KUVEMPU UNIVERSITY

"Isolation and Characterization of Antimicrobial Compounds from Medicinal Plants and Their Effect on Pathogens of Dental Caries"

Thesis submitted to Kuvempu University for the award of degree of

Doctor of Philosophy

in

Microbiology

Submitted by

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DECLARATION

I, **Mr. Venugopal T.M** hereby declare that the thesis entitled **"Isolation and Characterization of Antimicrobial Compounds from Medicinal Plants and Their Effect on Pathogens of Dental Caries"** embodies the results of bonafide research work carried out by me under the guidance of **Dr. N. Mallikarjun,** Associate Professor, Department of Microbiology, Sahyadri Science College (Autonomous), Shivamogga. I further declare that this or part thereof has not been the basis for the award of any other degree or diploma either in this university or any institution.

Date: 14/11/2016

Place: Shivamogga

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I, Certify that the thesis entitled **"Isolation and Characterization of Antimicrobial Compounds from Medicinal Plants and Their Effect on Pathogens of Dental Caries"** submitted to Kuvempu University for the award of the degree of Doctor of Philosophy in Microbiology by **Mr. Venugopal T.M** is the result of bonafide research work carried out by him under my guidance of at the Department of Microbiology, Sahyadri Science College (Autonomous), Shivamogga. Further, I certify that this or part thereof has not been the basis for the award of any other degree or diploma either in this university or any institution.

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LIST OF NOTATIONS AND ABBREVIATIONS

%	-	Percentage
µg	-	Microgram
µl	-	Microlitre
^{0}C	-	Degree centigrade
ABTS	-	2,2^I-Azinobs-(3-ethylbenzothiazoline-6-sulfonic acid
AEH	-	Aqueous extract of *H. mysoresence*
ATCC	-	American Type Culture Collection
ATP	-	Adenosine tri phosphate
BA	-	Blood agar
BHIA	-	Brain heart infusion agar
CEH	-	Chloroform extract of *H. mysoresence*
CFU	-	Colony forming unit
CLSI	-	Clinical and laboratory standard institute
Cm	-	Centimetre
CRP's	-	C-reactive proteins
DLVO	-	Derjaduin and landau abd Verwey and overbeek theory
DMSO	-	Dimethyl sulfoxide
DPPH	-	1,1-Diphenyl-2-picrylhydrazyl
EDTA	-	Ethylene diamine tetra acetic acid
EO	-	Essential oils
EPS	-	Extrapolysaccharide
et al	-	And others (co-authors)
FC	-	Folin ciocalteu's reagent
G	-	Gram (s)
Gbps	-	Glucan binging proteins
GC/MS	-	Gas chromatography mass spectroscopy
GTase	-	Glucosyl yransferase
HPLC	-	High pressure liquid chromatography
hr.	-	Hour (s)
IR	-	Infra red
kDa	-	Kilo Dalton
Kg	-	Kilogram

KOH	-	Potassium hydroxide
LC/MS	-	Liquid chromatography mass spectroscopy
LDL	-	Low density lipids
MBC	-	Minimum bactericidal concentration
MEH	-	Methanol extract of *H. mysoresence*
MFC	-	Minium fungicidal concentration
mg	-	Milligram
MHB	-	Muellor hinton broth
MIC	-	Minimum inhibitory concentration
Min	-	Minutes
MIS	-	Mitis salivarius agar
mM	-	Mili molar
MRS	-	De Mann rogosa and sharpe agar
MRVP	-	Methyl red voges prokeur
MS	-	Mutans streptococci
MSA	-	Mutans sanguis agar
MTCC	-	Microbial type Culture Collection
NCD's	-	Non communicable diseases
Nm	-	Nanometer
NMR	-	Nuclear magnetic resonance
OD	-	Optical density
PAC	-	Cell surface protein antigen
PCR	-	Polymerasee chain reaction
PEH	-	Petroleum ether extract of *H. mysoresence*
PRP's	-	Proline rich proteins
RNS	-	Reactive oxygen species
ROS	-	Reactive nitrogen species
Rpm	-	Revolution per minute
SD	-	Standard deviation
SDA	-	Saborauds dextrose agar
Sec	-	Second
TFC	-	Total flavonoid content
TLC	-	Thin layer chromatography

