 5 mm in diameter with dark red color containing single seed. Peppercorn is derived from them that may be referred to as black pepper, green pepper or white pepper.

At present black pepper is observed as native to Kerala of Southwestern India (Sen Colleen Taylor 2004, Hajeski and Nancy 2016) and is cultured elsewhere in tropical areas. Vietnam is the largest producer and exporter of pepper in the world that produced 34% of the world in 2013. This peppercorn had been cultivated since the antiquity and it is both for flavour and traditional medicine. Black pepper is the most traded spice in the world and is one of the most common spices all over the world.

Scientific classification

Kingdom : Plantae
Clade : Angiosperms
Clade : Magnoliids
Order : Piperales
Family : Piperaceae
Genus : Piper
Species : *P. nigrum*
Binomial name
Piper nigrum L. (ARS,USDA 2008)

Etymology

The word 'pepper' derived from Old English 'pipor', Latin 'piper', and Sanskrit 'pippali' (Douglas Harper 2016). In the century of 16th, people started to use pepper to mean new Word 'chili pepper' (genus Capsicum) (Douglas Harper 2016).

Varieties

Black pepper

Black pepper is produced from the green plant. The drupes are processed and cooked properly in warm water and prepared these for drying. Because the heat technique ruptures the cell walls of the pepper and accelerate the work of browning enzymes during the process of drying. Then the drupes are dried in the sun or machine for several days where the pepper skin become shrink and darken into a thin wrinkling the black layer. Thus the spice is called black peppercorn. Once the peppercorns are dried, pepper spirit and oil can be extracted from the berries by crushing them. Pepper spirit is applied in the medicinal and beauty purposes. Pepper oil is also used as ayurvedic massage oil and in certain beauty and herbal treatments.

White pepper

White pepper solely consists of the seed of the ripe fruit in the pepper plant where the thin darker skin is removed. This process is called retting in which ripe red pepper berries are soaked in water around 7 days for making the flesh of the peppercorn soften and easy decomposition and then the naked seed is dried (The Hindu Business line 2008).

Ground white pepper is popularly applied in Chinese and Thai cuisine and also used in salads, cream sauces, light-coloured sauces and mashed potatoes as the substitutes.

Green pepper

Green pepper is prepared from unripe drupes. Dried green peppercorns retain the green colour by using sulfur dioxide, canning, or freeze-drying processes. When peppercorns are pickled it becomes green and preserved in brine or vinegar.

The flavour is described as "bright aroma" (ManjunathHegde and Bomnalli 2013). Unpreserved peppercorns are spoiled quickly making the products unsuitable in foreign markets.

Wild pepper

Wild pepper grows more in the Western India. The wild pepper vines were reported from the forests in the 19th century by the Scottish physician Francis Buchanan (also a botanist and geographer) as mentioned in his famous of book of ‘A journey from Madras through the countries of Mysore, Canara and Malabar’ (Volume III) (Manjunath Hegde and Bomnalli 2013). However, deforestation limited wild pepper production only in the forest patches from Goa to Kerala in the wild environment that gradually decreased the quality and yield of this variety. The commercial pepper grafting was not accomplished (ManjunathHegde and Bomnalli 2013).

Orange pepper and red pepper

These types of the peppers usually consist of ripe red pepper drupes that are preserved in brine and vinegar. Ripe red peppercorns are dried and green color peppers are produced (Katzer and Gernot 2006).

Pink pepper and other plants

Pink peppercorns are the fruits of the Peruvian pepper tree. These types of peppers may have allergic effects. The bark of Drimyswinteri is popularly used as the substitute for pepper in cold and temperate areas such as Chile and Argentina where it is easily found. Kawakawa seeds (*Macropiper excelsum*) and the leaves of mountain horopito (*Pseudowintera colorata*) are used as the replacements for the peppers. Several other plants are also used in the United States for the substitutes of the pepper.

Phytochemicals, folk medicine and research

The pepper was historically used as folk medicine. Long pepper was often preferred to use in the medication. It is blindly believed that black pepper can cure several illnesses like sunburn, constipation, oral abscesses, insomnia and toothaches (Lawless Harry and Heymann Hildegarde 2010). It was also thought that the pepper is able to cure eye problems. But the study of Jirovetz *et al*. (2002) reported that the pepper did not have the medical evidences for these kinds of the treatments. Pepper is mostly known to cause sneezing. The piperine substance existing in the black pepper can cause irritation in the nostrils for the sneezing (Steinhaus Martin and Schieberle Peter 2005). Many studies are undertaken to find out the potentiality of piperine to accelerate absorption of selenium, vitamin B12, beta-carotene and curcumin as well as other chemical compounds (Siebert Tracey *et al*. 2008). Pepper is popularly used in the Buddhist Samaññaphala Sutta as folk medicine (Montagne Prosper 2001). Pepper contains various phytochemicals (Collings Emma *et al*. 2018) including amides, piperidines, pyrrolidines and trace amounts of safrole (Dalby Andrew 2002).

Various efforts are undertaken to find out the possible physiological effects of piperine (Davidson Alan 2002).

CHAPTER 4
MATERIALS AND METHODS

Biological Investigation

In developing countries there are so many plants, which are widely used as herbal medicine for the disease treatment and health improvement (Martinez *et al*. 1996). About 80% population of the developing countries uses the traditional medicine for the primary treatment (Esther and Staden 2003). Because of suitable temperature and humidity, the infectious diseases are very common in Bangladesh.

The synthetic antibiotic therapy is nearly impossible in Bangladesh because of the high cost. As the microbes are being the rapidly developing the drug resistance, it needs to explore and develop new antimicrobial drugs especially from the plant materials. The antimicrobial compounds extracted from plants may inhibit the bacterial growth and control the bacteria by different mechanisms than those are presently used as antimicrobial agents and may have a important clinical aspect for the treatment of resistant microbial strains. Recently, traditional medicine is getting popularity by investigating the antimicrobial potentiality of medicinal plants and serving as an alternative measure of medication as well as overcoming microbial resistance (Austin *et al.* 1999).

The method used in the present work is to isolate the cariogenic bacteria from the patients having dental caries and tested against the rectified spirit extracts of the selected plants or plant parts for their antibacterial activity. *Streptococcus mutans* is found as a major bacteria causing dental caries in the people. Isolation of *S. mutans* and culture to increase their growth and subjected to test against the selected plant or plant parts having strong antibacterial activity. Isolation of the bio-active principle was done and structure of the isolated compounds was determined using spectroscopic methods of analysis.

In vitro Antimicrobial screening

The antimicrobial screening of crude extracts or pure chemical or biological agent is very much crucial to determine the potency against different types of pathogenic microorganisms. Antimicrobial activity of any plant can be measured by examining the growth response of various microorganisms to crude extract from plant, which is placed in contact with them. Generally antimicrobial screening is done in two ways:

1. Primary Qualitative assay: This type of qualitative assay is used to detect whether the compounds have the potency to kill or inhibit the growth of microorganism and
2. Secondary Quantitative assay: This type helps to determine relative potency such as Minimum Inhibitory Concentration (MIC) value.

The antimicrobial activity in the plant can be traced by antimicrobial screening test which determines the ability to inhibit in vitro microbial growth by following three techniques:

a) Disc diffusion method

b) Serial dilution method and

c) Bioautographic method.

Among the three, the disc diffusion method is widely used popular method for the preliminary determination of antimicrobial potency. Baur *et al.* (1966) conducted standardized single disc method for determining the antimicrobial susceptibility. Some researchers used the diameter of the zone of inhibition or the minimum weight of the extract that inhibits the growth of a microorganism. The principle factors that determine the size of the zone of inhibition are

1. Intrinsic antimicrobial susceptibility of the sample,
2. Growth rate of the microorganism,
3. Diffusion rate of the sample and its relation to the water solubility,
4. Concentration of organism inoculated in the medium and
5. Thickness of the test medium in the petridish.

Principle of Disc Diffusion Method:

In disc diffusion classical method, diffusion of antimicrobial agent like antibiotics depends upon the ability of agent like antibiotics to diffuse from applied source through the nutrient agar medium and a concentration gradient is generated. The nutrient agar medium before solidified is taken in petridish and uniform thickness of the agar gel is assured. Then the culture of test microorganism is seeded uniformly in the nutrient agar medium. Dried and sterilized filter paper discs (4-6 mm diameter) containing the test samples of known concentration are placed on surface of the foregoing nutrient agar medium of the petridish. Standard antibiotic (Amoxycillin) disc and blank disc are also placed as positive and negative control. These plates (Petridish) are kept at low temperature ($4^{o}C$) for one day for allowing maximum diffusion of the test sample for the surrounding media (Barry 1976). The plates are then inverted and kept in incubator ($37^{0}C$) for 24 hours to allow optimal growth of the microorganism used. If the test sample has any antimicrobial activity it will inhibit the growth of microorganism in the media surrounding the disc containing the test sample and as a result a clear, distinct area surrounding the disc is created. This clear area is defined as zone of inhibition. The diameter of the zone of inhibition is measured in terms of millimeter. The antimicrobial activity of the test sample is expressed by this diameter of the zone of inhibition. The larger the diameter, the greater is the activity of the test material against the test organism (Barry 1976, Bauer *et al.* 1966). The size of the zone of inhibition is based on the following factors:

i. Properties of the test sample
ii. Amount of test sample
iii. Uniformity in thickness of agar medium
iv. Rate of diffusion of test sample
v. Size of inoculums
vi. Incubation time
vii. Incubation Temperature
viii. Composition of culture medium
ix. Rate of growth of the test organism

Microbiological Experimental work:

Apparatus and Reagents:

To perform microbial experimental work the following types of apparatus and reagents are needed.

1. Blank filter paper discs (5 mm in diameter)
2. 12-14 hours old culture of the test bacteria
3. Test tubes
4. Petri dishes (120 mm in diameter)
5. Sterile forceps and cotton
6. Ethyl acetate
7. Inoculating loop
8. Bunsen burner
9. Micropipette (10 and 100 μL)
10. Laminar air flow unit (BIOCRAFT and SCIENTIFIC INDUSTRIES, INDIA)
11. Autoclave (ALP Co. Ltd. KT-30L, Tokyo, Japan)
12. Incubator (OSK 9639 A, Japan)

13. Nutrient agar media (DIFCO), LB agar media and Mueller Hinton agar media
14. Nutrient Agar Plates, LB agar and Mueller Hinton agar plates
15. Alcohol (95%)
16. Methanol

Selection of Patient:

Patients having dental plaque with various oral complications attending at the Dental unit of Rajshahi Medical College and Hospital were examined and investigated. The diagnosis was based on clinical history, physical examination and relevant oral examination associated with dental plaque. Questionnaires for each patient were filled up to obtain various information such as age, sex, oral hygiene status, occupation, educational qualification, socio-economic condition which give a profile of oral condition with oral examination detect how much dental plaque can be accumulated. Dental plaque are common in both children and adults. During the research period, 480 patients (both male and female) were included in the study. Male were 289 and females were 191. The isolated organisms were characterized for their anti biogram. Two gram positive coccal forms of bacteria have been implicated in dental caries from over a century– *L. acidophilus* and *S. mutans.*

Oral Health Status among The Patients of Major Non-Communicable Diseases Attending in Different Hospitals and Clinics of Rajshahi City.

Socio-demographic characteristics of the major non-communicable disease patient.

Distribution of the respondents according to their age group.

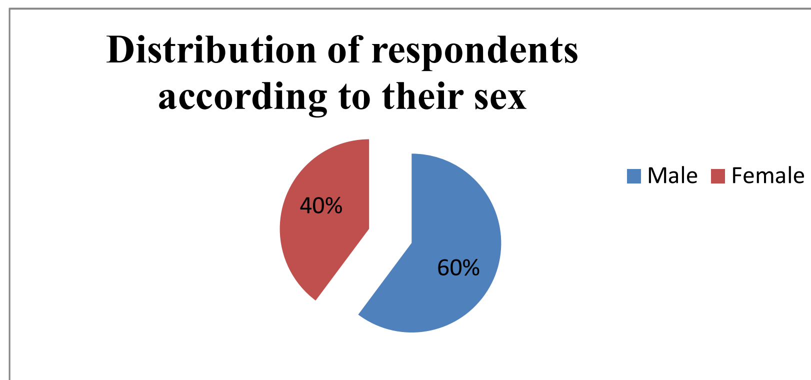
Table 2. Shows the distribution of respondents according to their age group. [n=480]

Age group of the respondents	Frequency	Percentage
≤20	31	6.46
20-30	66	13.75
31-40	57	11.88
41-50	142	29.58
51-60	133	27.71
61 through above	51	10.62
Total	480	100.00

Table 9 illustrates among the 480 respondents, age were divided in groups. 142 (29.58%) respondents were in 41-50 age group, 133 (27.71%) respondents were in 51-60 age group, 66 (13.75%) respondents were in 20-30 age group, 57 (11.88%) respondents were in 31- 40 age group, 51 (10.62%) respondents were in 61 through above age group and 31 (6.46%) respondents were in the age group of ≤20. Minimum age of the respondent was 12, maximum age of the respondent was 73.

Distribution of respondents according to their sex

Figure 10. Distribution of respondents according to their sex. [n=480]



The figure reveals that among the 480 respondents male were predominant 60% and female were 40% of the total respondents.

Collection of Dental Plaque bacteria from infected tooth:

Collection of dental plaque specimens

Sample : Dental plaque infected teeth

Study site : Dental Unit of Rajshahi Medical college Hospital

Study place : Entomology and Insect Biotechnology Laboratory, Institute of Biological Sciences, RU

Dental plaques were collected from 480 patients with different ages and sex. Initially anti-bacterial activity was done on 480 patients of dental plaque bacteria against two crude ethanolic extract (1 mg/disc, 2 mg/disc and 3mg/disc). Dental plaques were collected from the patients and work was done with the plaques for the isolation of *Streptococcus mutans* and screening was done against the isolated compounds. Antibacterial screening was done against the compounds of *Cynodondactylon* and *Piper nigrum* on the isolated *Streptococcus mutans.*

Distribution of respondents according to their marital status

Table 3. Shows the distribution of respondents according to their Marital Status. [n=542]

Marital status	Frequency	Percent
Unmarried	59	12.29
Married	383	79.81
Widow/ widower	38	7.9
Total	480	100.00

Table shows the distribution of marital status of the respondents which were 383 (79.81%) married, 59 (12.29%) unmarried and 38(7.9%) widow/widower.

Distribution of respondents according to their educational status group.

Table 4. Shows the distribution of respondents according to their educational status group. [n=480]

Educational status	Frequency	Percent
Illiterate	67	13.96
Can sign only	35	7.29
Non-formal	114	23.75
Completed year 1-12	75	15.62
Others (Bachelor and above)	189	39.38
Total	480	100.00

Table expresses the educational status of the respondents, overall 67 (13.96%) respondents had no education, 35 (7.29%) respondents could sign only, 114 (23.75%) respondents had no institutional education, up to HSC there were 75 (15.62%) respondents and Bachelor and above status were predominant; that was 189 (39.38%)

Distribution of respondents according to their occupational status group.

Table 5. Shows the distribution of respondents according to their occupational status group [n=480]

Occupation	Frequency	Percent
Unemployed	12	2.5
Service holder	135	28.13
Agriculture worker	29	6.04
Business	82	17.08
house wife	114	23.75
Others	108	22.5
Total	480	100

Table presents distribution of occupation. Among 480 respondents, 135 (28.13%) were service holder, 114 (23.75%) respondents were housewife, 82 (17.08%) were in business, 29 (6.04%) were agriculture worker, 12 (2.5%) were unemployed and 108 (22.5%) were from different occupations.

Figure 11. Distribution of respondents according to their Time of Oral Hygiene practice group.

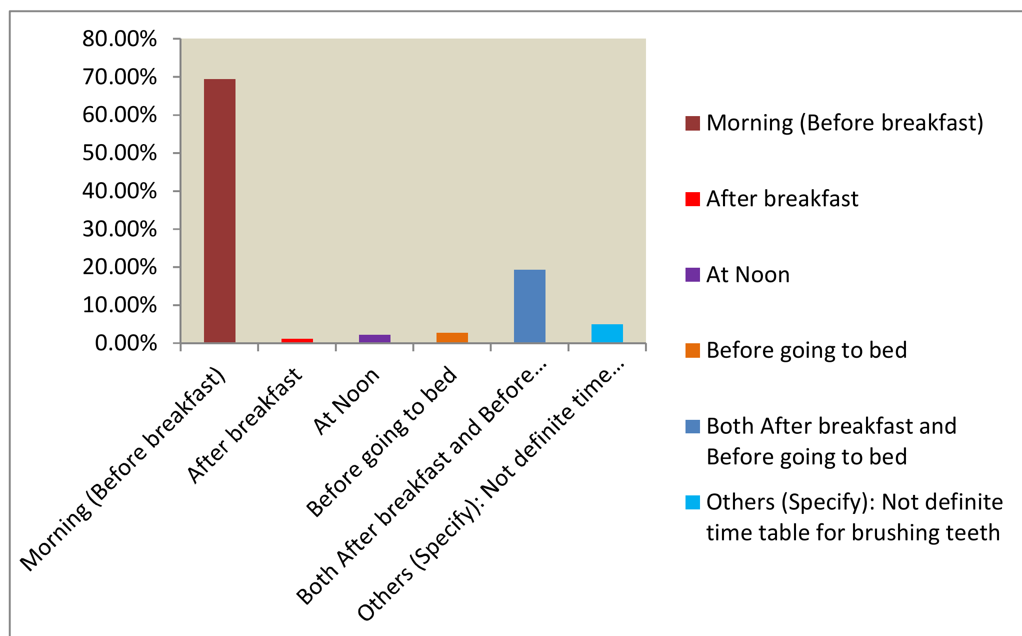


Figure shows the distribution of respondents according to their time of oral hygiene practice. 334 (69.6%) respondents used to brush tooth at morning before breakfast,17 (3.54%) respondents had no definite time for brushing, 104 (21.7%) respondents used to brush before going to bed, 17 (3.54%) respondents used to brush at noon, and 8 (1.7%) respondents used to brush after breakfast.

Distribution of respondents according to their Oral Hygiene practice

Table 6. Shows the distribution of respondents according to their Oral Hygiene practice group [n=480]

Oral hygiene practice	Frequency	Percent
Tooth brush	374	77.92
Neem stick	15	3.13
Finger	68	14.16
Others	23	4.79
Total	480	100

Table expresses distribution of respondents according to their oral hygiene practice. 374 (77.92%) respondents used tooth brush, 68 (14.16%) respondents used finger for brushing, 15 (3.13%) respondents used neem stick and 23 (4.79%) respondents used other tools for brushing.

Distribution of respondents according to their Material use for oral hygiene practice group.

Table 7. Shows the distribution of respondents according to their Material use for oral hygiene practice group. [n=480]

Material used for oral hygiene practice	Frequency	Percent
Tooth paste	368	76.66
Ash	53	11.04
Charcoal powder	8	1.67
Nothing	6	1.25
Others (specify)	45	9.38
Total	480	100

Table expresses distribution of respondents according to their material use for oral hygiene practice. 368 (76.66%) respondents used tooth paste, 53 (11.04%) respondents used ash for brushing, 8 (1.67%) respondents used charcoal powder, 6 (1.25%) respondents used nothing and 45(9.38%) respondents used other substances for brushing.

Distribution of the frequency of visit of the respondents to their Dentist for regular care

Table 8. Shows the distribution of the frequency of visit of the respondents to their Dentist for regular care [n=480]

Visit to dentist	Frequency	Percent
Not at all	122	25.42
Some times when pain arises	316	65.83
Yearly once a time	6	1.25
Visited to village untrained doctor	28	5.83
Every 6 monthly	8	1.67
Total	480	100

Table expresses the distribution of the frequency of visit of the respondents to their Dentist for regular care. 316 (65.83%) respondents visited their Dentist sometimes when pain aroused, 122 (25.42%) respondents never visited to their Dentist, 28 (5.83%) respondents visited to Dental quacks, 8 (1.67%) respondents visited to Dentist twice a year, 6 (1.25%) respondents visited to their dentist once a year.

Preparation of culture media

Mitis Salivarius Agar Base

Mitis salivarius Agar is recommended technique for the selective isolation of *Streptococci,* especially *Streptococcus mitis*, *Streptococcus salivarius* and *Enterococcus faecalis* from the samples.

Table 9. Composition

Ingredients	Gms / Litre
Casein enzymichydrolysate	15.000
Peptic digest of animal tissue	5.000
Dextrose	1.000
Sucrose	50.000
Dipotassium phosphate	4.000
Trypan blue	0.075
Crystal violet	0.0008
Agar	15.000
Final pH (at 25°C)	7.0±0.2

Directions

90.07 grams of the medium were suspended in 1000 ml of distilled water and the medium was heated to boil and dissolve the properly. It was sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes and then cooled to 50-55°C as well as added 1 ml of steriled 1% Potassium Tellurite Solution (FD052). After proper mixing, the medium was poured into sterile petridish.

Principle and Interpretation

Streptococcus species are the most commensal microbes inhabiting in the human mouth and throat, though there are several microbes found as opportunistic and primary pathogens (Collee *et al*. 1996). "Viridans" group of *Streptococcus* consists of *Streptococcus salivarius* and *Streptococcus mitis*. They showed various kinds of haemolysis when glowed on Blood Agar medium. Therefore it is very difficult to isolate the organisms existed in the saliva from the other inhabiting flora. Mitis salivarius Agar medium is used for the isolation of *S. mitis*, *S. salivarius* and *Enterococcus faecalis* from mixed cultures. *E. faecalis*is is the most common member of the Enterococci which is able to infect humans and is also responsible for human endocarditis (Balows *et al.* 2nd Ed., Springer-Verlag). Mitis salivarius Agar medium is formulated as per Chapman's guidelines (Chapman 1944,

Chapman 1946, Chapman 1947). This (with 1% potassium tellurite) is a highly selective medium, which isolates streptococci from highly contaminated samples exuded from body cavities and faeces etc. Synder and Lichstein (1940) used sodium azide in this medium that inhibits the gram-negative bacterial growth. Peptic digest and casein enzymatic hydrolysate used in the medium provide the required nutrients for the growth of the microorganisms. Dextrose and sucrose are the fermentable carbohydrates as well as ipotassium phosphate buffers the medium. Trypan and diazo blue dyes are the acidic and crystal violet is a basic dye, which inhibit the growth of many gram positive organisms. Potassium tellurite makes the medium selective for streptococci. The high concentration of trypan blue in Mitis Salivarius Agar medium inhibits the growth of *Streptococcus mutans* strains. Some of *Streptococcus mitis* strains might be easily identified by the longer incubation period (MacFaddin 1985).

Appearance of the medium

Appearance was observed as light yellow to light blue with homogeneous form.

Gelling

Firm and comparable with 1.5% of Agar gel

Colour and Clarity of prepared medium

Dark blue colour with clear to slightly opalescent gel formed in Petri plates.

pH

pH was recorded as 6.80-7.20.

Cultural Response

Cultural characteristics were observed after the incubation period of 35-37°C for 18-48 hours adding 1% of Potassium Tellurite (FD052).

Colony characteristics

The use of a standard medium will be of value in the development of colonies. The rate of growth, pattern, changes in colour, on the surface and reverse side of the colony were observed for identification. The colonies of *Streptococci* species are brownish or greenish zone around the colony, Gray-white glistening in colour (Christopher Hale *et al*. 2013).

Stores

After screening *Streptococcus mutans,* stored in a refrigerator at -80ºC.

Composition of culture medium

The media, which are used for demonstration of the antimicrobial screening and for making subcultures of the test organisms, are as follows:

i. Nutrient broth medium
ii. Nutrient agar medium
iii. Muller Hinton medium
iv. Tryptic soya broth (TSB) medium
v. Trypticase soya agar medium
vi. Staphylococcus defined medium (SDM)
vii. Adams and Roe medium
viii. NIH agar or broth medium

Among these media, nutrient agar medium was used in the current study for determining the sensitivity of the microorganisms for selected sample. The nutrient agar medium is the most popular medium used in the microbiology. The composition of nutrient agar medium is given below-

Table 10. Composition of Nutrient agar medium

Ingredients	Amounts
peptone	0.5 grams
Sodium chloride	0.5 grams
Yeast extract	1.0 grams
Agar	2.0 grams
Distilled water	100 ml
pH	7.2-7.6 at 25ºC

Preparation of culture media

Commercially available nutrient agar medium (DIFCO), LB agar media and Mueller Hinton agar media were used to demonstrate the antimicrobial activity of test preparations. For nutrient agar medium 2.8gm of the nutrient agar was used to dissolve in 100 ml of distilled water for the media preparation. This medium was sterilized properly by autoclaving at 121ºc temperature and 15-lbs /sq. inch pressure for 20 minutes.

Preparation of test plates

Petridishes and the glass wares were sterilized by autoclaving at 121ºC temperature and 15-lbs /sq inch pressure for 20 minutes. The selected sample was transferred from the subculture to the test tube containing 20 ml of culture medium with the inoculating loop. The test tube was rotated to attain the homogenous suspension of the microorganisms. The bacterial solution was immediately shifted to the sterile petridishes and was rotated several times to get the homogeneous distribution of the microorganisms. After cooling the medium, it was preserved in the refrigerator at 4ºC.

Preparation of discs

Sterilized discs were prepared with punch machine and were taken in the petridish. Discs were first shifted in the petridish and then subjected to dry and heated sterilization at 180ºC for 1 hour. After that these were transferred into the laminar

air flow for 30 minutes. For preparation of different antibiotic discs, we take different concentration of each solution were taken by a 10µl micropipette. Thus the discs were prepared for the test.

Placement of the discs, diffusion and incubation

Antibacterial investigation was done in a laminar hood and all kinds of precautions were adopted to remove the contamination of the microorganism under this test. The sample discs impregnated with the bacterial samples were kept gently on the solidified agar plates with a sterile forceps. Then the plates were inverted carefully and placed in incubator for 24 hours at 37ºC to obtain the maximum zone of diffusion. Finally, after the incubation period, the zone of diffusion was determined by measuring the zones' diameter in mm scale and the result was recorded. Thus antibacterial activity of the sample was determined.

Measurement of the zone of Inhibition

After the incubation, antibacterial activity was determined by measuring the inhibition zones' diameters in millimeter with the transparent scale.

Selection of the experimental medicinal plants

Three crude extracts (ethyl acetate) isolated from the plants *Cynodondactylon, Piper nigrum* were taken for the determination of antibacterial potency against a dental plaque bacteria *Streptococcus mutans*. Standard antibiotic discs of kanamycin were adopted for the comparison.

All of the crude extract of ethyl acetate from these plants exhibited the antibacterial potency against the most of the selected bacterial strains. In this research, 2 different medicinal plants were selected. Final aim is to find out the efficient medicinal plant which has a medicinal value for the prevention of dental plaques were given below-

Table 11. Selected plant specimen for dental plaques prevention.

Specimen no	Scientific name	Local Name	Using part
1.	*Cynodondactylon*	True grass (Durbaghash)	Whole plant
2.	*Piper nigrum*	Black pepper	Seed

Antibacterial activity test

Antibacterial activity of the plants can be determined by the observation of the growth response of different bacterial strains to the plant extracts. Generally, antibacterial screening is done in following phases:

1. a primary qualitative assay to detect the existence of activity and
2. Secondary assay, which quantities the relative potency, expressed as the Minimum Inhibitory Concentration (MIC) value.

In the present study ethyl acetate extracts of above 2 plants or plant parts were tested against dental plaques bacteria from dental plaques of patients are cited in the following table-

Table 12. Antibacterial activity assay-initial/ primary anti bacterial sensitivity test Result (1 mg/disc): zone of inhibition (mm)

Patient sample / Specimen (Plant)sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Cynodondactylon	8	10	11	13	15	17	11	11	14	10	11	10	11	11	9
Piper nigrum	7	12	13	13	12	14	12	14	16	15	14	10	13	8	10

Initial antibacterial sensitivity test were shown in the table-20. Out of 2 plants screened for antibacterial activity against those bacteria causing dental plaques, were rearranged according to the chronology of sensitivity. The *Streptococcus mutans* bacteria along with others causing dental plaques, were isolated and tested

with the above plant extracts. Among these medicinal plants showed anti-microbial activity. Out of these plants were selected for their highest antimicrobial activity against *Streptococcus mutans* (shown in the results and discussion section).

Thus the above two plants were used for the isolation of the active components and tested against the isolated major dental plaque forming bacteria. For further clarification, the crude ethyl acetate extracts of the above two plants were subjected to further antibacterial activity test using 1 μg/disc, 2 μg/disc and 3 μg/disc against the isolated plaque forming bacteria *Streptococcus mutans* from patients. Further work is to isolate the bioactive principle from the above two plants and again tested the isolated compounds against a major dental plaque forming bacteria *Streptococcus mutans* isolated from the specimen of the patients after culture and subculture method. About 6 compounds were isolated from the above two plants and were subjected to antibacterial activity and only 3 compounds showed strong to moderate activity.

Phytochemical Investigations:

General Methods

There are four major steps in chemical investigation of a plant.

a) Preparation of plant sample
b) Extraction
c) Isolation of the compound
d) Characterization / structure determination of compounds.

Preparation of the plant material

Generally, the crude samples were taken in the fresh condition. Then these samples were grinded into small pieces and sun dried as well as finally dried on the oven at

40-45^{0}C for 36 hours. The collected samples were grinded into the powder with the grinder and preserved in an air tight container for future usage.

Solvent extraction

Extraction is the process where the plants materials are subjected to the specific solvent to dissolve the medicinally active components and the inactive components remain undissolved. Solvent extraction was done in two following ways-

a) Cold extraction and

b) Hot extraction.

a) Cold extraction

In this type of extraction procedures, the powder was dissolved in the solvent mixture in the bottom flask for few days. The main part of extracted compounds from collected plant samples were dissolved in the solvent properly. Then, the content was filtered by the means of filter paper.

b) Hot extraction

In hot extraction process, the sample was extracted by the organic solvent where the impurities were insoluble. This is done in Soxhlet apparatus. The extract thus obtained was concentrated to the small volume y the help of a rotator evaporator at reduced temperature and pressure.

Solvent-solvent partitioning of the crude extract

The isolation process of the organic compound from the aqueous solution of the selected plants with the appropriate organic solvent is referred to as extraction. This was done in a separating funnel. It was the process by which separation of the organic compound dissolved in aqueous phase affected using organic solvent, which is not miscible with water. This was solvent-solvent extraction. The

compounds were partitioned between organic and aqueous layers. The organic layer was isolated from the aqueous layer by using separated funnel. For maximum extraction, the process is repeated several times (not less than three times).

Isolation of compounds

Various chromatographic and other techniques are usually used as routine methods of isolation of compounds from crude extracts. Brief and general descriptions of these compounds were mentioned below.

Chromatography

Chromatography is a commonly applicable and convenient method for the isolation and purification of the compounds from sample mixtures. It involves the selective principle of partition and absorption of the mixed components of a between the stationary and mobile phases. The stationary phase is described as solid or liquid while the mobile phase is the liquid or gas. The stationary phase was found solid on the basis of absorption and it was observed liquid on the basis of partition.

Different types of chromatographic procedure were as follows:

1. Thin Layer Chromatography (TLC)
2. Column Chromatography (CC)
3. Preparative Thin Layer Chromatography (PTLC)

1. Thin layer chromatography (TLC)

Thin layer chromatography is the most popularly used separation method for the determination of organic compounds in a mixture, which involves an adsorbent (usually silica gel, alumina etc) as stationary phase and a solvent or solvent mixture as a mobile phase and a support of glass plate. The solvent is drawn up the absorbent layer by capillary action. Due to the differential rate of adsorption on the

adsorbent, the components in a mixture migrate differentially along with the TLC plates. In other words, due to difference in mobility of the components they are separated from each other. The mobility of the components often depends on their polarity and on that of solvent(s) used.

For the plate preparation, the glass plates measuring 5cm x 20cm was thoroughly washed to remove any dirt or fatty material present and are dried at 100°C for 5-8 minutes. The plates are then placed over aligning tray specially made for TLC. A slurry made of silica gel (1.5 gm/plate) and distilled water (2 ml/gm of silica gel) is uniformly distributed over the plate with the help of spreader. Before distribution, the plates are washed with cotton soaked in acetone to remove fatty materials. The plates are then allowed to stand for sometimes to dry and activated at a temperature of 100°C for one hour.

Airtight cylindrical glass chamber was used for chromatoplate development. 20 ml of the selected solvent was poured onto the tank and smooth filter paper sheet was taken and subjected to soak in the solvent. The airtight tank was kept for few minutes to make the internal components saturated with the vapor of the solvent. The small amount dried extract was dissolved in the solvent to get the solution of about 1%. The small spot of the solution was used to activate the silica plate by the capillary tube just 2 cm above the lower plate edge. The spot was dried in the hot air blower.

Spotted plate was taken in the tank to keep used spot on the solvent system surface. The plate was kept for the development. The solvent reached to the certain limit, the plate was taken out and then air-dried. The developed plates were observed under the UV light of the wavelengths and treated with the reagents for identification of the compounds.

2. Column chromatography (CC)

Column chromatography technique involves the separation of components into several fractions according to the affinity or solvating capacity of the components to the solvent used. The components having high affinity or dissolving property eluted faster than less solvating components. Column chromatography had been done by using conventional method.

The normal chromatographic column was packed with silica gel (kiesel gel 60), the most commonly used stationary phase. Silica gel is suspended in a suitable solvent or solvent mixture. It was then poured into appropriate column of height and diameter. The desired absorbent height of the bed was obtained; the mixture of few hundred milliliter solvent was run for proper colunm packing. After packing, the sample was applied into the column in two ways.

A. Dry column

The extracts, which are sticky, in such cases dry column preparation are necessary. The extract is ground finely with a little amount of silica gel and mixed well using mortar and pestle in order to obtain a non-sticky free flowing mass. The amount thus obtained is placed on the packed column.

B. Wet column

In wet column the sample is applied as a concentrated solution in a suitable solvent. For packing the column, Silica gel (70-230 mesh) is taken in a beaker and solvent is poured. The content is covered and kept at 20^0c for at least 2 hrs. to make slurry. A glass column is first plugged with a piece of clean cotton at the bottom and fitted with a stand. A stopcock is opened and solvent is passed through the column. After setting some solvent is allowed to dry out. However, care must be taken so that the solvent sufficiently covered the adsorption bed. The column is packed at temperature 20^0c to avoid cracking. The sample is mixed with a small

portion of silica gel using mortar and pestle in order to obtain non-sticky free flowing mass. The amount thus obtained is placed on disturbing the upper layer of the bed. Different solvent systems are passed through the column with progressively increased polarity. The flow is maintained at a constant rate (2ml/min). The elutes are collected in a different conical flasks. The volume of elute in each conical flask is 100 ml. after completion of elution of one solvent system; the next solvent system is poured to the column. The fractions having identical compounds are then kept undisturbed to evaporate to dryness.

C. Preparative thin layer chromatography (PTLC)

The principle of preparative TLC is same as that of TLC. Here larger plate (20cm ×20cm) is used. The sample is applied as a narrow band rather than spot. In some cases, multiple development technique is adopted for improved separation. Compounds are separated into bands in the developed plates. After visualization, the required bands are scrapped from the plates, and the compounds are eluted from the silica gel by treating with suitable solvents.

Detection of compounds

Different methods were applied for the identification of compounds to resolve the chromatogram development.

a) Visual detection

The developed chromatogram is observed visually to determine the existence of color compounds.

b) UV light

The developed dried plates were observed under UV light (254 and 366 nm) to locate UV absorbing compounds.

c) Iodine vapor

Developed chromatogram was used in a closed jar which contained the iodine crystals and allowed for few minutes. The compounds were appeared as brown spots, which were marked properly. Unsaturated compounds absorbed iodine. Bound iodine was eliminated from the plate by the process of air blowing.

d) Determination of R_f values

The R values of the compounds are calculated on a developed chromatogram using the pre-established solvent system. The R_f values were determination by using the following formula:

$$R_f \text{ value} = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the compound}}$$

TLC Spray Reagents

a) **Vanilline-Sulfuric Acid:** For higher alcohols, phenols, steroids and essential oil.
b) **Spray reagent:** 1gm vanillin was dissolved in sulfuric acid of 100 ml concentration.
c) **Treatment:** The chromatogram was treated with 1% of vanillin-sulfuric acid reagent and heated at 120° C, which was carried out until the spots attained maximum intensity.
d) **Modified Dragen dorff's reagent:** It is a solution of bismuth nitrate and potassium iodide in acetic acid and water. Alkaloids are detected by spraying the plates with this reagent. Some coumarins showed the positive results with Modified Dragendorff's reagent.
e) **Perchloric acid reagent:** 2% of aqueous perchloric acid produced brown spots with steroids after heating at 150ºC for 10 minutes.

Characterization of compounds

a) Physical characterization

The physical properties of the isolated compounds such as physical form, color, odor, solubility behavior were studied carefully.

Solubility study of the compounds was performed by adding different solvents (3 ml) to 2 mg of each of the dried compounds in the beaker and shading well to allow thorough mixing change in the solid form represented the extent of solubility. Pet-ether (60°-80°), diethyl ether, chloroform, ethyl acetate, acetone, methanol and water were used to detect the compound solubility.

b) Chemical characterization

Tests for Steroids and Triterpenoids

a. Libermann-Burchard test:

Extract solution was mingled with several drops of acetic anhydride that was boiled and cooled where concentrated sulphuric acid was added from the test tube. The brown ring was appeared at the junction of two layers where the upper layer was turned into green color that indicated the existence of sterols as well as the deep red colour formation was found to observe triterpenoid existence.

b. Salkowski's test

The extract was dissolved in chloroform solution by adding concentrated Sulfuric acid. The red colour appeared in the lower part of suspension that indicated the existence of sterols where the yellow colour formation in the lower layer indicated triterpenoids presence.

Tests for Alkaloids

a. Mayer's test

Potassium mercuric iodide solution was used in this test. Several drops of Mayer's reagent were added to the extracted solution and creamy white precipitate was appeared.

b. Dragendroff's test

Potassium bismuth iodide and several drops of Dragendroff's reagent were added and intermingled properly to the extract solution and reddish brown precipitation was formed.

c. Wagner's test:

Potassium iodide and Wagner's reagent were mingled to the extract solution where reddish brown precipitation was found in this test.

d. Hager's Test:

Saturated Picric acid and Hager's reagent were mixed to the extract solution and yellow precipitation was found to observe.

Tests for Phenolic Compounds

Zinc-Hydrochloride reduction test:

Zinc dust mixture and concentrated HCl were added to the extract solution where it gave yellowish or yellow-orange and in some cases orange colour were appeared after few minutes.

Tests for Flavonoids

Zinc-Hydrochloride reduction test:

Addition of zinc dust mixture and concentrated HCl indicated the red colour after few minutes.

Test for Tannins

In general, it is the test for phenolic isolation. The plant extracts (1.0 g) were weighed and placed into a beaker where 10 ml distilled water was added. The mixture was allowed to boil for five minutes where two drops of 5% $FeCl_3$ were mingled properly. The existence of tannins was observed by formation of greenish precipitation. Evans, (1989) observed that blue or green color indicated the presence of tannins.

Test for the carbohydrate

Reducing sugars test:

There are two methods which are applied for reducing sugar test. First, 1ml water and several drops of Fehling's solution were taken and added to the ethanol extract in the test tube. The red-brick precipitation was formed in the bottom of the test tube which indicated the presence of reducing sugars. Second, addition of 2 ml aqueous solution and 5-8 drops Fehling's solution exhibited red-brick precipitation in the suspension which indicated the presence of reducing sugars.

^{1}H and ^{13}C-NMR (nuclear magnetic resonance) Spectra

^{1}H and ^{13}C-NMR spectrum of the pure compounds were recorded on the jeol-Ex 400 MHz and FT NMR spectrometers at the Strathclyde University, Glasgow, London in $CDCl_3$ as solvent with tetramethylsilane (TMS) as an internal standard and the chemical shifts were provided in δ-value.

IR Spectroscopy

IR spectrum of the compound was recorded with KBrdicsc, using the PERKIN ELMER 1600 FTIR spectrophotometer at the Beximco Pharmaceuticals Ltd., Tongi, Dhaka and data are given in cm^{-1}

UV Spectroscopy

UV spectrum of the compounds were recorded in methanol solution using the BECKMAN double beam spectrophotometer at the Beximco Pharmaceuticals Ltd., Tongi, Dhaka and data are given in λ max.

Mass Spectroscopy

Mass spectrum were recorded at low resolution in mass spectrometer at the Strathclyde University, Glasgow, London and the data are given in m/z values.

Others Methods and Instruments

a) Melting Point

Melting point was determined by clariton (PIC) melting point apparatus that was uncorrected.

b) Rotary Evaporator

Evaporation was done by the Rotary evaporator (Janke and Kunkel, RV 05-ST), with the help of a advanced water bath.

PHYTOCHEMICAL STUDY ON *Cyndondactylon*.

Material

The matured bulb of the plant *Cyndondactylon (*Family: Poaceae) was selected for the chemical investigation.

Collection of Plant Material

The plant samples were collected from different areas of Rajshahi University campus during the month of March to April.

Drying and Pulverization

The collected plant samples were washed with tap water to remove the dirt and then the samples were subjected to sun drying treatment for seven days. Finally these samples were dried in an electric oven for 36 hours at below 60ºC temperature. The dried materials were pulverized into coarse powder by grinding on the machine (FFC-15, china) and were preserved in an air tight container for future use.

Plant Material Extraction

About 500 gm of powdered plant materials were extracted with rectified spirit (3 L) in a Soxhlet apparatus for 24 hours at 70-80°C temperature.

Whole plant **Pulverized**

Figure 12.