TPC	-	Total phenol content
UV	-	Ultra violet
VA	-	Vanderwaals attractive energy
VR	-	Pepulsive electrostatic energy
VT	-	Total interactive energy
w/v	-	Weight per volume
B	-	beta
A	-	alpha

1

Introduction

Natural product is considered as a backbone of traditional medicinal system for healing various ailment including infectious diseases of microbial origin. Eventhough the use of natural products dates back thousands of years ago, their utilization and initiation as a new lead to drug discovery is started from 19th century. Several studies suggested that, the natural compounds played a critical role in the modern drug discovery, particularly for infectious diseases and anticancer agents. Although the reputation of chemically synthesised products amplified due to its potentiality, rapid effect and manufacture cost, but their side effect and resistance (as antimicrobials) remained debatable. These aspects shaped the use of natural products by more than 80% of people residing in developed and underdeveloped countries. The use of natural compounds in various stages of clinical research in the development of new lead phytochemicals highlighted the active feasibility and importance of natural products.

Plants are considered as imperative source of novel pharmacologically active compounds and they are, derived directly or indirectly from natural source as a blockbuster compounds. Eventhough, in current situation the concern on synthetic chemical as a vector to find out and manufacture of new drug, the input of herbal based product to cure various diseases is still under consideration and it is also alarmingly high. WHO considered 11% of the 252 drugs are basic and essential for humans and they are fully derived from plant origin. It is no doubt that, herbal based drugs noticeably continues to be important source for new plant based drug discovery.

Many of the plant based medicines found, are directly used as new drug entities in treatment of various ailments, and many of them are used or acts as a template for new drug design. The plants products are also used in combination with synthetic drugs which forms basics of combinatorial chemistry. They are also used as a template for computational studies like, molecular modelling design and molecular docking. The application of these plant based medicine, with its safe utilization and

consideration, the usage of synthetic drugs are gradually declining due to their cost effectiveness and its adverse effects.

Most of the drugs obtained from plants are used in the treatment of several humankind diseases. Up to 50% of permitted drugs from last 30 years for the treatment of various ailments are from either directly and indirectly derived from natural product. These have been extensively studied in the area of cancer and in treatment of various infectious diseases (as antimalarial and antituberculosis drugs).

With respect to microbial infections, potential utilization of plant products as antimicrobial agents is increasing due to its safety efficacy and low cost effectiveness. Like other infectious diseases, oral diseases can also be managed by the utilization of plant products. India, being an example of rich plant diversity, natural products has been utilized by traditional peoples in diverse form of medicine in maintaining oral hygiene. Several studies suggested that, natural products have the potential to interfere with the pathophysiology of oral diseases such as, dental caries and periodontitis. These two diseases are considered as important and global oral health problems. Some of the studies have reported that, there is a link between poor oral health and chronic diseases. There is a evidence that, the presence of these two diseases may influence the general quality of life and linked to several systematic disease, such as bacterial endocarditis, diabetes, rheumatoid arthritis, osteoporosis and risk of pregnancy complication.

Eventhough, general advances in the overall health status of the people living in industrialized countries is increasing, the prevalence of dental caries in school aged children is up to 90% and the majority of adults are also affected. Earlier reports suggest that, dental caries is the most important global health problem because of its ubiquitousness among civilized populations.

Dental caries is a multifactorial disease characterized by the dissolution of dentine (hard tissues of mouth). This disease is caused by the interplay of three factors- host (teeth and saliva), microorganism (present in dental plaque) and substrate (in the form of diet). Thus, dental caries requires a susceptible host, cariogenic flora along with suitable substrate, which must be present in sufficient length of time.