The extract was first sieved through cotton and then filtered through whatsman filters paper where the materials were concentrated with the rotary evaporator under reduced pressure to afford a semi-solid mass (13.2 gm). Water (50 ml) was added to the above extract in a separating funnel and n-Hexane (50 ml) was added and partitioned by shaking vigorously and stayed for sometimes until two distinct layers were formed. Separation of the organic layer and the process was continued with n-Hexane (3 times). Organic layers were separated and combined. After drying with anhydrous sodium sulphate, the solvents (n-Hexane) was evaporated off under reduced pressure in a rotary evaporated to afford (0.144gm) oily mass.

Solvent-solvent Partitioning of Crude Extract

Solvent-solvent partitioning was performed by applying the protocol designed by Kupchan and modified by Wagenen *et al*. (1993)

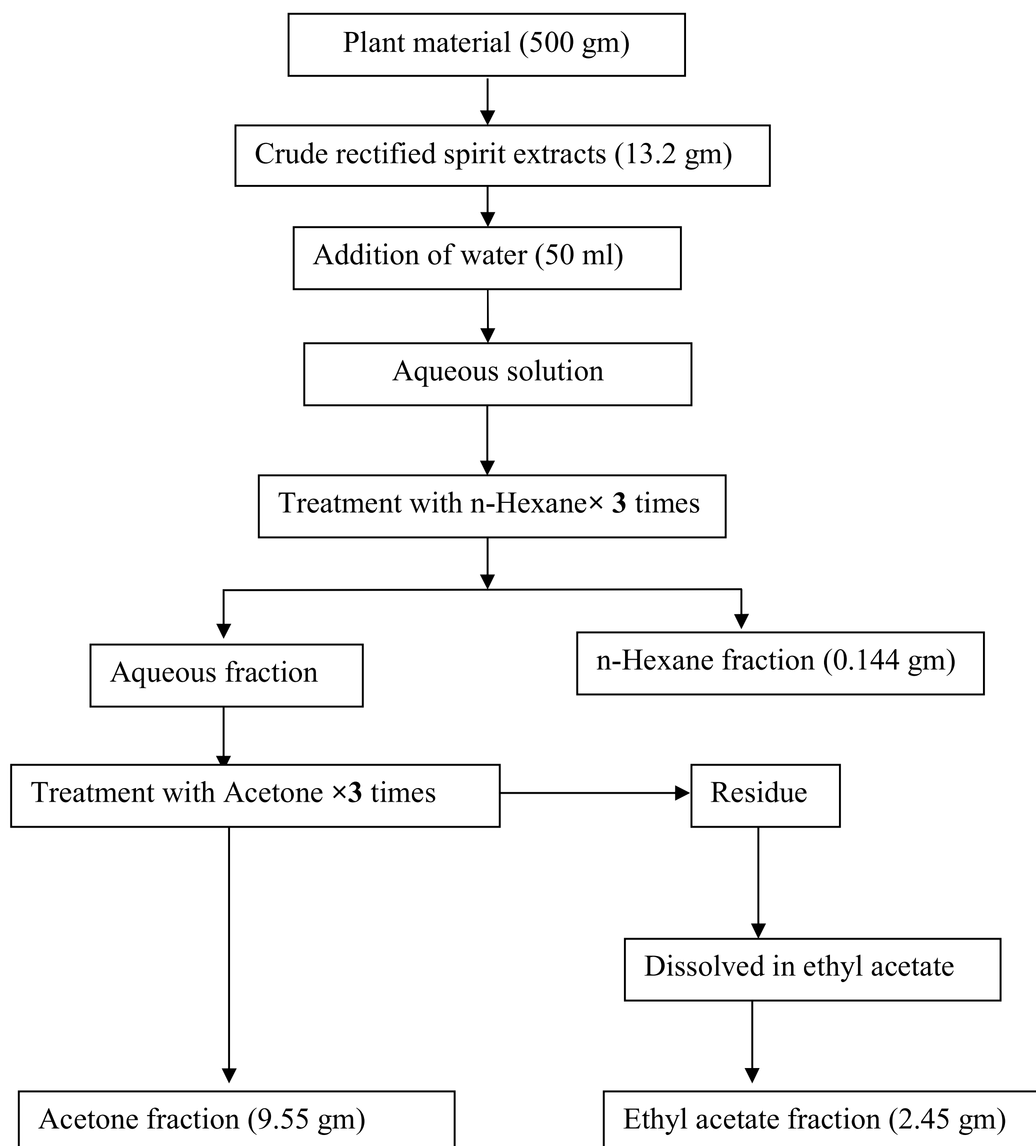


Figure 13. Schematic representation of solvent partitioning of the crude extract of *Cyndondactylon.*

The aqueous layer in a separating funnel was again extracted with acetone (3 times) similarly as before. After drying, the solvents was evaporated off under reduced pressure to afford a light grey colour (oil, 9.55gm) and was termed as acetone extract.

Water of the rest aqueous fraction (3.65gm) was evaporated off under reduced pressure in a rotary evaporator to dryness. Ethyl acetate was added to the mass, shaking and filter in whatmann filter paper.

The residue was again two times shaken with ethyl acetate and filtered. Combined filtrate was dried and ethyl acetate was evaporated off under reduced pressure to afford ethyl acetate extract (2.45gm).

Investigation of n-Hexane Extract

Since the amount of the extract was poor (0.144 gm) and did not show any remarkable spots on TLC, so, further investigation was not proceeded.

Investigation of the Acetone Extract

The chloroform extract showed several spots along with 3 prominent spots on TLC eluted with n-Hexane : Acetone (1:4). Sprayed with vanillin-sulphuric acid reagents on heating. This also showed the similar spots under UV and Iodine vapour.

A dry column was prepared with silica gel. A portion of the chloroform extract (10 gm) was taken in a morter and few amount of silica gel was added and mixed well with spatula. After mixing and drying, the mass was grind with pistle to a free flowing mass. This mass was then poured on the column bed very carefully without disturbing the upper surface of the bed and solvent n-Hexane was poured down very slowly without affecting the smooth surface of the column. The column was then eluted with n-Hexane and then with acetone and finally with acetone according to the following table.

Table 13. Solvent system used in column analysis of chloroform fraction.

Fraction no.	Solvent system	Proportion	Volume collected(ml)
1	n-Hexane	100%	75
2	n-Hexane	100%	75
3	n-Hexane : Acetone	100%	75
4	n-Hexane : Acetone	25 : 1	50
5	n-Hexane : Acetone	25 : 1	50
6	n-Hexane : Acetone	25 : 1	50
7	n-Hexane : Acetone	25 : 1	50
8	n-Hexane : Acetone	20 : 1	75
9	n-Hexane : Acetone	20 : 1	75
10	n-Hexane : Acetone	20 : 1	75
11	n-Hexane : Acetone	20 : 1	75
12	n-Hexane : Acetone	10 : 1	100
13	n-Hexane : Acetone	10 : 1	100
14	n-Hexane : Acetone	10 : 1	100
15	n-Hexane : Acetone	10 : 1	100
16	n-Hexane : Acetone	10 : 1	75
17	n-Hexane : Acetone	10 : 1	75
18	n-Hexane : Acetone	5 : 1	75
19	n-Hexane : Acetone	5 : 1	75
20	n-Hexane : Acetone	5 : 1	75
21	n-Hexane : Acetone	5 : 1	75
22	n-Hexane : Acetone	3 : 1	75
23	n-Hexane : Acetone	3 : 1	75
24	n-Hexane : Acetone	1 : 1	75
25	n-Hexane : Acetone	1 : 1	75
26	Acetone	100 %	75

The fractions 11 to 15 eluted with n-Hexane:Acetone (20:1 and 10:1) showed similar very close two spots on TLC eluted with n-Hexane : Acetone(1:4) and sprayed the chromatogram with vanillin-sulphuric acid reagents. Under UV the fraction also showed the similar spots (similar very closed two spots on TLC). Under UV and Iodine vapour treatment, these fraction were combined and dried with anhydrous sodium sulphate. The solvent was evaporated off under reduced pressure to afford compoundCD-1(2.4 gm). No other compounds with good amount was not isolated from *C. dactylon*

A

B

A

Figure 14: A (Materials used for TLC test of Cyndondactylon)
B (TLC Plate showing number of visualized bands or spots)
C (Cylindrical glass column containing silica gel with liquid solvent and extract sample)

Analysis of the fraction

TLC, UV and Iodine vapour treatment, they also showed very close two spots resembled single spot tried to separate these two spots using various methods like chromatographic separation, preparative thin layer chromatographic separation and partial crystallization method. But unable to separate these two compounds and these was such retained for spectroscopic analysis.

Table 14. Developing solvent system and Rf value of the compounds.

Sample	Solvent	Rf values	Color produced
C. dactylon	n-hexane : Acetone(1:4)	0.73	Light grey

Properties of the Isolated Compounds

The isolated compounds were characterized by their physical, chemical as well as spectral data. In physical properties physical form color, solubility, melting point and R_f values were recorded. Chemical properties included color reaction of the isolated compound with specific spray reagent. This compound is sent for spectral analysis.

1. Properties of Compound

a) Physical Properties

Physical form : Oil

Color : Light grey

R_f Values : 0.73

Solubility : Acetone

b) Chemical Properties

The compound mixture (CD-1) showed positive test for alcoholic OH group and unsaturation with bromine water reagent. Moreover, this fraction showed test for primary amine and carbonyl function with 2,4-dinitrophenyl hydrogen reagent.

c) Spectral Characteristics

^{1}H-NMR spectral data of compound CD-1 (Solvent $CDCl_3$, δ value, TMS as internal standard, 400 MHz). Spectral characteristics discussed in result and discussion section.

PHYTOCHEMICAL STUDY ON *Piper nigrum*

Material

The seed of the plant *Piper nigrum* (Family: Piperaceae) was selected for the chemical investigation.

Collection of Plant Material

The plant seed was collected during the month of March to April from adjacent areas of Rajshahi University campus.

Drying and Pulverization

The bark was first washed with tap water to eliminate the dirt and dried for seven days under the sun. Finally, the samples were taken for drying in an electric oven for 36 hours at below 60°C temperature. The dried materials were then pulverized into the powder by the grinding machine (FFC-15, china) and were kept in the preservation in an air tight container for the usage.

Extraction of the Plant Material

The powdered materials (about 500 gm) were extracted with rectified spirit (3 L) in a Soxhlet apparatus for 24 hours, at 70°C-80°C temperature.

The extract was first filtered then through Whatman No. 1 filter paper and was concentrated in a rotary evaporator under reduced pressure at 50°C temperature to afford a semi-solid mass (17.6gm). Water (50 ml) was added to the above extract in a separating funnel and ethyl acetate (50 ml) was added and partitioned by shaking vigorously and stayed for sometimes until two distinct layers were formed. Separation of the organic layer and the process was continued with ethyl acetate (3 times). Organic layers were separated and combined. After drying with anhydrous sodium sulphate, the solvents (ethyl acetate) was evaporated off under reduced pressure in a rotary evaporated below 50°C to afford semi solid mass (1.45gm).

Solvent-solvent Partitioning of Crude Extract

Solvent-solvent partitioning was done by using the protocol recommended by Kupchan and modified by Wagenen *et al*. (1993)

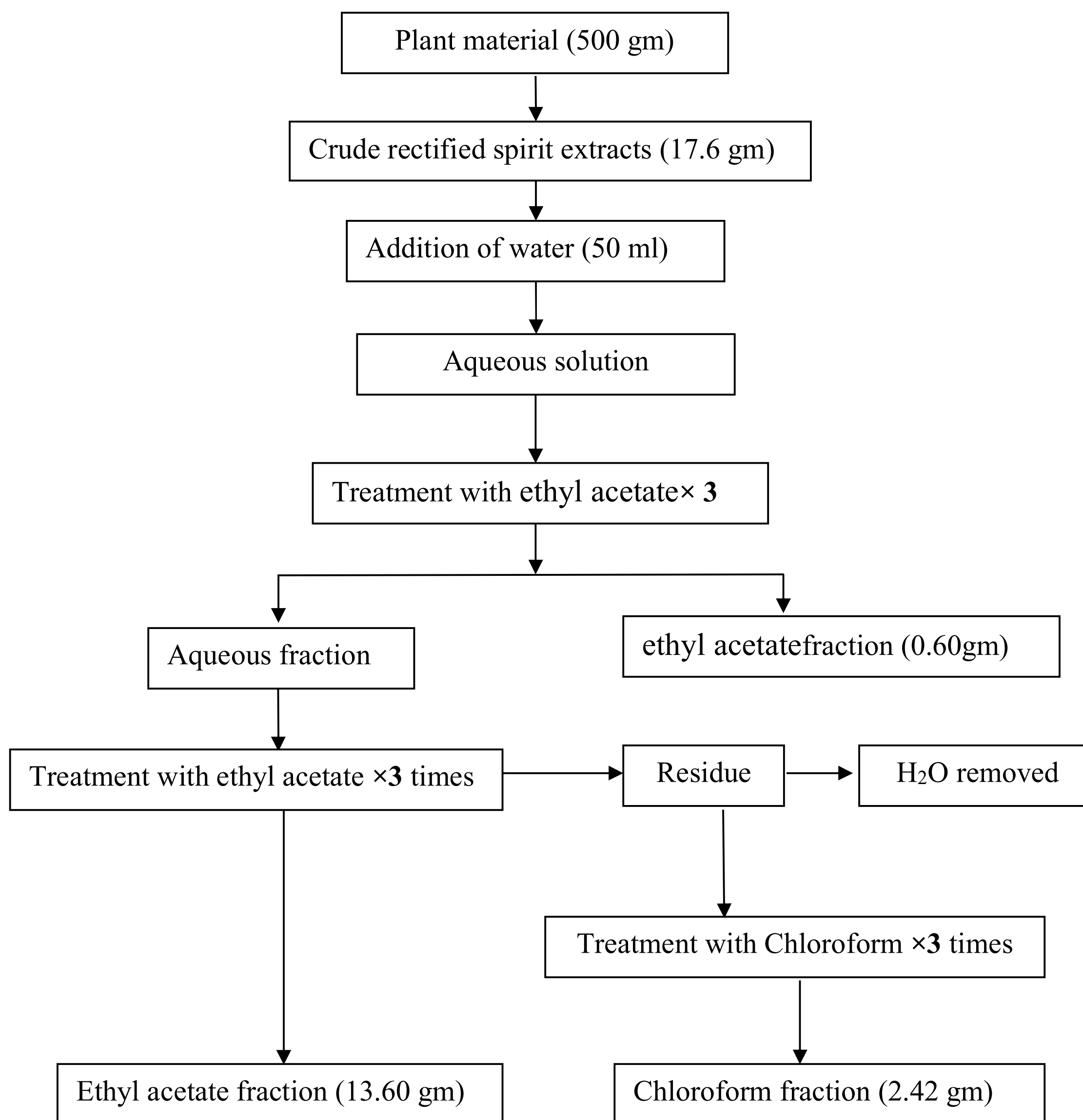


Figure 15. Schematic representation of solvent partitioning of the crude extract of seed of *Piper nigrum*.

The aqueous layer in a separating funnel was again extracted with ethyl acetate (3 times) similarly as before. After drying the solvents was evaporated off under reduced pressure to afford a blackish colour amorphous powder (13.60gm) and is termed as ethyl acetate extract.

Water of the rest aqueous fraction was evaporated off under reduced pressure in a rotary evaporator to dryness. Chloroform was added to the mass (3 times), shaking and filtered with whatmann filter paper.

Combined filtrate was dried and methanol evaporated off under reduced pressure to afford Chloroform extract (2.42gm) as a solid mass.

Investigation of Chloroform Extract

Since the amount of the extract was poor (6 mg) and did not show any remarkable spots on TLC. So, further investigation was not proceeded.

Investigation of the Ethyl acetate Extract

The ethyl acetate extract showed several spots along with one prominent spots on TLC eluted with Ethyl Acetate: Chloroform (7:1). Sprayed with vanillin-sulphuric acid reagent on heating. This also showed the similar spots under UV and Iodine vapour.

A dry column was prepared with silica gel. A portion (10 gm) of the ethyl acetate extract was taken in a morter and few amount of silica gel was added and mixed well with spatula. After mixing and drying, the mass was grind with pistle to a free flowing mass. This mass was then poured on the column bed very carefully without disturbing the upper surface of the bed and solvent Chloroform was poured down very slowly without affecting the smooth surface of the column. The column was then eluted with Chloroform, ethyl acetate and then with increasing portion of methanol and finally with methanol.

Table 15. Solvent systems used in column chromatography analysis of chloroform extract.

Fraction no.	Solvent system	Proportion	Volume collected(ml)
1	Chloroform	100%	100
2	Chloroform	100%	100
3	Chloroform: Et. Acetate	100 : 5	100
4	Chloroform: Et. Acetate	100 : 10	100
5	Chloroform: Et. Acetate	100 : 10	100
6	Chloroform: Et. Acetate	100 : 20	100
7	Chloroform: Et. Acetate	100 : 30	100
8	Chloroform: Et. acetate	100 : 40	100
9	Chloroform: Et. acetate	100 : 50	100
10	Chloroform: Et. acetate	100 : 50	100
11	Et. Acetate: Chloroform	100:10	100
12	Et. acetate: Chloroform	100 : 30	100
13	Et. acetate: Chloroform	100 : 50	100
14	Et. acetate: Chloroform	100 : 60	100
15	Et. acetate	100%	100

Fractions 3 to 7 eluted with Chloroform: Ethyl acetate (100:5 and 100:30) showed similar single spot on TLC. Solvent of these fractions were combined. Evaporation of the solvent under reduced pressure to afford amorphous powder (83 mg), compound PN-1

The other fractions 8 to 10 eluted with Chloroform and Ethyl acetate (100:40 to 100:50) afforded a mixture of the above compound with others. The next fraction also contaminated with more polar compound. Fraction 12 to 15 also gave an spot did not cross the base line. Compound PN-1 sent for spectral analysis.

Analysis of the fraction

TLC examination with multiple development compound PN-1 showed single spot examined under UV, Iodine vapour and vanillin H_2SO_4 reagent.

Table 16. Developing solvent system and Rf value of the compounds.

Sample	Solvent	Rf values	Color produced
Piper nigrum	Ethyl Acetate: Chloroform (7:1)	0.86	Blackish

Properties of Compound

a) Physical Properties of PN-1

Physical form : Amorphous powder

Color : Blackish

R_f Values : 0.86

Solubility : Ethyl acetate

b) Chemical Properties

It gave rise to blackish color on TLC with vanillin-sulfuric acid spray reagent on heating the plates at 110°C until the coloration took place. The compound PG-1, showed positive test for a carbonyl compound with 2,4-dinitrophenyl hydrazine test along with positive test for aromatic OH group.

c) Spectral Characteristics

^{1}H-NMR spectral data of compound (Solvent $CDCl_3$,δ value, TMS as internal standard, 500 MHz) (Fig:9) are given in a table-5.

Biofilm Production

General Methodology

Biofilms are responsible for the most chronic and recurrent infection diseases. Biofilm related infections reoccur in approximately 65-80% of cases. Bacteria associated with the biofilm are highly resistant to antibiotics (Venkatesan *et al*. 2015).

Biofilms are the bacterial colonies encased within extracellular polymeric matrix. Sessile (immobile) biofilm bacteria are phenotypically different from planktonic bacteria, conferring increased resistance to desiccation, antibiotics and the immune response. Antibiotics are able to kill the planktonic cells released by the biofilm after its maturation stages, but bacteria within the biofilm that can persist causing chronic infections. In biofilm formation, bacteria adhere reversibly to a surface begining to produce extracellular polysaccharides. As the bacterial number grows, quorum sensing allows a phenotypic change in the bacteria. The matured biofilm grows and eventually, proteins break down the matrix parts so that bacteria can spread within the biofilm. (Ulrey *et al*. 2014).

About 80% microbial infections are biofilm based. Biofilm architecture consists of structured and aggregated communities of the bacteria encased in a self-secreted exopolymeric substance. Several studies revealed that bacteria developed the resistance due to the prolonged treatment with conventional antibiotics possessing a broad range efficacy by toxic and growth inhibitory effects on target organisms rendering the traditional antibiotic treatment that is virtually ineffective. It was found that the bacteria living in the biofilm mode of growth were the resistant to antibiotics up to 1000 times more than the planktonic counterparts (Husain *et al*. 2015).

The presence of biofilms was mostly found in medical implants and urinary catheters. Various signalling events including two components signalling, extra cytoplasmic function and quorum sensing are involved in the biofilm formation. The presence of an extracellular polymeric matrix in biofilms makes it difficult for the antimicrobials to act on them and make the bacteria tolerant to antibiotics and other drugs (Claire *et al.* 2015)

Working Procedure for the Biofilm Formation

Microtitre plate based biofilm formation assay For detection and confirmation of biofilm formation capability of the clinical isolates *Staphylococcus aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa* and also in dental plaque forming bacteria *S. mutans*, we employed microtitre based technique. Freshly grown culture was diluted to 1:100 into fresh trypticase soy broth for biofilm assays. 100 µL of the dilution per well was added in a 96 well polystyrene microtitre dish. For quantification, 2-3 replicates were maintained. The microtitre plate was incubated for 24 hrs at 37°C. Upon incubation the cells were dumped by flipping the plate. The plate was cleared of any planktonic cells and media. To the wells 125µL of 0.1% crystal violet dye was added. For staining the plates were incubated at room temperature for 15 to 20 minutes. For quantification of biofilm, the stain was solubilized by addition of125 µL of 30% acetic acid to the wells. This was maintained static for 10 minutes. Thereafter the contents were added to a new microtitre plate and optical density was analyzed at 570 nm with 30% acetic acid as blank (O'Toole, 2011).Effect of the plant extracts on growth of biofilm isolates The action of plant extracts on the growth of biofilm formers were analysed by a concentration based study. 20-100µg/mL of methanol extract of *Pisonia alba* and ethyl acetate extract of *Cynodondactylon* and *Piper nigram* were prepared as working solutions. 100 µL of overnight cultures *S. mutans*, adjusted to McFarland standard 0.5 was added in to wells followed by 100 µL of the extracts. After incubation at 37ºC for 24 hours the plate was read spectrophotometrically at OD600 for any change in the growth of the biofilm isolates (Edeoga *et al*. 2005). DMSO and bacterial suspension served as blank and negative control respectively. All treatments and control were maintained in triplicates (Antunes *et al*. 2010).

Inhibition efficacy of plant extracts on biofilm formation 100µLof each working solution varying from the concentrations of 20-100µg/mL was added to the wells containing 100 µL of isolates in trypticase soy broth. The plate was incubated at 37º C for 24 hours. The plate was washed with sterile phosphate buffered saline to

remove all unattached cells and 0.4% crystal violet was added to stain the plate. The plate was de-stained for 30 minutes with 95% ethyl alcohol. At OD570, the contents were analysed spectrophotometrically. Percentage inhibition is determined by the same formula.

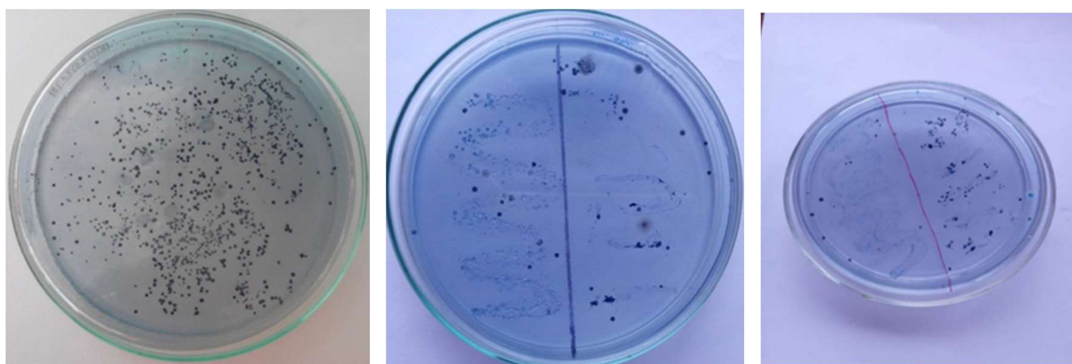


Figure 16. Identifications of *S. mutans* bacteria on mitis salivarius bacitracin agar media

Protocol of biofilm production

Dental plaque is also commonly referred to as microbial plaque, dental plaque biofilm, bacterial plaque, oral biofilm or biofilm dental biofilm. Bacterial plaque is the principal factors for dental decay and gum disease. Progression and dental plaque formation can cause the tooth decay and the destruction of the tooth local tissues by the acid production from the bacterial degradation of fermentable sugar and periodontal problems of gingivitis and periodontitis. Therefore, it is important for the disruption and removal of bacterial mass. The treatment and elimination of plaque can be accomplished by twice-daily tooth brushing and application of interdental aids like interdental brushes and dental floss.

The bulk of the microorganisms that form the biofilm is *Streptococcus mutans*. Other anaerobes varied by the location in the mouth though the specific composition. Anaerobes included *Fusobacterium* and *Actinobacteria*. *Streptococcus mutans* and other anaerobes are initially colonized the tooth surface

and play a significant role for the establishment of the early biofilm community. Flow chart for the formation of the biofilm was given below:

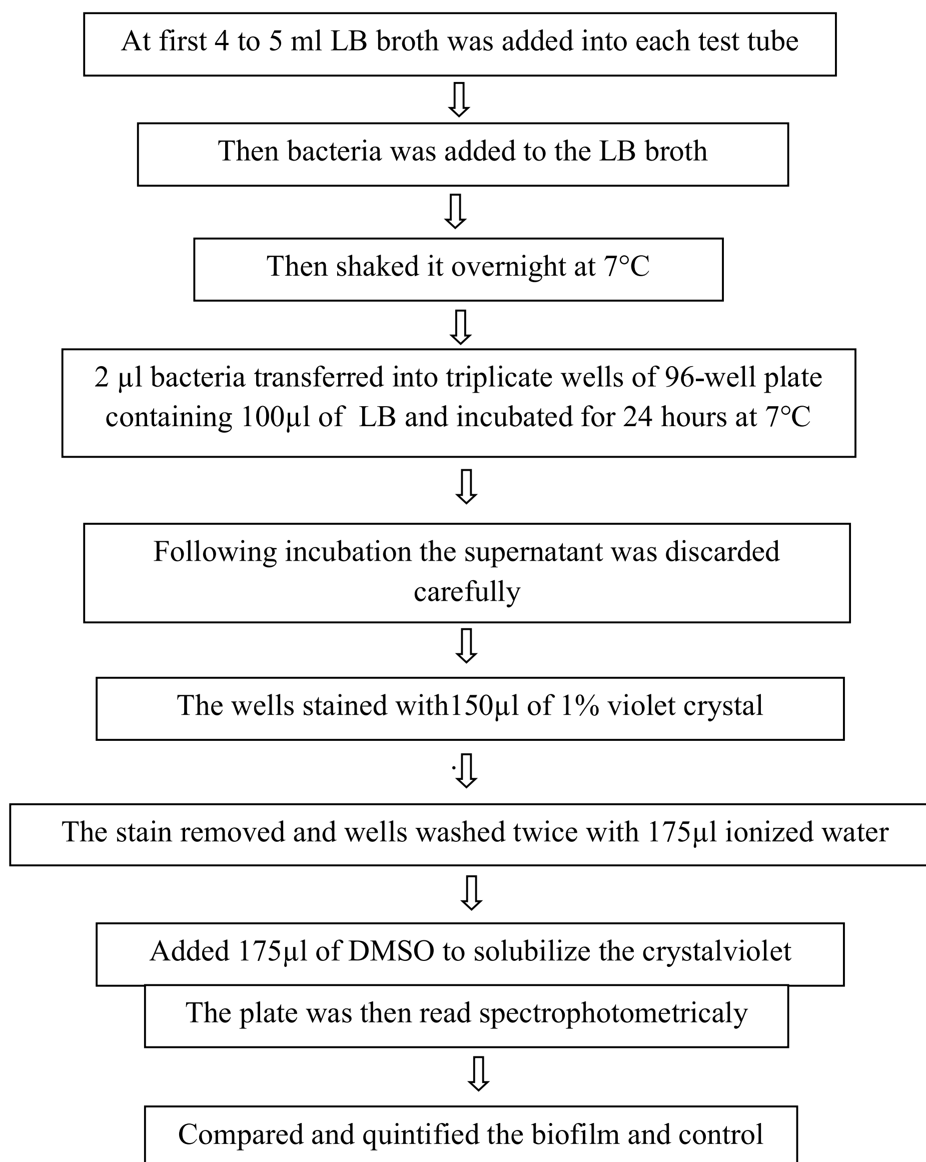


Figure 17. Flow chart for the biofilm production

Minimum biofilm inhibitory concentration (MBIC)

Minimum inhibitory concentration of biofilm (MBIC) of the extracts was decided on the basis of the visible destruction of the biofilm and noticeable decline in the absorbance in comparison with the control (Dhamodaran *et al*. 2010, Sanchez *et al*. 2016).

CHAPTER 5
RESULTS AND DISCUSSION

Microbiological Investigation

One of the most important and effective *in vitro* antibacterial screening of crude extracts or pure compounds is disc diffusion method. The method is simple, using discs of filter paper, put on petridishes containing nutrient broth media where test micro-organismswere cultured. Test samples were then applied on the filter paper discs in varying concentrations, kept in refrigerator for 12 hours at low temperature (4°C) for maximum diffusion and then incubated at 37°C for bacterial growth. In that case bacterial growth is not observed on the disc. This is known as zone of inhibition. The larger the zone of inhibition the greater the activity of the applied sample and is calculated by measuring the diameter of the zone of inhibition.

The *Streptococcus mutans* bacteria along with others causing dental plaque, were isolated from the sample of 480 patients having dental plaque and tested against the acetone and ethyl acetate extracts of two plants or plant parts for their antibacterial activity shown in the table-5.Among these two medicinal plants, showed from moderate to highest anti-microbial activity. These two plants were selected for their highest antimicrobial activity against *Streptococcus mutans.*

Table 17. Selected plants specimen showing antibacterial sensitivity.

Specimen (Plant) sample / **Patient sample**	***Piper nigrum* Zone of inhibition (mm)**	*Cyndondactylon* **Zone of inhibition (mm)**
1	13	12
2	12	15
3	14	16
4	19	14
5	22	18

All the two plants showed activity against the dental plaque causing bacteria. Here 1 mg/disc were used for the test. Upon antibacterial screening, the extracts showed good activity against the dental plaque causing bacteria. They were further used at a concentration of 2 mg/disc and 3 mg/disc. The results of the antibacterial screening measured in term of zone of inhibition (mm) as shown in table.

Table 18. Anti bacterial activity test result for selected specimen (2mg/disc) zone of inhibition (mm)

Specimen (Plant) sample / Patient sample	*Piper nigrum* Zone of inhibition (mm)	*Cyndondactylon* Zone of inhibition (mm)
1	15	13
2	16	11
3	13	16
4	14	15
5	17	18

Table 19. Anti bacterial activity test result for selected specimen (3mg/disc) zone of inhibition (mm)

Specimen (Plant) sample / Patient sample	*Piper nigrum* Zone of inhibition (mm)	*Cyndondactylon* Zone of inhibition (mm)
1	16	15
2	19	17
3	18	20
4	21	23
5	23	19

Plants screened for antibacterial activity against those bacteria causing dental plaque, *Streptococcus mutans* bacteria along with others causing dental plaque, were isolated and tested with the above plant extracts. Extracts of two plants showed highest antibacterial activity, namely *Piper nigrum*, *Cyndondactylon.*

Next work is to isolate the active compound from the selected two plants which will show highest activity against the isolated pathogenic bacteria and the structures of the isolated compounds will be determined using spectroscopic methods of analysis.

Upon antibacterial screening, among the crude acetone and ethyl acetate extracts, the crude acetone extracts of *Piper nigrum* extract showed highest activity against *Streptococcus mutans* than most of the other Gram-positive and Gram-negative bacteria cited in the above table-8. They were used at a concentration of 200 µg/disc and 400 µg/disc. The results of the antibacterial screening measured in term of zone of inhibition as shown in table-8.

Table 20. *In vitro* antibacterial activity of two fractions of the *Piper nigrum* **extract.**

Bacterial strains	Zone of inhibition, diameter in mm				
	Acetone extract		Ethyl acetate extract		Standard antibiotic kanamycin
	200 µg/disc	400 µg/disc	200 µg/disc	400 µg/disc	30µg/disc
Gram positive					
1. *Staphyloccus aureus*	11	13	12	18	11
2.*Streptococcus mutans*	13	19	11	16	14
3.*Bacillus subtilis*	12	15	13	15	10
4.*Bacillus cereus*	11	16	14	19	12
Gram negative					
1.*Escherichia coli*	11	14	11	15	13
2.*Shigella dysenteriae*	12	16	11	14	15
3.*Shigella shiga*	11	15	12	16	13
4.*Salmonella typhi*	10	15	13	15	12

A mixture of two compounds (very close spots on TLC) isolated from chloroform extract of *Allium sativum* was tried to separate using conventional methods, but failed. These mixture (PN-1) of two compounds showed strong antibacterial activity against *S. mutans*. The result of this test is tabulated below (Table-9).

From the table-9 it is seen that the zone of inhibition due to compound (PN-1) is much larger than that of the standard kanamycin. When 200 µg/ml of compound (PN-1) used, the zone of inhibition is 19 mm while in case of 400 µg/ml it is in 24 mm. But in case of Kanamycin (30µg/ml) the zone of inhibition is only 11 mm. Which indicates, perhaps Kanamycin that was used for the test my partially resistant to *S. mutans*. The mixture of compounds (PN-1) used as for spectral analysis.

Table 21. *In vitro* antibacterial activity of compound PN-1.

Bacterial strains	Zone of inhibition, diameter in mm		
	200 µg/disc	400 µg/disc	kanamycin 30µg/disc
Gram positive			
1. *Staphyloccus aureus*	12	16	10
2.*Streptococcus mutans*	19	24	11
3.*Bacillus subtilis*	13	15	12
4.*Bacillus cereus*	11	15	10
Gram negative			
1.*Escherichia coli*	12	12	10
2.*Shigella dysenteriae*	12	14	13
3.*Shigella shiga*	11	15	10
4.*Salmonella typhi*	13	16	12

For bacterial screening four Gram-positive and four Gram-negative bacteria was used against crude acetone and chloroforms extracts at a concentration of 200µg/ml and 400 µg/ml.

Upon antibacterial screening, the crud ethyl acetate extract of *C. dactylon* extract showed highest activity against *Streptococcus mutans* than most of the organisms which were used for the test. The results of the antibacterial screening measured in term of zone of inhibition as shown in table-10.

Table 22. ***In vitro*** **antibacterial activity of three fractions of the** ***C. dactylon*** **extract.**

Bacterial strains	Zone of inhibition, diameter in mm				
	Methanol extract		n-Hexane extract		Standard antibiotic kanamycin
	200 µg/disc	400 µg/disc	200 µg/disc	400 µg/disc	30µg/disc
Gram positive					
1. *Staphyloccus aureus*	11	14	10	15	10
2.*Streptococcus mutans*	12	15	13	17	14
3.*Bacillus subtilis*	11	12	12	14	12
4.*Bacillus cereus*	10	11	13	14	14
Gram negative					
1.*Escherichia coli*	10	13	12	14	10
2.*Shigella dysenteriae*	11	14	11	13	12
3.*Shigella shiga*	11	13	11	15	11
4.*Salmonella typhi*	10	12	12	14	13

A single compound (CD-1) isolated from the crude ethyl acetate extract of *C. dactylon*, having RF value 0.73 showed highest antibacterial activity against *S. mutans* among the four Gram positive and four Gram negative bacteria at a concentration of 200 µg/ml and 400 µg/ml.

The zone of inhibition are 16 mm and 24 mm when extract used at a concentration 200 µg/ml and 400 µg/ml, respectively. These zone of inhibition are more than that of the standard kanamycin which showed only 13 mm. This is perhaps due to the partial resistance of Kanamycin against *S. mutans*.

Table 23. ***In vitro*** **antibacterial activity of compound** CD-1.

Bacterial strains	Zone of inhibition, diameter in mm		
	200 µg/disc	400 µg/disc	kanamycin 30µg/disc
Gram positive			
1. *Staphyloccus aureus*	13	14	12
2.*Streptococcus mutans*	16	24	13
3.*Bacillus subtilis*	15	19	10
4.*Bacillus cereus*	12	16	12
Gram negative			
1.*Escherichia coli*	10	10	11
2.*Shigella dysenteriae*	12	14	12
3.*Shigella shiga*	10	16	12
4.*Salmonella typhi*	12	16	10

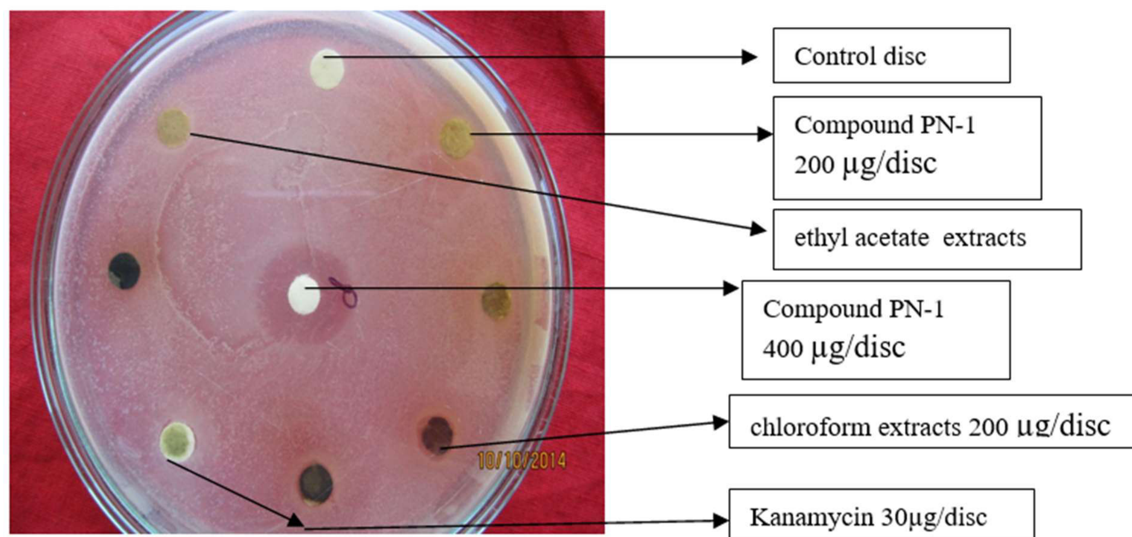


Figure 18. Effect of ethyl acetate and chloroform extract on Compound PN-1(200 and 400 µg/disc) on *Streptococcus mutans.*

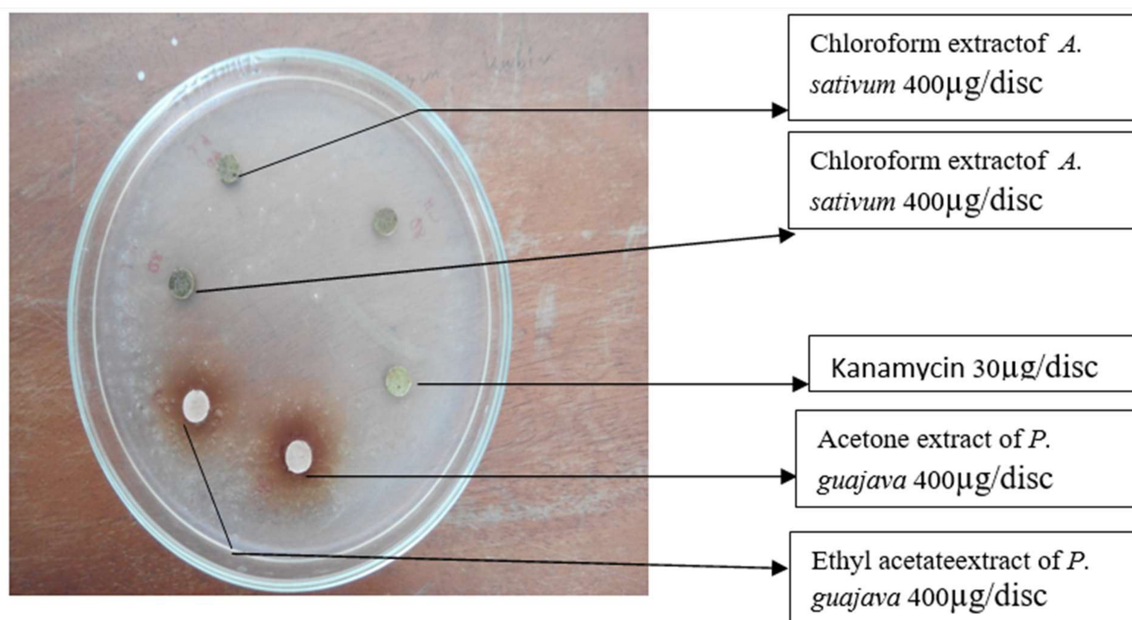


Figure 19. Inhibitory effect of chloroform, ethyl acetate and acetone extract on *Streptococcus mutans.*

Phytochemical work on *Cyndondactylon*

The matured bulb of the plant *Cyndondactylon* (Family: Poaceae) was selected for the chemical investigation. Plants were first washed with water to remove adhering dirt and then sun dried for seven days and finally dried in an electric oven for 36 hours at a temperature below 60ºC. The dried material was then pulverized into course powder with the help of a grinding machine (FFC-15, china) and were stored in an air tight container. The powdered materials (about 500 gm) was extracted with rectified spirit (3 L) in a Soxhlet apparatus for 4 days, at 70-80ºC temperature. The extract was first filtered through cotton and then through whatman No. 1 filter paper and was concentrated with a rotary evaporator under reduced pressure at 50ºC temperature to afford crude extract (13.2 gm).