In oral microbiology, oral streptococci have gained the considerable concern because, these groups of bacteria are involved in the initiation of dental caries by forming thin pellicle around the teeth surface. Eventhough, other group of organisms (*Lactobacilli sp., Actinomyces sp., Bifidobacteria, Fusobacterium and Candida sp.* etc.,) are also involved in the pathogenesis of dental caries, they have a role in the progression of dental caries rather than initiation.

The genus *Streptococci* is of great clinical interest due to their pathogenic potential because of its low virulence and shows variable reaction with Lancefield antigens. The most frequently isolated Streptococci species in association of dental caries are *Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguis, Streptococus mitis, Streptococcus downei* and *Streptococcus milleri* etc. Among these species, *Streptococcus mutans* is the major etiologic agent of this disease in man, followed by *Streptococcus sobrinus*. Several studies suggested that *S. mutans* utilizes dietary sugar (sucrose) to produce extracellular glucosyltransferase enzyme (GTase), which inturn synthesis insoluble glucan layer around the teeth. To this glucan layer, initial adherence of *S. mutans* takes place as a primary colonizer and gradually secondary colonizers or late colonizers usually binds to the salivary proteins or surface of *S. mutans* with the help of cell surface adhesions forming a complex biomatrix called dental plaque.

Dental plaque is a complex microbial consortium along with salivary proteins present around the hard surface of teeth, which form the barrier that prevents the diffusion of acids produced by the bacteria. These undiffused acids accumulate *insitu* and results in the demineralization of tooth surface. If this situation is uncontrolled or untreated, it may result in the changing its ecological niche (within plaque) by late colonizers like *Actinomyces species* and *Candida species* which makes the disease to attain vulnerable condition like soft tissue infection (periodontitis and other systemic diseases). Thus, *S. mutans,*glucosyltransferase and dental plaque are the main supporters for the initiation of caries, which continuously encourages the late colonizers providing a suitable environment for their growth and survivness of late colonizers within the oral cavity leading to the progression of dental caries. These three factors may become ideal targets for the control of dental caries.

Dental caries can be avoided by targeting with its virulence mechanisms like, inhibition of glucosyltransferase activity (enzyme inhibitors), inhibition of Streptococcal adherence to the tooth surface, by inhibiting coaggregation with other group of bacterial species and finally by inhibition of cell growth of *S. mutans* and other groups by antimicrobial agents. This third line of research in particular has attracted a great deal of attention. Effective use of antimicrobial agents against these cariogenic organisms may offer a better way to treat dental caries.

Several studies have been reported that penicillin, erythromycin and other antibiotics are administered for the prevention of dental caries. Due to their adverse effect like vomiting, diahhorea, teeth staining, suprainfection and hypersensitivity reactions has made limited use of these antibiotics by physicians. Even though, along with above said side effects, bacterial resistance among the oral pathogens is gaining much importance. Some investigations suggested that the misuse of antibiotics leading to gain antimicrobial resistance. This antimicrobial resistance has made several researchers to search alternative natural compounds to combat antimicrobial resistance. Several studies also suggest that, the natural products have the ability to reverse the antimicrobial resistance mechanism among the pathogens. This advantageous factor made several researchers to think on the utilization of herbal based components to treat dental caries.

Several studies have also focused on, antimicrobial susceptibility studies of dental caries isolates against antibiotics. Both resistant and susceptible organism have been found. As the consequence of side effects and bacterial resistant to antibiotics, it has been encouraged several researchers to carryout formulation based studies of plant products along with antibiotics. Several studies have explained about the synergistic activity of natural products in combination with antibiotic, where a single compound failed to show its complete potency. Development of these formulation based study may result in better and reduced usage of antibiotic, leading to a safe and effective utilization of natural product and also efficiently declines bacterial resistance among the pathogens.