Solvent-solvent partitioning of crude extract

To the semisolid mass obtained above (9.55 gm) water was added and taken in a separating funnel and extracted (3 times) with 100 ml portions of acetone and ethyl acetate. The respective organic layers were washed with water, dried over anhydrous Na_2SO_4 and evaporated off under reduced pressure to afford following amounts of each extracts.

Water of the aqueous layer (left after above extraction) was removed in vacuum and the mass was extracted with acetone (3 times). Solvents was filtrated, dried and evaporated off under reduced pressure to afford a reddish semisolid mass (9.55gm) termed as acetone extract.

Name of fraction	Weight of fraction
Acetone fraction	9.55gm
Ethyl acetate fraction	2.45gm

Work up with acetone extract

Fractions of the column 13-16, 19-22 and 25-28 showed very close similar spots on TLC. Solvent of the above combined fraction was evaporated off in a rotary evaporator under reduced pressure to obtain compound mixture CD-1 (9.55gm).

The compound mixture was tried to separate using PTLC and partial crystallization but failed.

TLC examination of this mixture is given as follows eluted with n-Hexane: chloroform (1:4) and sprayed the chromatogram with vanilline-H_2SO_4 reagent after heating.

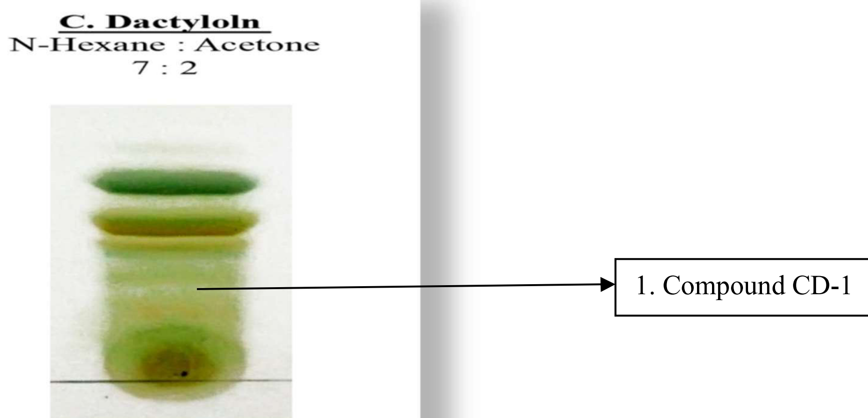


Figure 20. Characterization of *Cyndondactylon* on TLC spot

Characterization of the mixture of two compounds obtained from fraction (CD-1):

The compound mixture (CD-1) isolated as syrup from the fractions13-16, 19-22 and 25-28 tried to separate by various methods of separation like PTLC, and partial crystallization and was not possible to separate the two compounds.

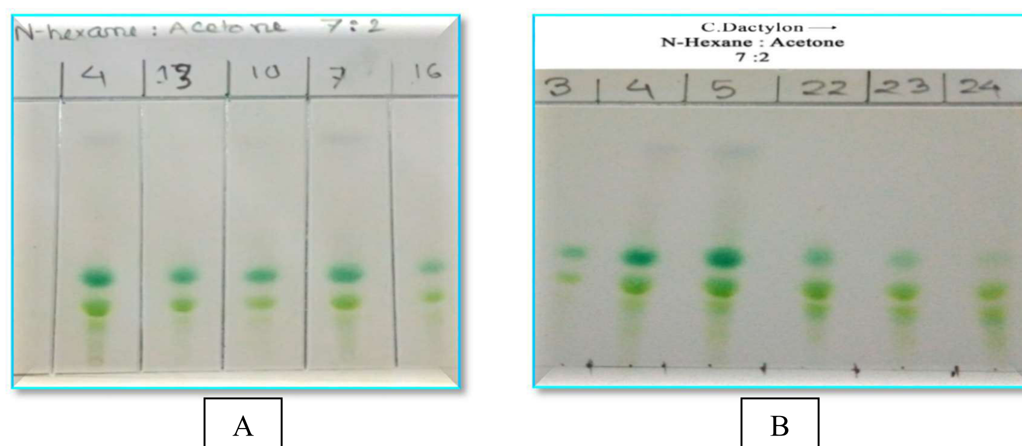


Figure 21. A, B (TLC plate visualized different types of compound bands which can be detected by using spray reagent)

The combined fraction showed positive test for alcoholic (OH^-) group and test for unsaturation with bromine water. Moreover, this fraction gave positive test for primary amine along with carbonyl function, with 2,4-Dinitrophenyl hydrazine.

Proton NMR spectrum of the compound (Figure 3) also resembles a mixture of two compounds. Chemdraw spectrum of the known compounds Allin and Allicin are given in Figure 4 and Figure 5.

Spectral analysis

Analysis of the chemdraw spectrum of A phytol derivatives (3,7,11,15, Tetramethyl-hexadeca-2-en-1-01), B phytol derivatives (3,7,11,15, Tetramethyl-hexadeca-2,4 dien-1-01)and C (Stigmasterol) (Figure 4) showed six olefinic protons at δ: (5.12, 5.03, 5.96 and 5.03, 5.70, 4.97) and two methylene protons at δ 3.23 and 3.24 (structure of 3,7,11,15, Tetramethyl-hexadeca-2-en-1-01) and 3,7,11,15, Tetramethyl-hexadeca-2,4 dien-1-01 showed a carboxic acid proton at 11.0 ppm as primary amine proton and δ 2.0 and generally disposed methyl proton at δ 1.33 as singlet. Three olefinic protons are disposed at δ 5.66, 5.92 and 6.52.

The ^{1}H-NMR spectrum of compound CD-1 (Figure 3) presumed to be indicated all the peaks present in 3,7,11,15, Tetramethyl-hexadeca-2-en-1-01 and 3,7,11,15, Tetramethyl-hexadeca-2,4 dien-1-01 with some impurities. Although all peaks were not identified due to the low resolution and expansion of the spectrum. Due to the lack of NMR facility it would be very difficult to structural elucidation of the isolated compounds.

The isolated mixture of compound CD-1 was tentatively assigned as mixture of 3,7,11,15, Tetramethyl-hexadeca-2-en-1-01 and 3,7,11,15, Tetramethyl-hexadeca-2,4 dien-1-01 and Stigmasterol showed strong activity against *Streptococcus mutans* with zone of inhibition (mm).

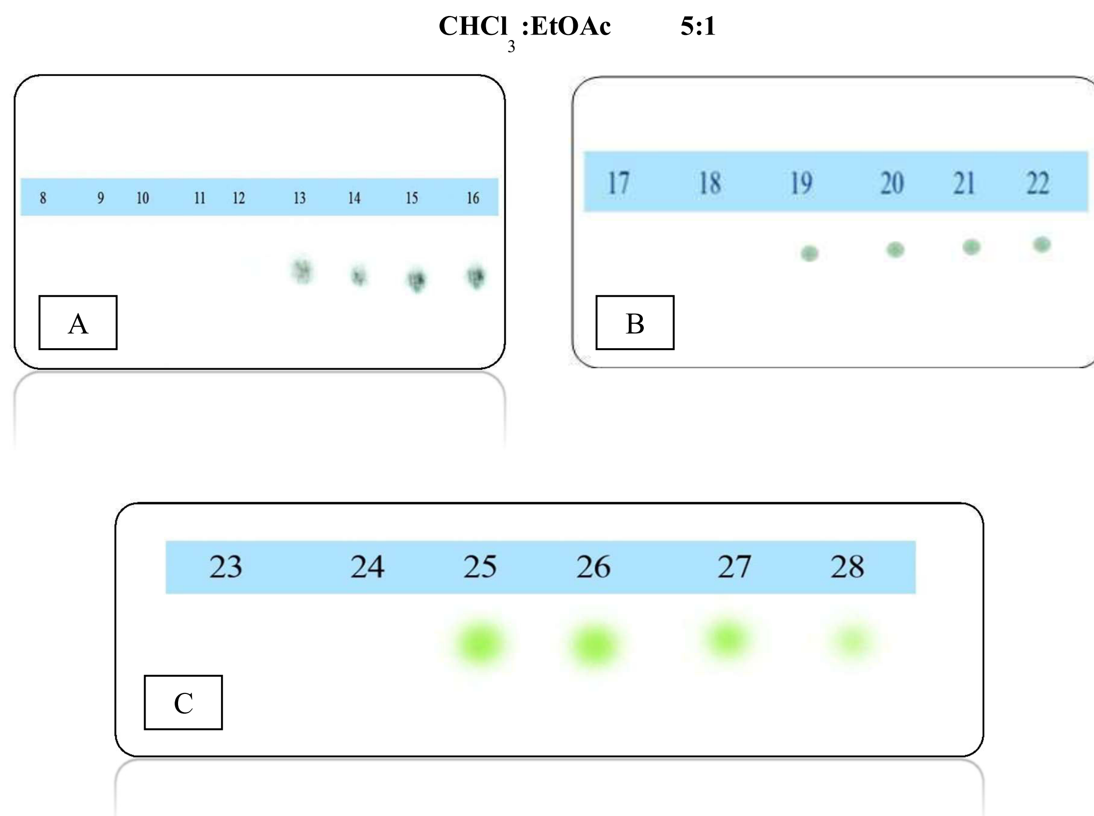
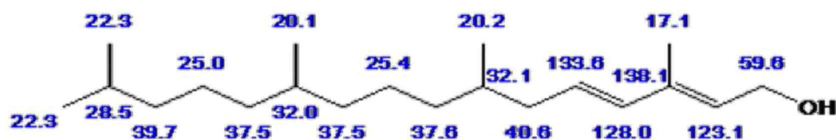


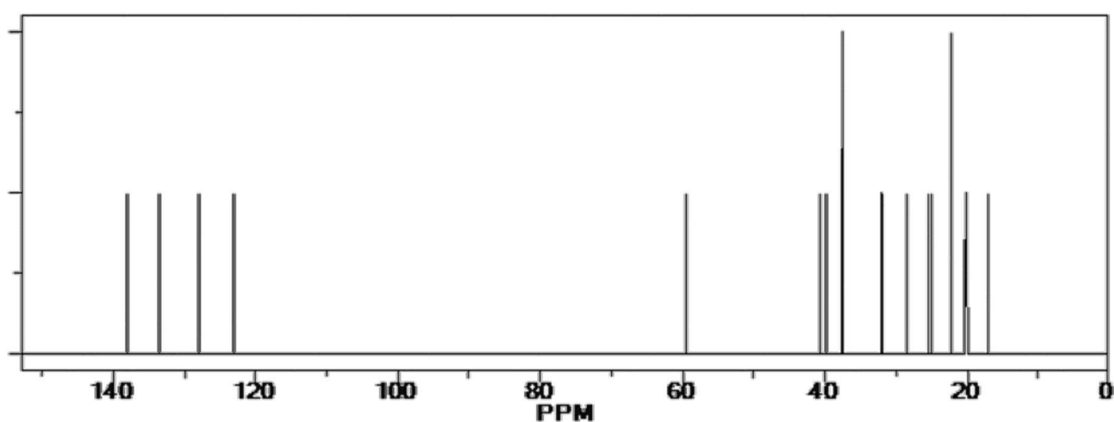
Figure 22. Isolation and purification of *Cyndondactylon* using column-Chromatographic techniques.

Structure elucidation of sub-fraction of two crystals

Chem NMR C-13 Estimation

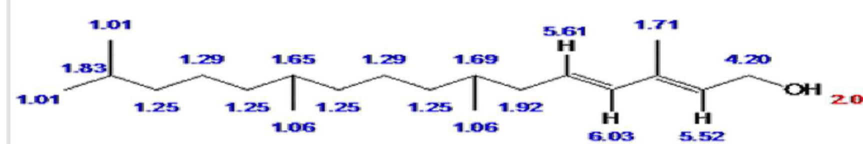


Estimation Quality: blue = good, magenta = medium, red = rough



Chem NMR H-1 Estimation

ChemNMR H-1 Estimation



Estimation Quality: blue = good, magenta = medium, red = rough

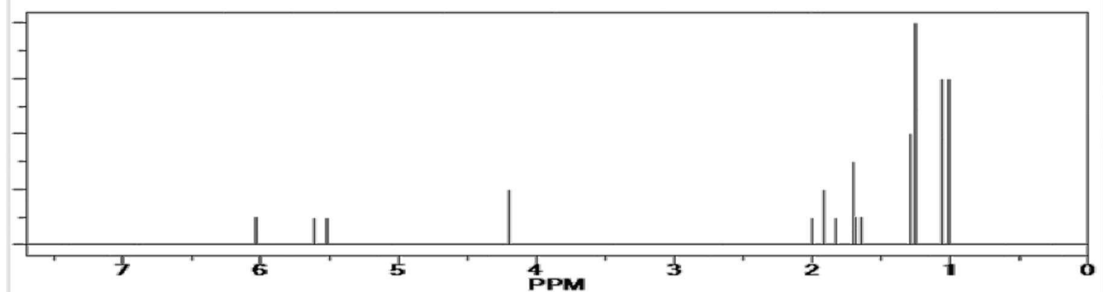
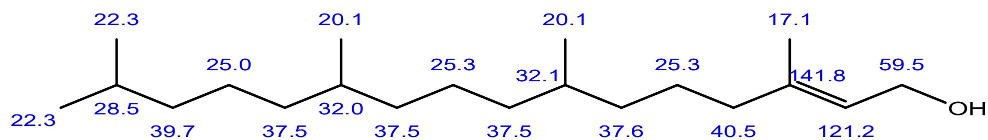
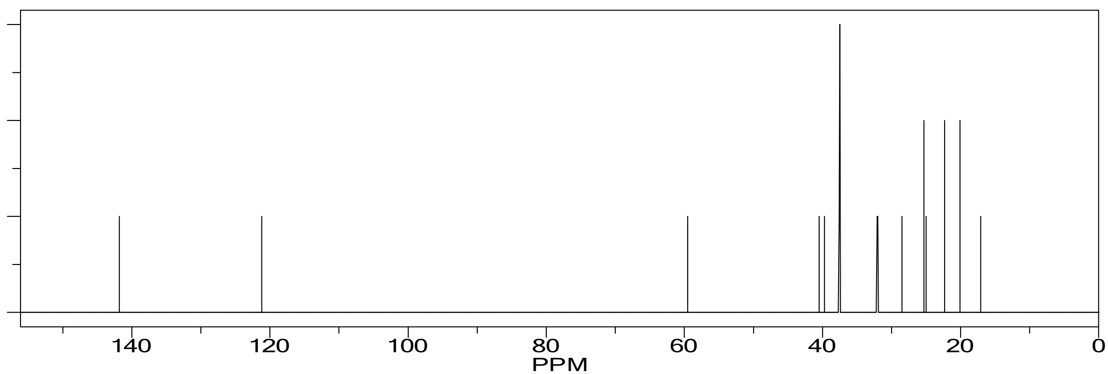


Figure 23. Phytol derivatives (3,7,11,15, Tetramethyl-hexadeca-2,4 dien-1-01)

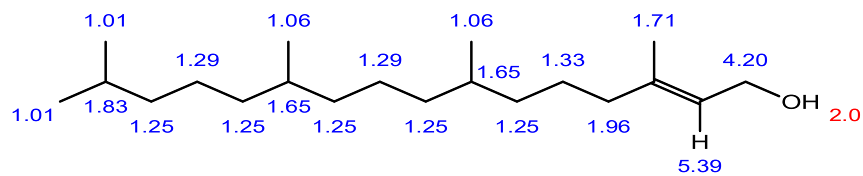
ChemNMR C-13 Estimation



Estimation Quality: blue = good, magenta = medium, red = rough



ChemNMR H-1 Estimation



Estimation Quality: blue = good, magenta = medium, red = rough

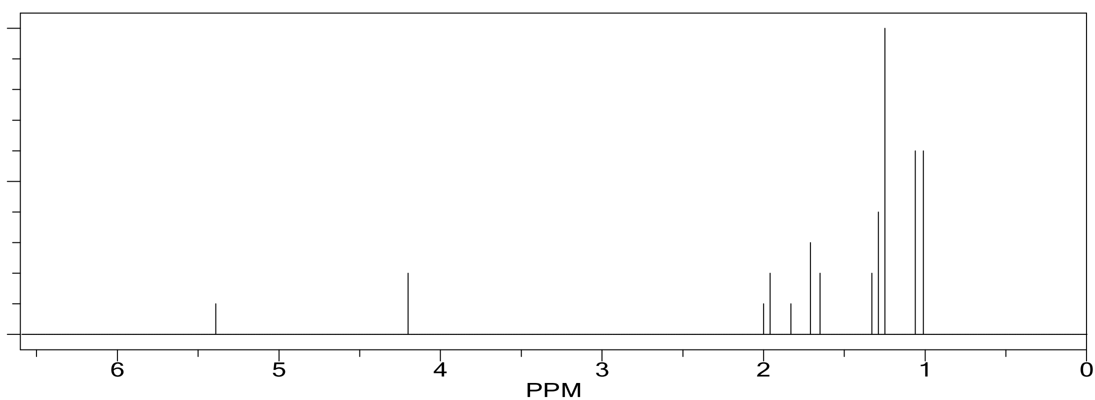
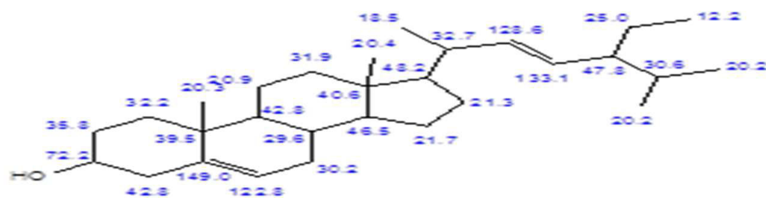
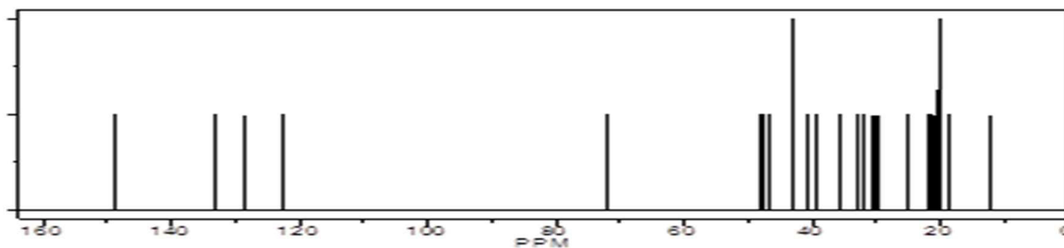


Figure 24. Phytol derivatives (3,7,11,15, Tetramethyl-hexadeca-2-en-1-01)

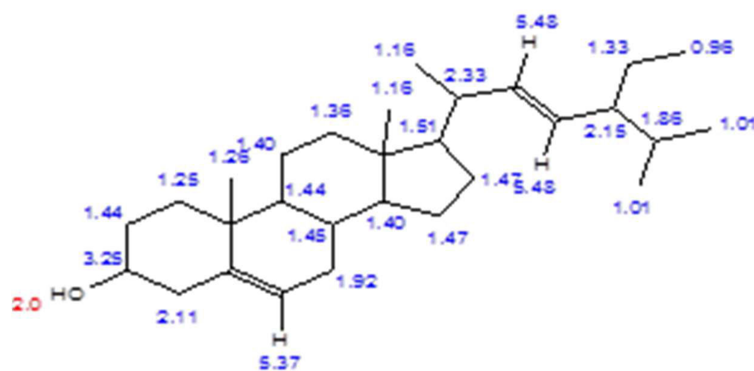
ChemNMR C-13 Estimation



Estimation Quality: blue = good, magenta = medium, red = rough



ChemNMR H-1 Estimation



Estimation Quality: blue = good, magenta = medium, red = rough

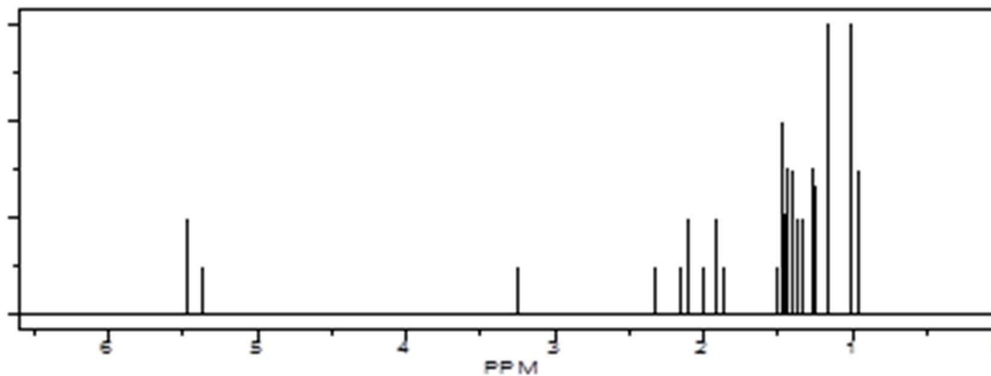


Figure 25. Stigmasterol

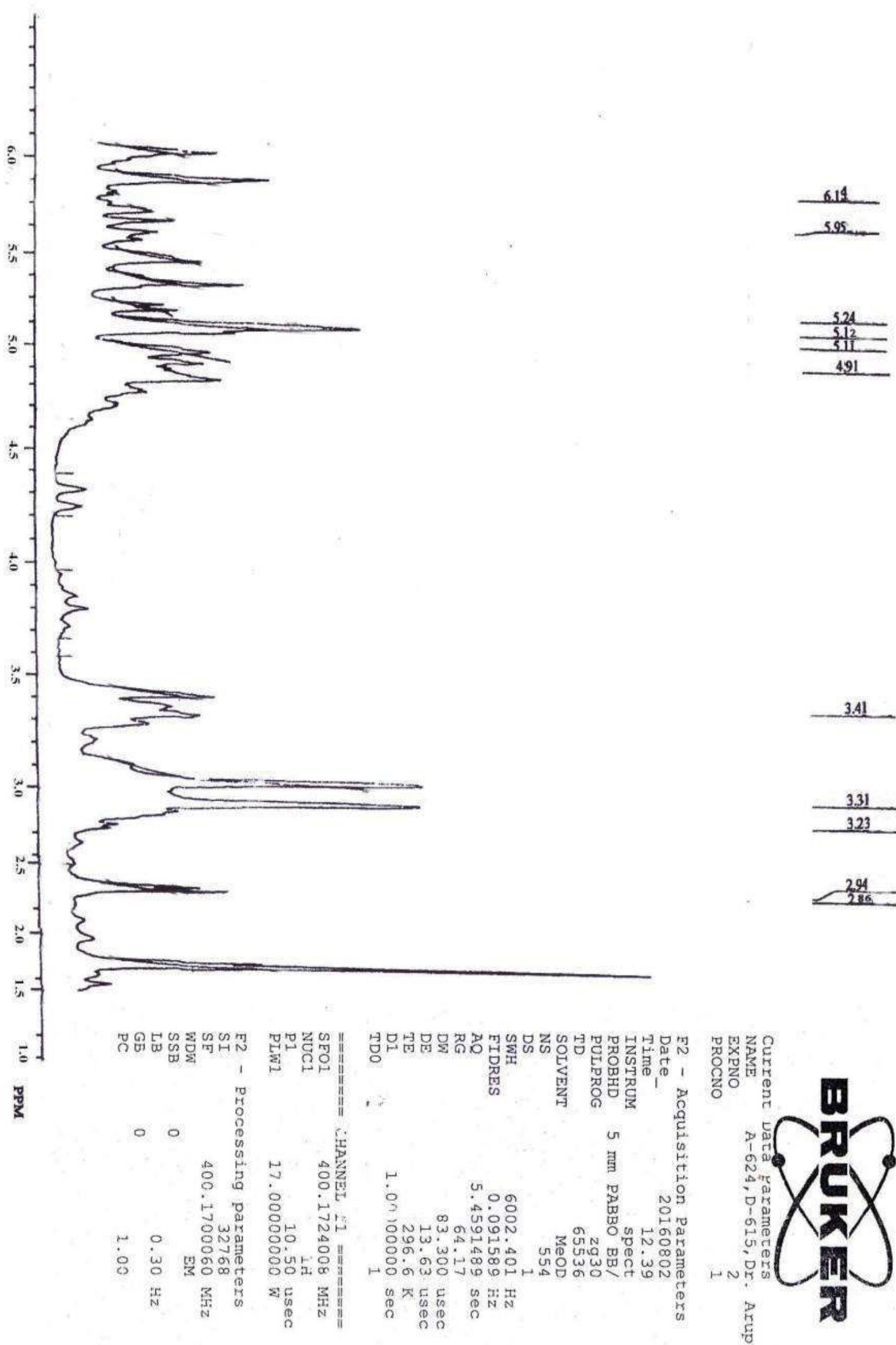


Figure 26. ^{1}H-NMR spectrum of the AS-1 in $CDCl_3$

Phytochemical work on *Piper nigrum*

Collection of Plant Material

Piper nigrum was collected during the month of March-April from adjoining areas of Rajshahi University campus.

Extraction of the Plant Material

The powdered materials (about 500 gm) were extracted with rectified spirit (3 L) in a Soxhlet apparatus for 24 hours, at 70ºC-80ºC temperature.

The extract was first filtered through cotton and then through whatman No. 1 filter paper and was concentrated with a rotary evaporator under reduced pressure at 50ºC temperature to afford a semi-solid mass (13.6 gm). Water (50 ml) was added to the above extract in a separating funnel and chloroform (50 ml) was added and partitioned by shaking vigorously and stayed for sometimes until two distinct layers were formed. Separation of the organic layer and the process is continued with n-Hexane (3 times). Organic layers were separated and combined. After drying with anhydrous sodium sulphate, The solvent (ethyl acetate) was evaporated off under reduced pressure in a rotary evaporated below 50°C to afford oily mass (13.6 mg).

Fractionation of the Crude Extract

The aqueous layer was then taken in a separating funnel was again extracted with chloroform (3 times) similarly as before. After drying the solvents was evaporated off under reduced pressure to afford a blackish colour (oil, 2.42 gm) and is termed as chloroform extract.

After separation the aqueous layer was again extracted with ethyl acetate three times as before. After drying solvent was evaporated off under reduced pressure to afford a semisolid mass of ethyl acetate (13.60 gm).

Name of fraction	Weight of fraction
Ethyl Acetate fraction	13.60 gm
Chloroform fraction	2.42 gm

Investigation of chloroform extract

Since the amount of the extract was very little (2.42 gm) and did not show any remarkable spots on TLC. So, further investigation was not proceeded.

Investigation of the ethyl acetate extract

The ethyl acetate extract showed several spots along with one prominent spots on TLC eluted with Ethyl Acetate: Chloroform (7:1). Sprayed with vanillin-sulphuric acid reagents on heating. This also showed the similar spots under UV and Iodine vapour.

A dry column was prepared with silica gel. A portion of the ethyl acetate extract was taken in a morter and few amount of silica gel was added and mixed well with spatula. After mixing and drying, the mass was grind with pistle to a free flowing mass. This mass was then poured on the column bed very carefully without disturbing the upper surface of the bed and solvent n-Hexane was poured down very slowly without affecting the smooth surface of the column.

Water of the aqueous layer (left after above extraction) was removed in vacuum and the mass was extracted with methanol (3 times). Solvents was filtered, dried and evaporated off under reduced pressure to afford a reddish semisolid mass (1.46gm).

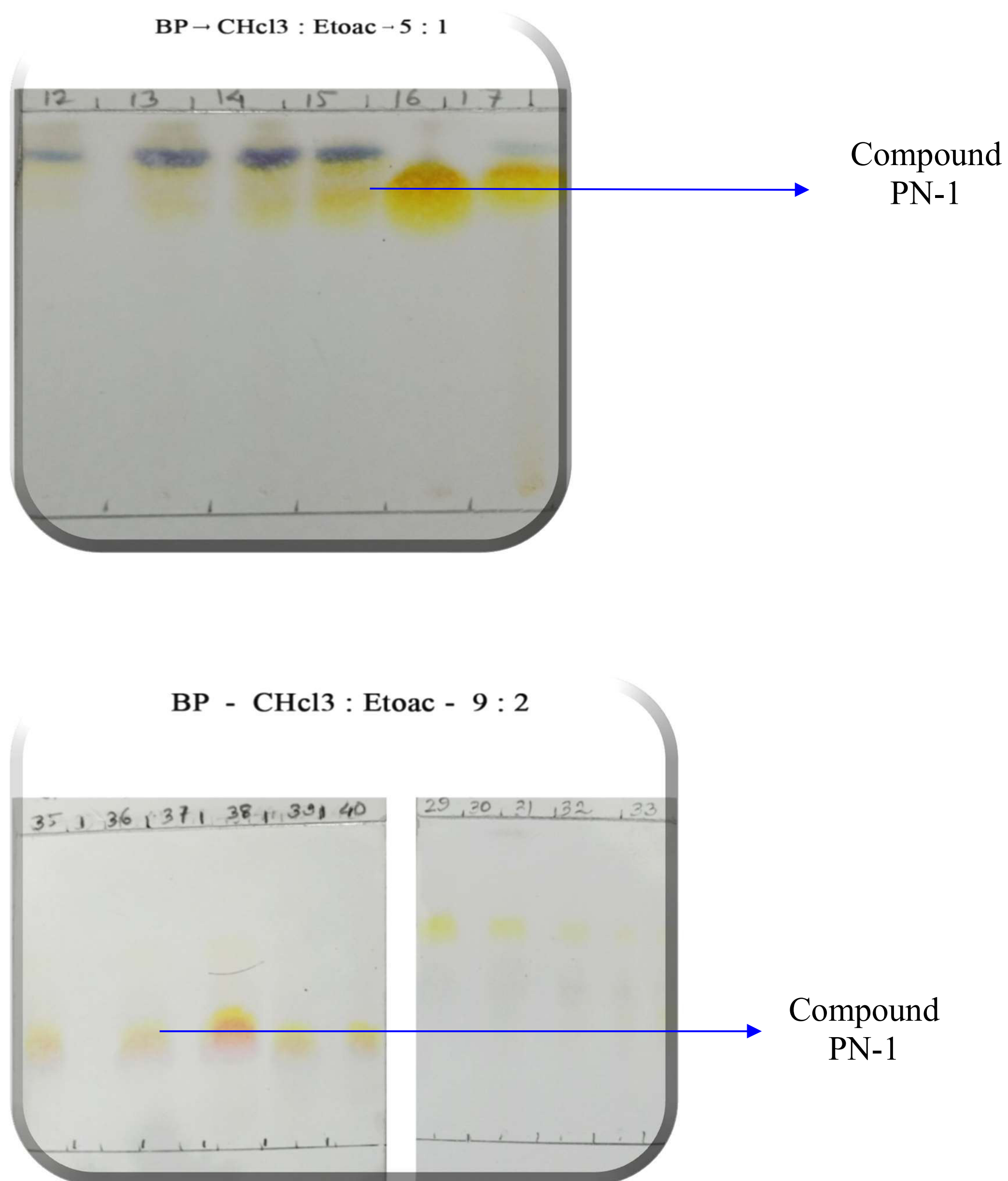


Figure 27. Showed TLC spot

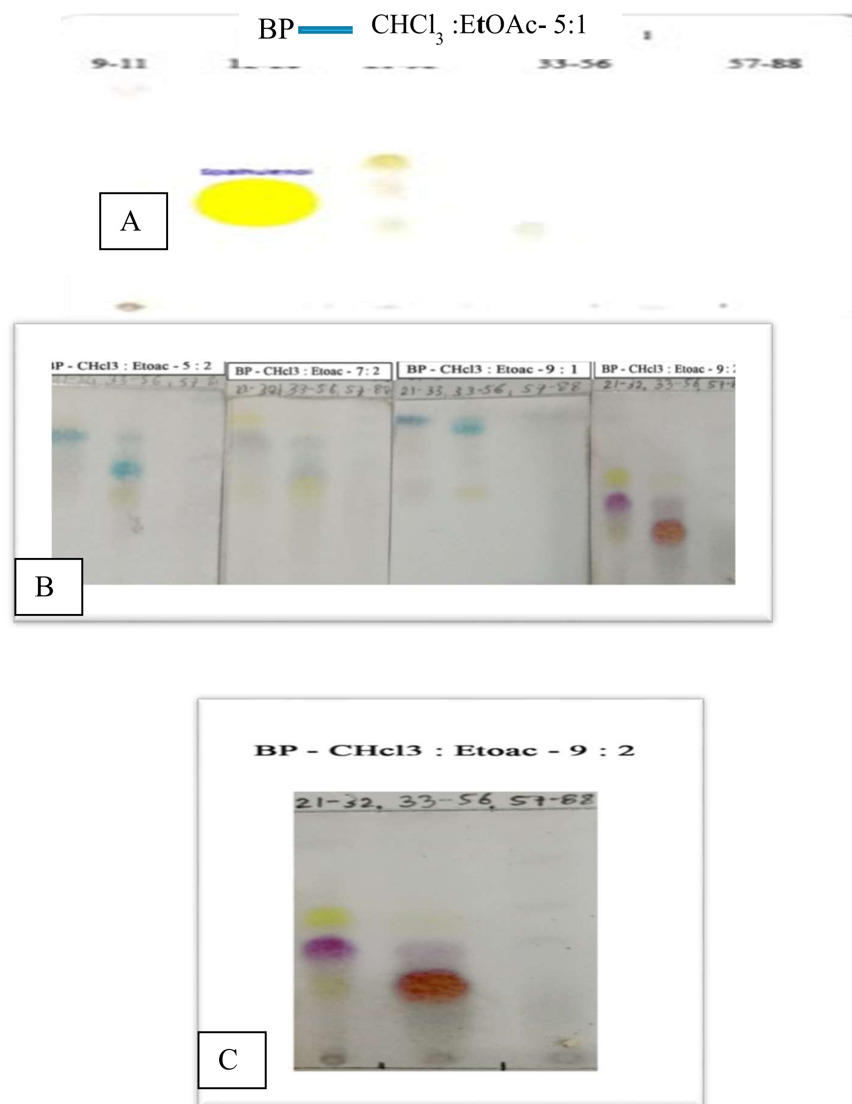


Figure 28. A (12-20), (21-2), (-56) No. test tubes are mixed with each other and dried, a single sport is detected after doing the TLC of 12 to 20 test tubes).

B, C (TLC) was done of the dried sample of (21-32), (33-56), No. test tube in different solvent ratio and ($CHCl_3$ EtOAc-9 : 2) give the best result.

Characterization of Compound

Chemical study of the compound showed the negative test for alkaloid and steroid but showed positive test for flavonoid with Salkowski and Liebermann-Burchard reagent. Presence of carbonyl function was indicate with 2,4-dinitrophenyl hydrazine reagent. The compound also showed the positive test for aromatic OH group along with a test for unsaturation.

^{1}H-NMR spectral analysis of the compound PN-1 run with 400 MHz instrument Bruker using CD_3OD as solvent and TMS as an internal standard was presumed to be identical with Spathulenol and Piperin known flavon type compound isolated previously from the plant *Piper nigrum.*

The structure of the compound is given below-

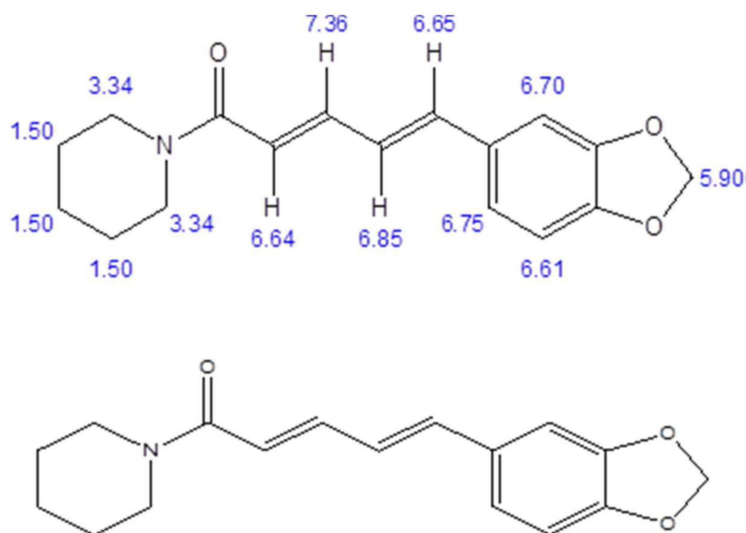


Figure 29. Piperine

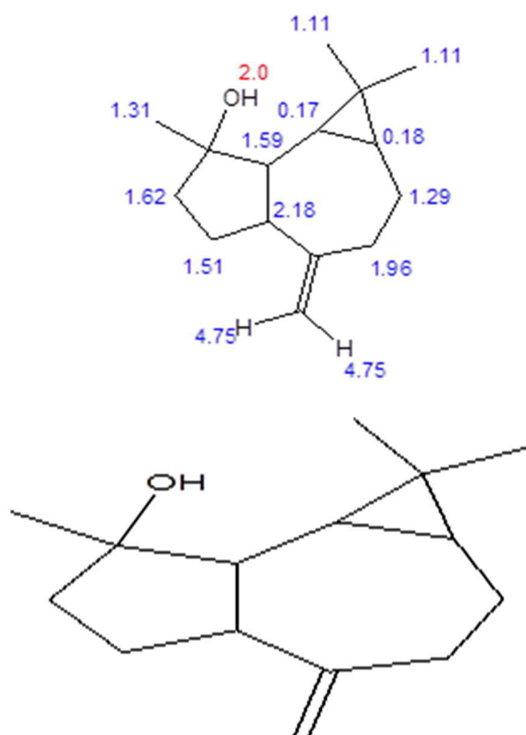


Figure 30. Spathulenol

From the chemdraw of proton NMR spectrum of the compound pinfaensin 2-CH_3 protons appeared as doublet at δ1.30 by coupling with 2-H germinal proton which gives a quarted very small peaks at δ3.85. Another Ar-Me group disposed at 1′ position gives singlet at δ2.35. Four protons of aromatic OH group give a broad singlet at δ5.0. An olefinic proton disposed at 4 position also gives a singlet at δ7.20. Four aromatic proton of this compound disposed at 4′, 6′ and 1″, 3″ positions with chemical shifts at δ6.53, 6.61 and 6.53, 6.13 respectively.

The compound PN-1, isolated from *Piper nigrum* also showed a br S for 2-CH_3 group instead of a doublet and its germinal proton 2-H did not identified as quartet due to low resolution of the spectrum but several small peak are appeared there. Aromatic methyl group disposed at 1′ position gives a singlet at δ3.20. Four protons of four aromatic OH groups showed a broad singlet with high intensity at δ5.0. The olefinic proton disposed at 5position showed a singlet at δ7.0. The rest

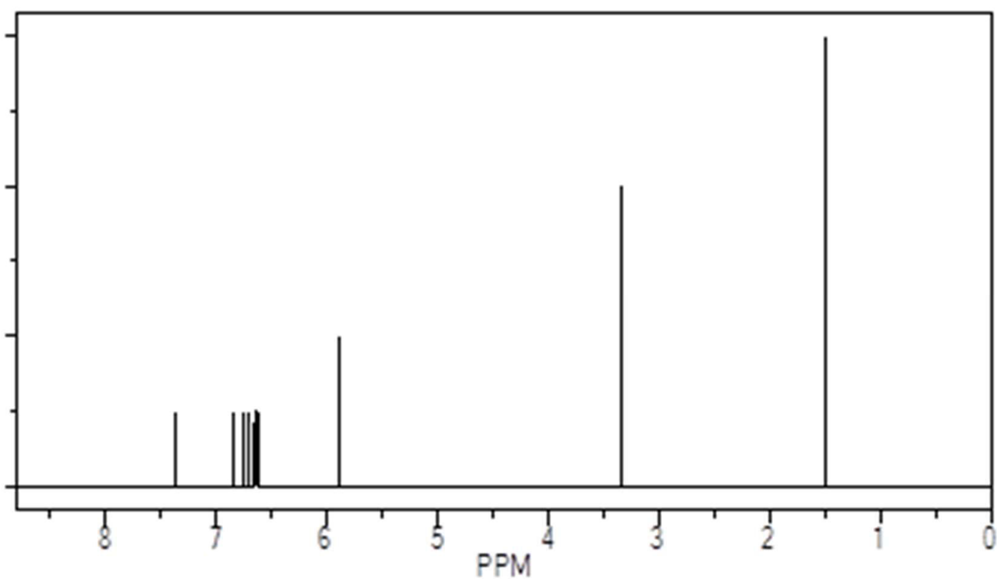
four aromatic protons at H-4′, H-6′ and H-1″, H-3″ showed their presence in the aromatic region at δ6.45, 6.60 and 6.45, 6.15 as singlets respectively.

From the above analysis and comparing the data (Table-5) of the isolated compound with the chemdraw spectrum, the compound PN-1 was found to be identical with the chemdraw structure (table-5). Hence it can be concluded that the isolated compound from the *P. nigrum* were piperine and Spathulenol.

Table 24. The compound PN-1 was found to be identical with the chemdraw structure.