Moreover, many studies have been reported about the utilization of plant product towards the treatment of dental caries. But, the investigation performed is limited to examination of crude aqueous or organic extracts. In most cases, the

investigators have basically wanted to validate the traditional medicinal use of the plant. There is a lacunae in the utilization of purified phytochemical in inhibiting the specific oral pathogens. A lot of research has to be carried in this field, to validate the potentiality of purified chemicals by number of clinical trials. Further, investigations are needed to study on safety and efficacy of these natural products, whether they can find their way by offering therapeutic benefits, such that they can help us to reduce the overall burden of oral diseases world wide. Thus with this brief introduction, the present work is designed with following objectives,

2. Objectives of the present study

I. Collection and extraction of bioactive compounds from traditional medicinal plants.
II. Purification and identification of potent purified compounds.
III. Collection and screening of clinical samples for dental caries isolates.
IV. Biochemical characterization and determination of antibiotic sensitivity pattern of dental caries isolates against standard antibiotics.
V. Bioassay of extracted compounds of different medicinal plants on selected dental caries isolates.
VI. Bioassay of crude and purified compounds of *H.mysoresence* on selected dental caries isolates.

3

Review of Literature

3. 1 Medicinal plants

Medicinal properties of the plants have been recorded in many pharmacopoeias and have a long history. The plant and their products have been utilized by many ancient peoples to treat various ailments. This system of medicine has transformed from one generation to successive generation in various parts of the world and played a pivotal role in the development of different traditional systems of medicines (Payne *et al.,* 1991). Plant and their constituents, which forms a major part of our food were conclusively studied for many years (Giovani *et al.,* 2014). These plants which form a therapeutic value have been studied and are utilized as a rich source of phytochemical agents and finds their way as an antimicrobial and antioxidants and also, they were used as powerful tools in drug discovery by several researchers around the globe. The plants products are extracted in the form of crude drugs, as they possess diverse medicinal properties, which may find a new lead for the exploration of new phytochemical drug with potential therapeutic benefits to mankind.

World health organization (WHO) has estimated roughly that, 80% of the global population relies on traditional medicaments as part of health care standards (Foster *et al.,* 2005). At present number of physicians prescribes drugs isolated from plant origin or chemically synthesized modified version of natural products (Payne *et al.,* 1991).

In the early nineteenth century, scientific methods turn out to be more developed and have become ideal one, due to this reason the practice of botanical therapy was exculpated as quackery. In 1960s, with considerations over the iatrogenic effects of traditional medicine and wish for extra self-reliance, curiosity in "natural health" has gained the interest in herbal medicine and has reconstituted the use of herbal medicine for treating various ailments and expanded herbal products. In 1992, by cognizance, of the rising use of herbal drugs and other non traditional remedies to cure various ailments. This improvements has led the endowment of office of

alternative medicine by the National Institute of Health (NIH) in USA. From this onwards, global herbal medication has received a boost and also encouragement shown by WHO for the usage of herbal products influenced the use of traditional medicine to satisfy needs were unmet by the modern system of medicine (Winslow and Kroll, 1998). As a consequence, plant and plant derived products have utilized with various success to heal and restrict diseases throughout the history. This development has resulted in a shift in universal trend from synthetic to herbal medicine, shortly 'Return to Nature' (Aok, *et al.,* 2008).

Traditional medicine is the synthesis of curative knowledge of generations of practicing physicians of indigenous medicine system. Herbal preparations comprise of plant based products, minerals and organic matter, etc. Herbal medicinal drugs constitute only these usual drug treatments, which mainly use medicinal plant preparations for a cure. The earliest recorded evidence of their use by Indian, Chinese, Egyptian, Greek, Roman and Syrian, texts dates again to about 5000 years. The classical Indian texts comprise Rigveda, Atharvaveda, Charak Samhita and Sushruta Samhita. The herbal medicaments have therefore shown the history that they have been derived from earlier knowledge of ancient civilizations and scientific heritage (Kamboj, 2000).

The herbal compounds have a long history of clinical use, due to its higher patient tolerance and acceptance. So far, 35,000 - 70,000 plant species have been segregated for their therapeutic uses. Plants peculiarly, those with ethanopharmacological uses are the principal sources of medicine for early drug discovery. Fabricant and Farnsworth (2001) stated that, 80 % of 122 plant derived drugs were connected to their unique ethnopharmacological purposes.