Number of protons	Groups of protons	Chemical shift of protons	
		Chemdraw	Isolated compound
2	Me	1.38 (d)	1.30 (3H, d)
2	H	3.9	3.85
4	=H	7.20 (s)	7.00
1′	Ar Me	1.38 (s)	3.20 (s)
2′,3′ and 2″,4″	Ar OH	5.0 (bs)	4.90(bs)
4′	Ar H	6.53 (s)	6.45 (s)
6′	Ar H	6.61 (d)	6.60 (s)
1″	Ar H	6.53 (s)	6.45 (s)
3″	Ar H	6.13 (s)	6.15 (s)

^{1}H NMR of Piperine from CHEMDRAW



C-13 NMR of Piperine from CHEMDRAW

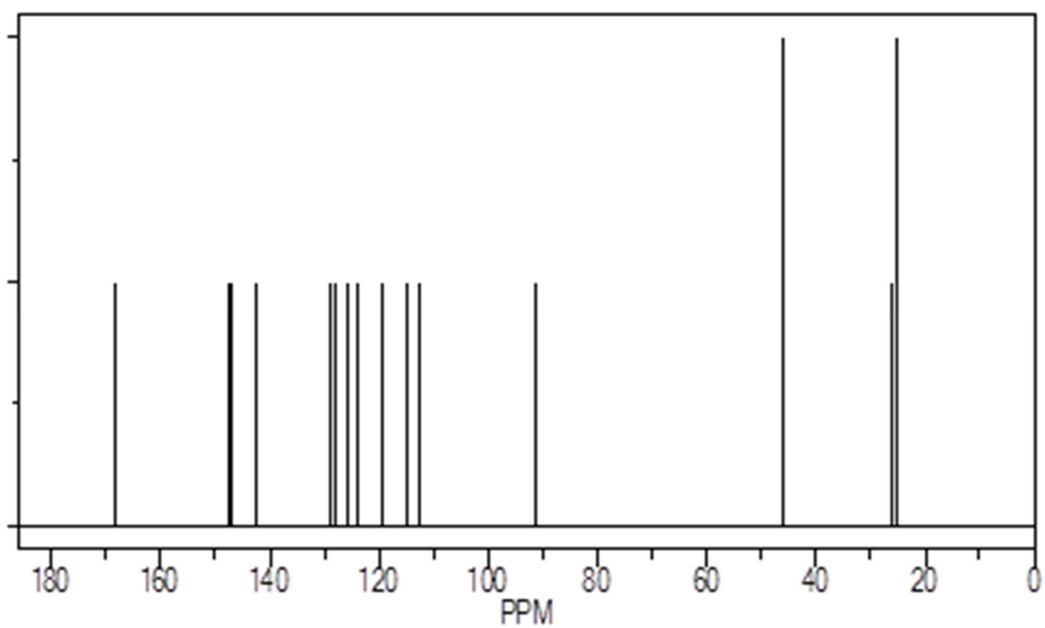
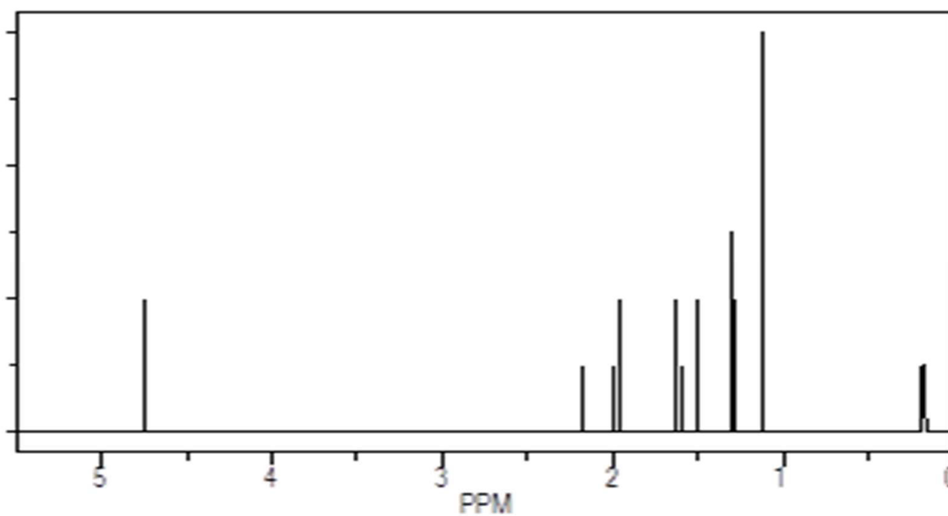


Figure 31. Structure of the compound Piperine

^{1}H NMR of Spathulenol from CHEMDRAW



C-13 NMR of Spathulenol from CHEMDRAW

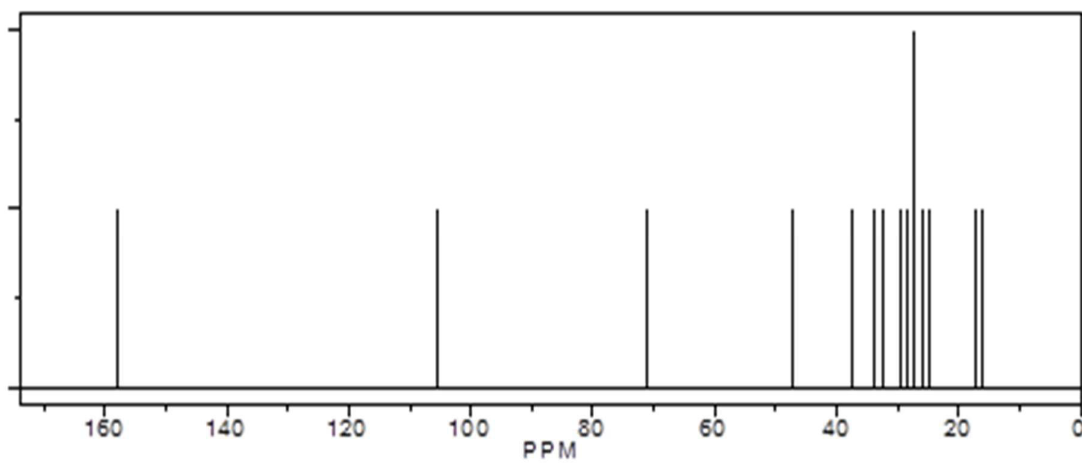


Figure 32. Structure of the compound Spathulenol

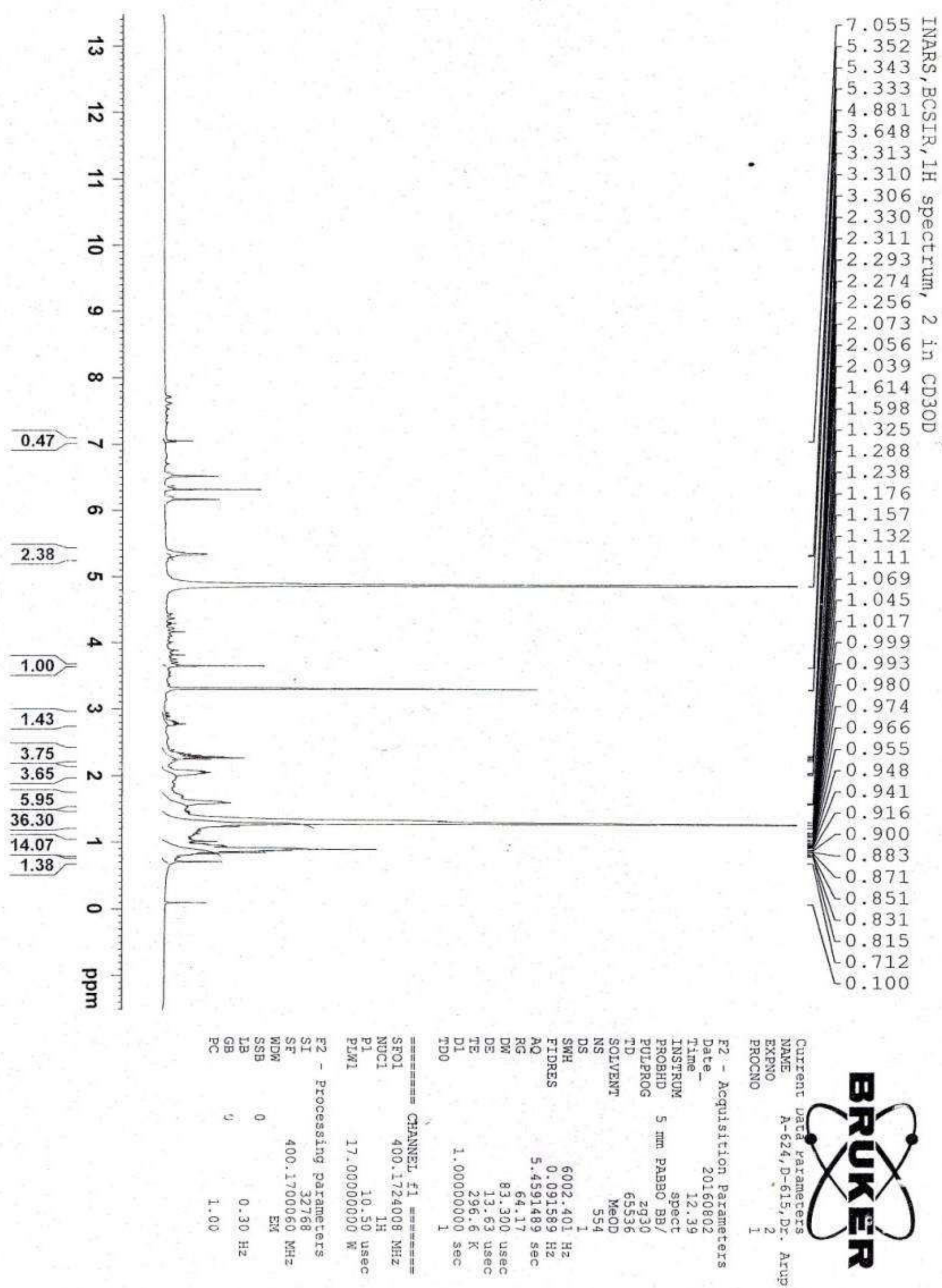


Figure 33. ^{1}H-NMR spectrum of the compound PG-1 in $CDCl_3$

Biofilm Production

Biofilm may be defined as aggregation of microorganisms in which cells are embeded within self-produced matrix of extra cellular polymeric substances such as DNA, protein and long chain polysaccharides and the cells are stick to each other on a surface.

Streptococcus mutans biofilm or dental plaque is a pale yellow biofilm that develops naturally on the tooth surface.

Protocol of biofilm production:

:

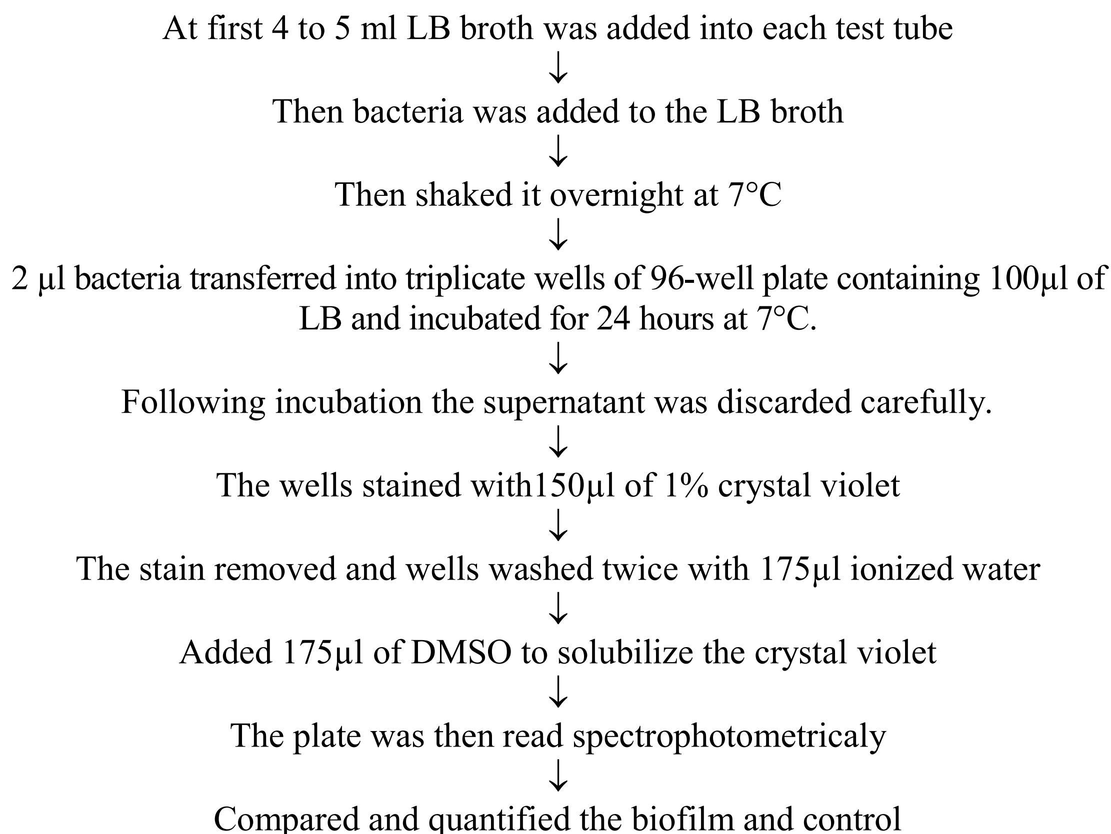


Figure 34. Flow chart for the Protocol of biofilm production

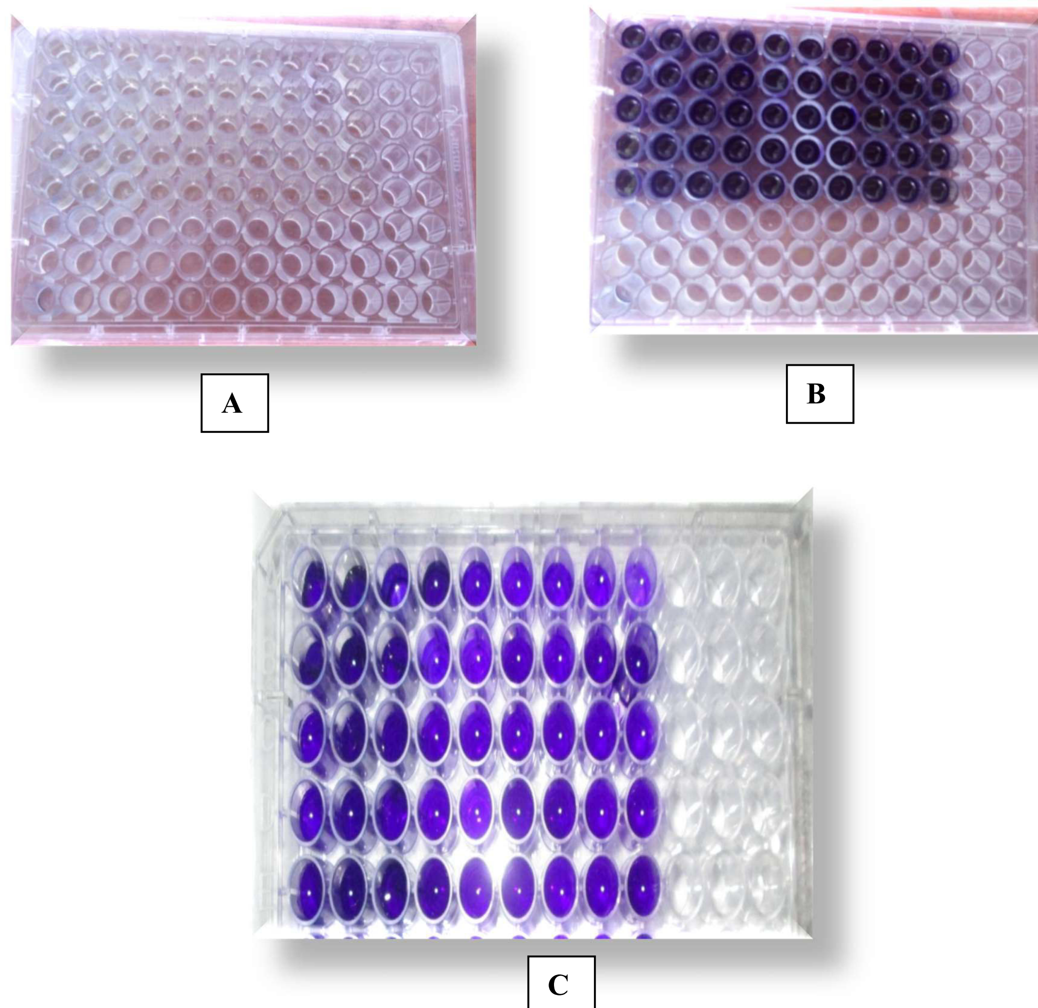


Figure 35. Process of biofilm formation

A (Adding LB broth on 96 well plate), B (Adding 150 µL crystal violet) and C (Stain washed with deionized water)

Two medicinal plants (*Cyndondactylon* and *Piper nigrum*) were selected based on their remarkable activities and Ethyl acetate showed best performance than any other solvent.

Table 25. Effect of 3,7,11,15-tretramethyl-hexadeca-2,4dien-1-01 of Cynodondactylon on the biofilm of *P. aeruginosa*, *E. coli* and *S. mutans*.

Organisms	No Treatment	25 UL	50 UL
P. aeruginosa	0.484	0.2	0.26
E. coli	1.42	1.229	1.01
S. mutans	4.12	1.95	1.27

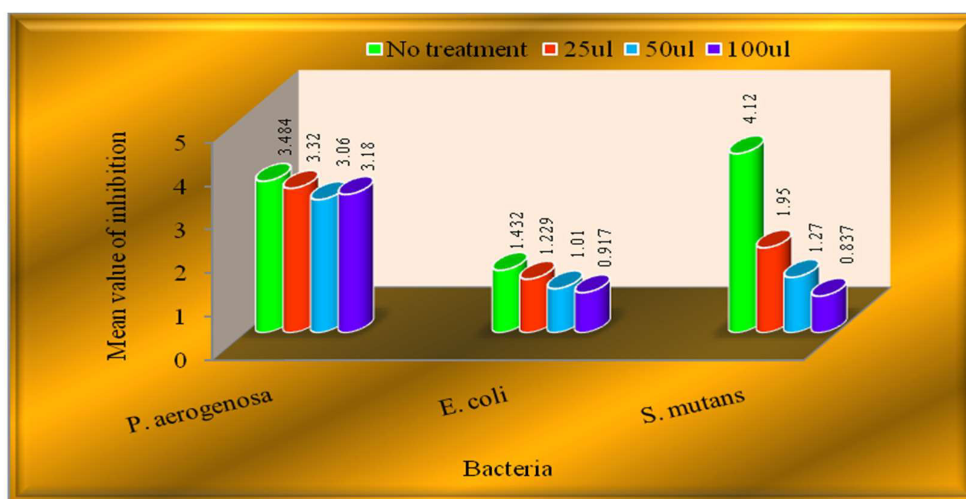


Figure 36. Mean value of inbhibition of *P. aeruginosa, E. coli* and *S. mutans.*

Table 26. Effect of , 7,11,15-tretramethyl- hexadeca-2 en 1-01 of *Cynodondactylon* on the biofilm of *P. aeruginosa, E. coli* and *S. mutans.*

Organisms	No Treatment	25 UL	50 UL	100 UL
P. aeruginosa	0.484	0.127	2.625	2.765
E. coli	1.42	1.02	0.901	0.756
S. mutans	4.12	1.87	1.2	0.816

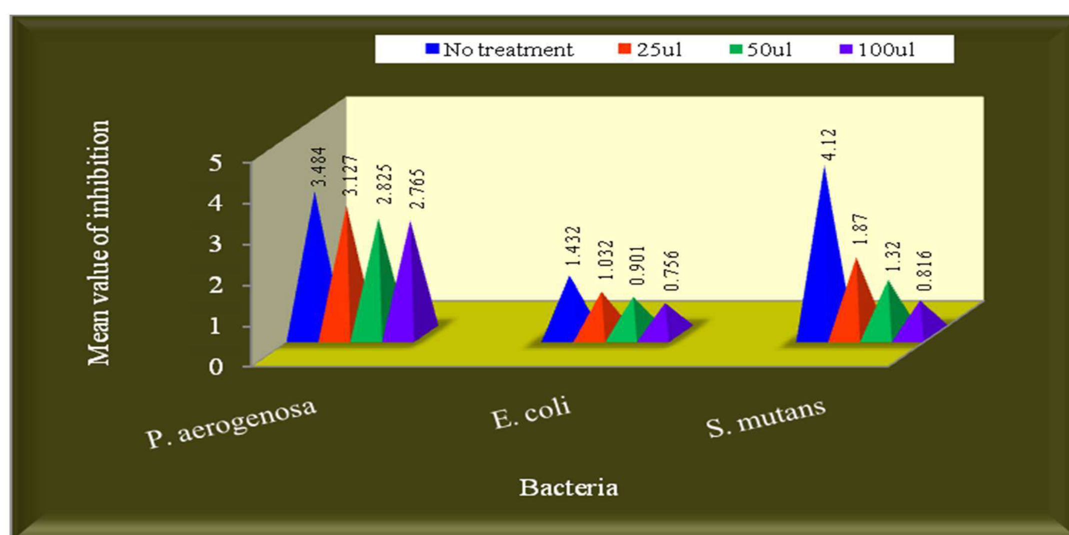


Figure 37. Mean value of inhibition of *P. aerginosa, E. coli* and *S. mutans*.