Current drug discovery mainly works on bioactivity guided fractionation which has given the way to discover many anticancer drugs like paclitaxel, camptothecin etc. In 1896, German Merck company introduced the drug morphine, which was first commercially pure natural product and aspirin as a semisynthetic drug. In 1899, the Bayer Company, introduced a natural compound salicin isolated from *Salix alba*. This resulted in the isolation of the early drugs such as cocaine, digitoxin, codeine, quinine and pilocarpine. Other than this, there are several plants

derived drugs such as Paclitaxel from *Taxus brevifolia* for breast, lung and ovarian melanoma, Artemisinin from *Artemisia annua* to fight against multidrug resistant malaria, Silymarin is used to treat liver disorders which were isolated from *Silybum marianum*.

3.1.1. Need for the study of plant products:

The ethnobotanical study of plant is important for modern day medicine, but its usefulness cannot be over emphasized if the methods are not standardized to obtain comparable and reproducible results. The potential use of plant derived chemicals as a source of new drug has been poorly explored. It is estimated that, among the 250,000–500,000 plant species, only a small percentage of plants have been phytochemically explored and an even smaller percentage has been utilized and studied for their pharmacological properties. In most of the situations, plant products, have been studied only for their pharmacological property and preliminary screening has been investigated (Hamburger and Hostettman, 1991), but little work has been undergone in treatment of particular infectious diseases of microbial origin.

It is known that plants are having the capability of synthesizing two types of metabolites, primary and secondary metabolites. Primary metabolites (Carbohydrates, lipids and proteins) are the metabolites produced by the plant cell which involves direct role in growth and metabolism. Secondary metabolites are an overwhelming variety of low-molecular-weight organic compounds produced by plants as defense chemicals. These secondary metabolites are usually unique and complex in their structures and are not involved in any metabolic activities.

Secondary metabolites can have a remarkable effect to other plants, on microorganisms and animals because of their immediate availability in the environment. These secondary metabolites, apart from determining unique plant traits, such as: color and scent of flowers and fruits, characteristic flavor of spices, vegetables, they also complete the functioning of plants on organism, showing both biological and pharmacological activity of a plant. Therefore, medicinal properties of plants can be attributed to secondary metabolites (Hartmann, 2008). Plants are estimated to produce over 200,000 metabolites and the yield of these compounds is often low (≤ 1% dry weight) and depends greatly on the physiological and

developmental stage of the plant. Of these metabolites, only a small percentage has been studied for their biological activities.

At present, researchers are investigating the plant products which are having antimicrobial properties. It would be advantageous to standardize the methods of extraction and *in vitro* antimicrobial screening, so that the search for new biologically active plant products could be more systematic and interpretation of the results would be facilitated. Thousands of phytochemicals which are having inhibitory effects on all types of microorganisms *in vitro,* it should be subjected to *in vivo* screening to evaluate the efficacy in controlling the incidence of diseases in crop plants and humans. Efficient collaborations with pharmacologists, medical practionars, plant pathologists and microbiologists are crucial to see the complete development of an lead compound into an exploitable product.

3.1.2 Phytochemicals or Bioactive compounds

Phytochemical are defined as a large group of bioactive compounds hypothesized to reduce the risk of major chronic diseases (Liu, 2004). They naturally occur as non-nutritive chemicals and appear to work alone and in combination with vitamins and other nutrients in food to prevent, arrest or minimize disease. These phytochemicals are produced by plants to acquire resistance against bacterial, fungal and pesticidal pathogens and indirectly these phytochemicals are utilized for the treatment of various human disorders (Abo *et al.,* 1991; Nweze *et al.,* 2004).

Based on the nature and structure, they have been categorized into five main groups and further subdivided as shown in the figure 3.1

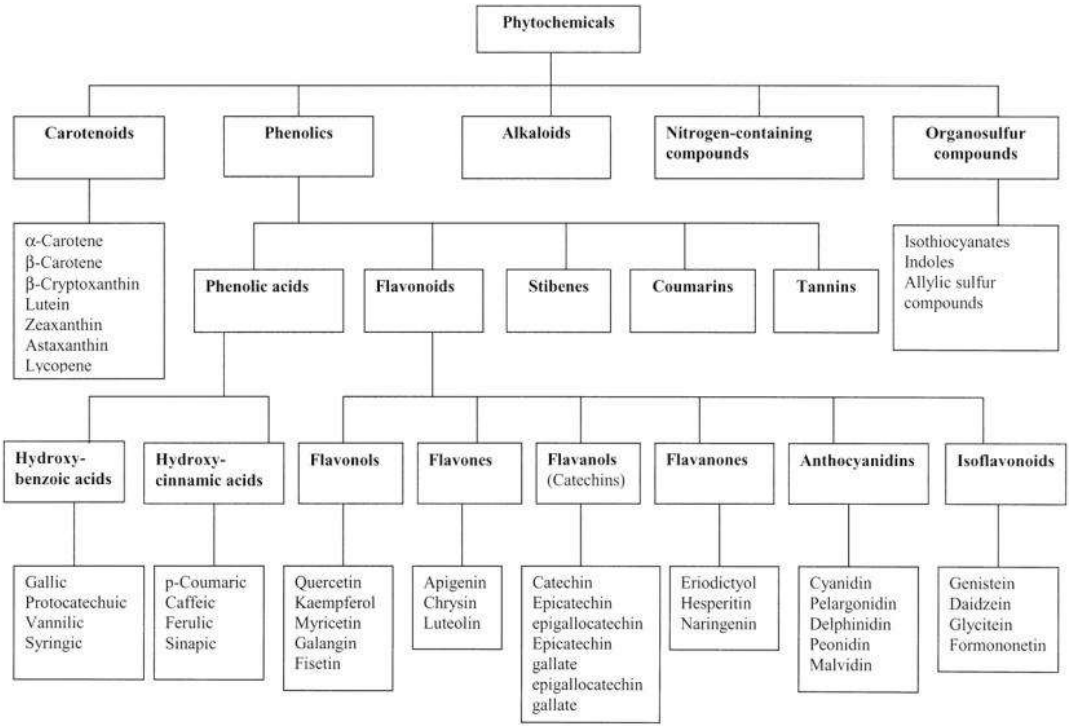


Figure 3.1: Classification of phytochemicals based on functional groups

3.1.3 Isolation, purification and characterization of the bioactive compound:

Phytochemicals are usually isolated by making use of solvents. There's a necessity to build up a standard method to extract, purify and characterize active compounds and eventually to test its efficacy to treat various ailments. Extraction of phytochemicals, is the first step in the utilization of these bioactive compounds by plant based pharma industries in the preparation of herbal drugs, dietary suppliments or as nutraceuticals. Plant samples are usually treated by milling, grinding and homogenization. Due to wide efficiency and applicability of solvent, solvent extraction procedure is employed to extract the bioactive compounds. The yield of extraction relies on the type of solvents used with varying polarities, sample to solvent ratio, extraction time and temperature, etc.

Extraction of bioactive compounds from plant can be carried out by both conventional and non conventional extraction methods. Non conventional methods consists of ultrasound, pulse field, enzyme digestion extrusion method, supercritical fluids extraction methods etc. Zulema *et al.* (2016) used ultra sound assisted extraction process for the isolation of 'stilibenes' from grape canes. Dong *et al.* (2016) isolated polysaccharides from Mulberry leaves, following the ultrasound