Table 27. Effect of Stigmasterol of *Cynodondactylon* on the biofilm of *P. aeruginosa, E. coli* and *S. mutans.*

Organisms	No Treatment	25 UL	50 UL	100 UL
P. aeruginosa	0.484	0.01	2.45	2.65
E. coli	1.42	1.2	1.12	1.146
S. mutans	4.12	0.45	2.74	0.01

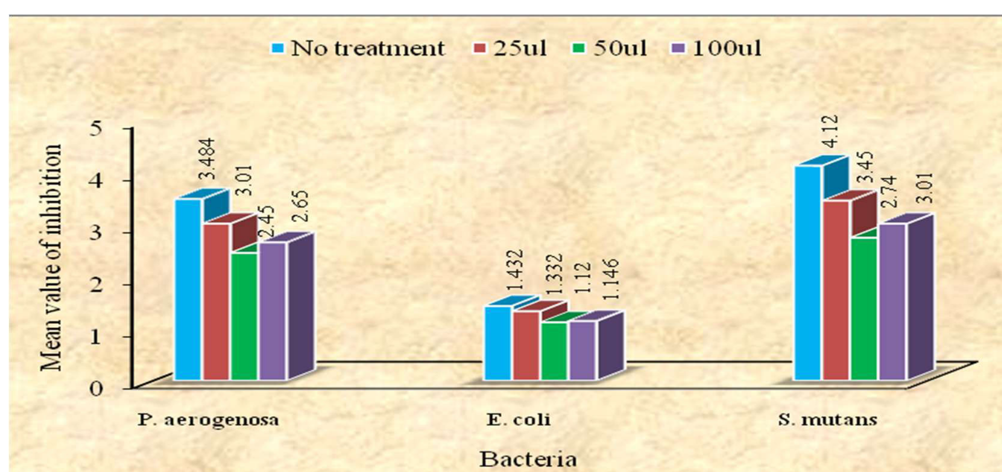


Figure 38. Mean value of inbhibition of *P. aeruginosa, E. coli* and *S. mutans.*

Table 28. Effect of Spathulenol of *Piper nigrum* on the biofilm of *P. aeruginosa, E. coli* and *S. mutans.*

Organisms	No treatment	25ul	50ul	100ul
P. aeruginosa	**0.484**	**0.21**	**2.85**	**3.101**
E. coli	**1.42**	**1.21**	**0.77**	**0.921**
S. mutans	**4.12**	**2.54**	**1.47**	**1.727**

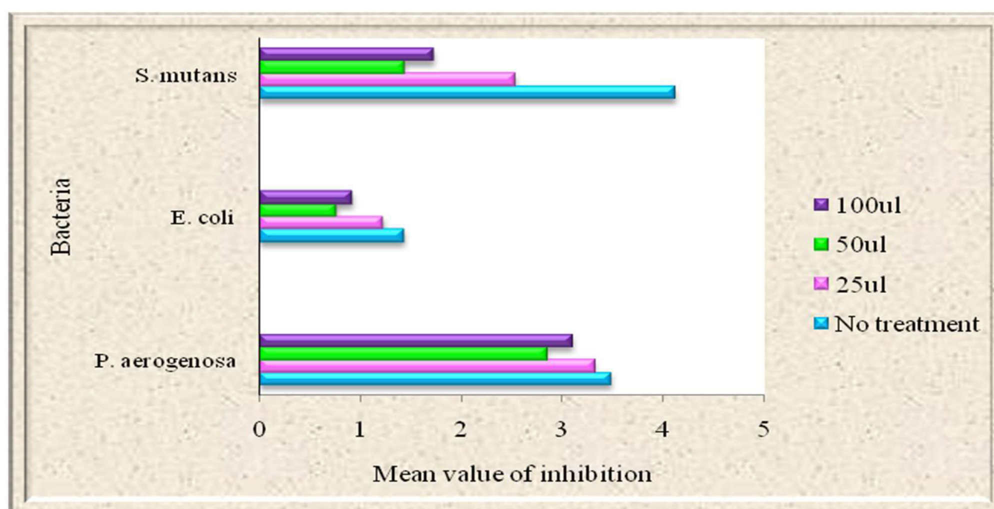


Figure 39. Mean value of inbhibition of *P. aeruoginosa, E. coli* and *S. mutans*

Table 29. Effect of Piperine of *Piper nigrum* on the biofilm of *P. aeruginosa, E. coli* and *S. mutans.*

Organisms	No treatment	25ul	50ul	100ul
P. aeruginosa	**0.484**	**0.084**	**2.256**	**2.495**
E. coli	**1.42**	**1.278**	**1.06**	**1.21**
S. mutans	**4.12**	**0.416**	**2.4**	**2.651**

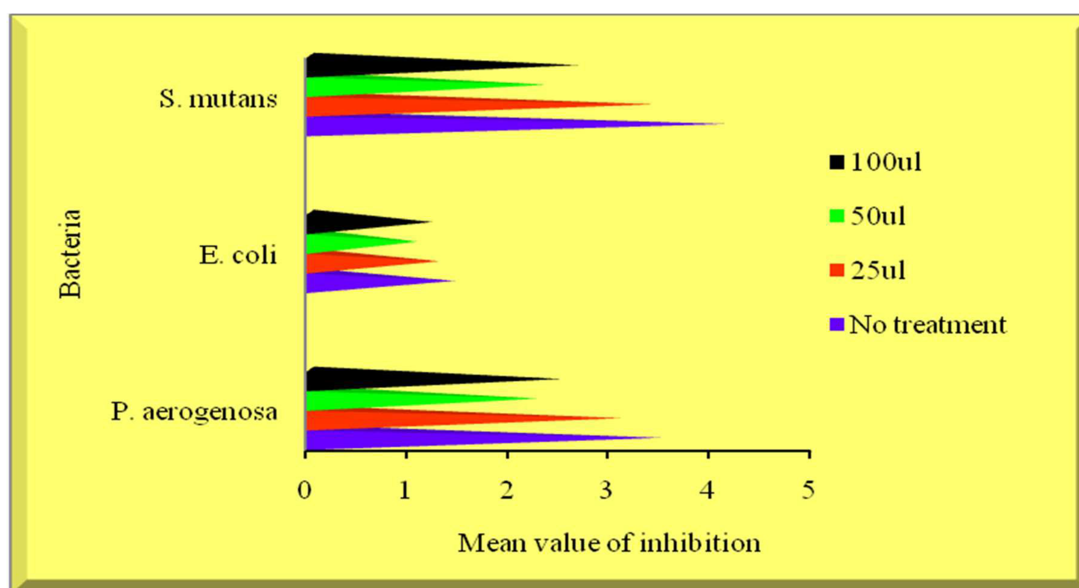


Figure 40. Mean value of inbhibition of *P. aeruginosa, E. coli* and *S. mutans*.

Cyndondactylon: Results indicated that compound, 7,11,15-Tretramethyl-hexadeca-2-en-1-ol (phytol derivatives) showed highest antimicrobial activities for the growth of inhibition on the biofilm of *S. mutans* among all the compounds of *Cyndondactylon*.

Table 30. Effect of growth of inhibition different compound of *Cynodondactylon* on the biofilm of *P. aeruginosa, E. coli* and *S. mutans*

Compound	Mean value of different kinds of bacteria											
	P. aeruginosa				*E. coli*				*S. mutans*			
	No treat	25ul	50ul	100ul	No treat	25ul	50ul	100ul	No treat	25ul	50ul	100ul
T_1	.484 ±0.21	.2 ±0.19	.26 ±0.14	.18 ±0.21	1.42 ±0.12	1.229 ±0.11	1.01 ±0.08	0.917 ±0.09	4.12 ±0.41	1.95 ±0.08	1.27 ±0.08	0.87 ±0.06
T_2	.484 ±0.21	.127 ±0.1	2.825 ±0.08	2.765 ±0.1	1.42 ±0.12	1.02 ±0.09	0.901 ±0.0	0.756 ±0.11	4.12 ±0.41	1.87 ±0.09	1.2 ±0.12	0.816 ±0.04
T	.484 ±0.21	.01 ±0.16	2.45 ±0.11	2.65 ±0.17	1.42 ±0.12	1.2 ±0.10	1.12 ±0.04	1.146 ±0.08	4.12 ±0.41	.45 ±0.06	2.74 ±0.16	.01 ±0.2

No treat = No treatment; T1 = ,7,11,15tretramethyl hexadeca-2-4 dien-1-01; T2 = ,7,11,15 tretramethyl hexadeca-2-en-1-01;T = Stigmasterol

Piper nigrum: Results indicated that compound Piperine showed highest antimicrobial activities for the growth of inhibition on the biofilm of *S. mutans* among all the compounds of *Piper nigrum*.

Among all the compounds of these two plant extracts only ,7,11,15 tretramethyl-hexadeca-2-en-1-01 (Compounds of cyndondactylon) were highly significance of targeted bacterial species.

Table 31. Effect of growth of inhibition of different compound of *Piper nigrum* on the biofilm of *Pseudomonas aeruginosa, Escherichia coli* and *Streptococcus mutans* bacteria.

Com	Mean value of different kinds of bacteria								
	P. aeruginosa			*E. coli*			*S. mutans*		
	25ul	50ul	100ul	25ul	50ul	100ul	25ul	50ul	100ul
T1	**.21 ±0.18**	**2.85 ±0.2**	**.101 ±0.19**	**1.21 ±0.11**	**0.77 ±0.06**	**0.921 ±0.07**	**2.54 ±0.2**	**1.47 ±0.12**	**1.727 ±0.14**
T2	**.084 ±0.2**	**2.256 ±0.11**	**2.495 ±0.16**	**1.278 ±0.06**	**1.06 ±0.04**	**1.21 ±0.08**	**.416 ±0.2**	**2.4 ±0.16**	**2.651 ±0.2**

Com = Compound; No treat = No treatment

T1 = Piperine; T2 = Spathulenol

The present results revealed that three separate compounds were recovered from the ethyl acetate and methanol extracts of *Cyndon dactylon*. The isolated compounds were 3,7,11,15 tetramethyl hexadeca 2-4 di en 1-01, 3,7,11,15 tetramethyl hexadeca 2 en 1-01 from phytol derivatives and Stigmasterol. Among three compounds 3,7,11,15 tetramethyl hexadeca 2 en 1-01 from phytol derivatives showed highest antimicrobial activities. On the other hand two major compounds were isolated from *P. nigrum.* The compounds were Piperine and Spathulenol. The present results indicated that piperine showed highest antimicrobial activities.

In case of some oral diseases, bacteria resistance was increased to the antibiotics where the adverse effects of some antibacterial agents were currently found out in dentistry. It is necessary to explore the alternative prevention and treatment options that are safe, effective and economical. Hence, the research for alternative measures continues for natural phytochemicals isolated from plants. The phytochemicals of the plant extracts can inhibit the oral pathogenic growth that mitigates the biofilms and dental plaque developments.

Palombo (2011) studied dental caries and periodontal diseases as the major health complexities among the oral infectious diseases. The acidogenic and aciduric gram-positive bacteria (mutans streptococci, lactobacilli and actinomycetes) are involved in the development of dental caries. Palombo and Semple (2002) studied ethanolic extracts from Australian medicinal plants showed antibacterial activity against gram positive bacteria of *Staphylococcus aureus* and *Enterococcus faecalis* to investigate the inhibition abilities of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). The leave extracts from *Eremophila alternifolia* (Myoporaceae) and *Acacia kempeana* (*Mimosaceae*) exhibited the inhibition activity against MRSA and VRE respectively. Leave extracts from *Amyema quandong* (Loranthaceae) and *Eremophila duttonii* (Myoporaceae) showed the activity against both types of bacteria. Where stem

extracts from *Lepidosperma viscidum* (Cyperaceae) were found to show effective against MRSA and exhibited VRE incomplete inhibition. All active potential extracts were estimated by time-kill assay procedures. Most of the extracts was investigated to show the bactericidal effects and decreased the viable cell numbers by 4–6 logs within four hours whereas the leave extracts of *Acacia kempeana* and stem extracts of *Lepidosperma viscidum* showed bacteriostatic activity against VRE. The extracts from *Musa* sp. exhibited the moderate antibacterial potentialities with the zones of inhibition that ranged from 8.0 to 18.6 mm (Karuppiah and Mustaffa 2013). The extracts from the species of *Musa paradisiaca* showed the highest effectivity against *E. coli*, *P. aeruginosa* and *Citrobacter* sp. The minimum inhibitory concentrations were found within 15.63-250 µg/mL values where the minimum bactericidal concentrations were recorded within the ranges of 31.25- 250 µg/mL. Dwivedi and Singh (2016) studied to find out the effectivity of embelin and piperine on the biofilm formation of *Streptococcus mutans.* Total 30 isolates were identified as *S. mutans* and screened for the formation of biofilm by the microtiter plate method. The strongest biofilm producer (SM03) was used to identify both minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC).

This concentration was subsequently used against the strong biofilm producing isolates at optical density (OD) of $A_{492} < 0.5$. Among which 30 isolates were screened for biofilm formation where 18, 9 and 3 isolates exhibited strong, moderate and poor biofilm formations respectively. The MIC for embelin was recorded 0.55 ± 0.02 showing the strongest biofilm producer (SM03), whereas piperine exhibited 0.33 ± 0.02. The MBIC for embelin was found as 0.0620 ± 0.03, whereas that of piperine was reported as 0.0407 ± 0.03, which was lower than embelin. At $OD_{492} < 0.5$, the MBIC of both compounds significantly inhibited biofilm formation of all 18 strong biofilm-forming isolates. The results of this study demonstrate a significant antibiofilm effect of the natural compounds

embelin and piperine, which can contribute towards the development of a database for novel drug candidates for treating oral infections caused by *S. mutans*.

Nallathambi and Bhargavan (2019) recorded that *Cynodon dactylon* is a common grass in Tamil Nadu, India. Its health and nutritional benefits are properly documented but there is very little knowledge about its bioactive components. The bioactive compounds were determined existing in the aqueous extracts of *Cynodon dactylon* by GC/MS tool. Hot aqueous extract of *C. dactylon* was accurately analyzed for the isolation of these bioactive compounds by the application of gas chromatograph of JEOL GC MATE II and mass spectrometer of quadruple double focusing mass analyzer. Eight bioactive compounds were isolated from hot aqueous extract of *C. dactylon* by GC-MS analysis. The compounds were recorded as Alanine, 9,12-Octadecadienoic acid (ZZ), n-Hexadecanoic acid, Oleic acid, Oxiraneoctanoic acid, 3-octly-, methyl ester, trans-, Phytol, Coumarine, 3-(2,4 - dinitrophenyl), 2-Cyclohexen-3,6-diol-1one, 2-tetradecanoyl.

Sharma and Singh (2017) studied the hidden specific antimicrobial property of *Cynodon dactylon* (L.) Pers., Cow dung powder, leaves of *Achyranthes aspera*, Wheat and Rice plant extracts against pathogenic strain isolated from dental plaque of caries active and caries free mouth from total 500 subjects. They examined and compared the effect of extracts from different selected sources. Extraction process was completed by using solvent like ethanol, chloroform, methanol and water. The efficiency of extracts was studied and determined by applying different extract concentration onto the cultured bacteria strain using the disc diffusion method. *Streptococcus sanguis* and *Streptococcus mitis* were the most susceptible bacteria to all plant extracts as compared to *S. mutans* and *Enterococci*. The extracts showed significant activity against the investigated dental plaque strains, which is promising they reported. The present investigation agrees with all above findings.

SUMMARY AND CONCLUSION

The present study revealed that after proper preparation, extraction, isolation in different solvent systems and characterization of the structure determination of the two medicinal plants such as *Cyndon dactylon* and *Piper nigram*. Three compounds were found from *C. dactylon*: i) 3,7,11,15 tetramethyl hexa-deca 2-4dien-1-01, (Phytol derivatives), ii) 3,7,11,15 tetramethyl hexa-deca 2-en-1-01 (Phytol derivatives) and iii) Stigmasterole (Sterole derivatives). Two compounds were recovered from *P. nigrum.* The compounds are i) Spathulenol and ii) Piperine. These five compounds showed the highest antimicrobial activities against *Streptococcus mutans* biofilm or dental plaque. Nallathambi (2019) recorded the existence of bioactive compounds in the moist extract of *C. dactylon* by using the gas chromatograph and mass spectrometer of quadruple double focusing mass analyzer and detected eight compounds which were-Alanine, 9,12-octadecadienoic acid, n-Hexadecanoic acid, oleic acid, 3-octyl- methyl-ester trans, phytol derivatives and coumarine, 3-(2-4-dinitrophenyl) etc. The compounds are of phytol derivates and are used as anticancer, anti inflammatory, antioxidant and diuretic properties. The present study reported that three specific compounds were reported from *C. dactylon* and two specific compounds from *P. nigrum.* The antimicrobial activity for the growth of inhibition was assessed. Three bacteria were used which were- *Pseudomonas aeuro ginosa*, *Escherichia coli and Streptococcus mutans*.

Both *P. aeuroginosa* and *E. coli* are acts as a control group and *S. mutans* acts as a sample group. At first, we have to detect the adhesion strength of *S. mutans* bacterial biofilm and plaque. The saliva sample were collected from 500 patients of Rajshahi Medical College Hospital (RMCH). Three different compounds of *C. dactylon* (3,7,11,15 tetramethyl-hexa-deca-2,4dien in 14-01, 3,7,11,15 tetramethyl

hexa-deca 2 dien 1-01 and stigma sterole) were applied in different concentrations on the biofilm of *P. aeurogenosa*, *E coli* and *S. mutans*. The adhesion strength is reduced from 3.484 to 1.180, 3.484 to 1.765 and 3.484 to 2.650 in case of the biofilm of *P. aeuroginosa*, 1.42 to .917, 1.42 to .756 and 1.42 to 1.116 in case of the biofilm of *E. coli* and 4.12 to 0.87, 4.12 to .716 and 4.12 to 1.01 in case of the biofilm of *S. mutans* bacteria. From this study it was showed that the compound 3,7,11,15 tetramethyl hexadeca-2en-1-01 (phytol derivatives) possess highest antimicrobial activities for the growth of inhibition on the biofilm of *S. mutans*.

Sharma and Singh (2017) found that *S. sanguis* and *S. mitis* were the most susceptible bacteria to all plant extracts as compared to *S. mutans* and *Enterococci*. The reported plant extract showed significant activity against the investigated microbial strain. The present study was similar to the study of Sharma and Singh (2017). It was also reported that the extracts of selected species contain a good potential antimicrobial component that can be utilized for the preparation of powerful and potent drugs for the treatment of dental problem and *Streptococcus* related health infections.

The second plant was black piper and after isolation two specific compounds were found (i) Spathulenol and (ii) Piperine. These compounds were applied on the biofilm of *P. aeuroginosa*, *E. coli* and *S. mutans* in different concentrations and the adhesion strength is reduced from 3.484 to 2.101 and 3.484 to 2.204 in the biofilm of *Pseudomonas aeuroginos*, 1.42 to 0.921 and 1.42 to 1.21 in the biofilm of *E. coli* and 4.12 to .987 and 4.12 to 1.201 in the biofilm of *Streptococcus mutans*. It was confirmed that compound piperine of the black piper showed highest antimicrobial activities for the growth of inhibition on the biofilm of *P. aeurogenosa*, *E. coli* and *S. mutans*. Hikal (2018) were used piperine and black pipper in different concentrations for antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtillis*, *Salmonella* sp and *E. coli* through agar well diffusion

method. He found Piperine and black piper that showed significant antibacterial activity with all tested gram positive bacteria with zone of inhibition ranges from 8.23 to 18 mm and 3.14 to 10.43 mm. He further mentioned that the result showed that the piperine was an excellent antibacterial agent with all tested bacteria. Similar study was conducted by Dwivedi (2014) and showed that the effect of natural compound embelin and piperine of black piper on the formation of biofilm property of *Streptococcus mutans*. A total 30 isolates were identified as a *S. mutans* and screened for the biofilm formation. The strongest biofilm producer (SM03) was used to identify both minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC) of the natural compounds are analyzed for the strongest biofilm producer isolated SM03 are presented and the significant differences in the MIC and BIC of the natural compounds were noted: the MIC of embelin was 0.55±0.02, where as that of piperine was 0.33±0.02 (MIC, Piperine<embelin). The MBIC of embelin was .0620±0.03 whereas that of piperine was 0.047±0.03. The results confirm that piperine is the most potent antimicrobial and antibiofilm compound. The authors also assure that the MBIC of embelin and piperine had reproducible biofilm inhibitory activity against each of the strong biolfilm producing isolates of *S. mutans*. The comparative effect of minimum biolfilm- inhibitions concentrations of embelin (0.0620±0.03) and piperine (0.47±0.03) shows the significant inhibtion of biofilm he noted. The MBIC and MIC values indicate that piperine has better ability to inhibit biofilm formation than embelin. The present study also showed similar results.

The present research was carried out in order to identity the effect of medical plants such *Cyndon dactylon* and *black pipper* isolated compounds used for the prevention of *S. mutans* biofilm isolated from dental plaque sample. Three kinds of bacteria were used for this study: *P. aeurogenosa*, *E coli* and *S. mutans*. *S. mutans* was the most susceptible bacteria to all compounds compared with *P. aeuroginosa* and *E. coli*.

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