extraction method. Corralesa *et al.* (2008) recovered phytosterols from maize, isoflavonoids (genistein and daidzein), soybeans anthocyanins and grape by-product, using the pulse electric field. Young *et al.* 2016, extracted phenolic compounds such as, catechin, ferulic acid, p-coumaric acid and chlorgenic acid by enzymatic digestion method from *Hordeum vulgare*. Pan *et al.* (2003) and Chiremba *et al.* (2012) extracted phenolics from coffee, tea waste, bran, flour fractions of sorghum and maize by microwave assisted extraction techniques. Mroczek and Mazurek (2009) and Erdogan *et al.*, (2011), extracted lycorine and galanthamine from *Narcissus jonquilla* and gallocatechin (GCT), catechin, epicatechin gallate, caffeic acid, chlorogenic acid and myricetin and total phenolic contents were recovered from various parts of *Anatolia propolis* using pressurized liquid extraction proceedure. Saldana *et al.* (1999) and Khorassani and Taylor (2004) extracted pure alkaloids (caffeine, theobromine, and theophylline) from *Ilex paraguaryensis*, naringin (flavonoid) from citrus fruits and polyphenols and procyanidins from grape seeds using super critical fluid extraction process.

Other conventional extraction methods include maceration, infusion, decoction, digestion, percolation, cold and hot extraction methods (soxhalet extraction). Extraction with soxhalet method is most and widely used method for the isolation of bioactive compounds. This process includes repeated use of solvents by recyclization of large amount of single solvent. When compared to conventional methods, non conventional extraction methods are reliable methods. These non conventional methods make use of less chemical, energy efficiency machineries and pollution free equipments. Isolation and identification of plant compounds depend upon the type of solvent used in extraction procedure. The choice of the solvent depends upon the nature of the targeted compound to be isolated (Das *et al.,* 2010). Along with this, the other factors like affinity between solute and solvent, quantity of phytochemicals to be extracted, diversity of compounds to be extracted, ease of evaporation, rapid physiologic absorption of the extract and financial feasibility plays an important and critical role in the extraction procedure (Eloff *et al.,* 1998).

Solvents are selected for the extraction procedure based on the polarity index of the specific solvents. Usually solvents from non polar to polar solvents are used for the extraction of phytochemicals. The solvents like methanol, ethanol, acetone,

ethyl acetate, petroleum ether, chloroform, benzene and their combinations are used for extraction of different phytochemicals from the plant samples (Fuleki *et al.,* 1997; Jayaprakasha *et al.,* 2003; Ozkan *et al.,* 2004). Most of the identified phytochemicals are often obtained through ethanol or methanol as a choice of solvent (Serkedjieva and Manolova, 1992). In general, methanol is the choice of solvent for efficient extraction of low molecular weight polyphenols and other phytochemicals like flavonoids and terpenoids *etc.*. (Serkedjieva and Manolova, 1992). Literature reports suggest that ethanol is the best solvent to isolate polyphenols and is safe for human consumption (Brain and Turner 1975; Vanwyk and Wink 2004).

Polar solvents like methanol, ethanol or ethyl acetate were used for the extraction of hydrophilic compounds and non polar biological active compounds were isolated by using chloroform as a solvent (Gomez *et al.,* 1996; Baydar *et al.,* 2006; Kafka *et al.,* 2007; Harmala *et al.,* 1992). Tannins and other phenolics were extracted better by acetone rather than methanol. For the extraction of more lipophilic compounds, mixtures of dichloromethane/methanol in a ratio of 1:1 are used. For the extraction of anthocyanin rich phenolic groups, most commonly methanol or ethanol is used as a solvents, these solvents denatures the cell membrane and involves in the dissolution of anthocyanin compounds. For the extraction of phenolics, such as formic acid, acetic acid, citric acid, phosphoric acid and tartaric acid, low concentration of strong acids like trifluroacetic acid (3 to 5%) and hydrochloric acids (1%) are used for extraction (Jackman 1987; Revilla 1998; Nicoue 2007).

3.1.4. Phytochemical screening

After extraction, phytochemical screening assay were done, which helps us in preliminary identification of specific compound (s) present in the extract mixture or fraction. It is a simple, quick and inexpensive procedure , table 1 represents the different test performed for the detection of phytochemicals.

Sl No	Name of the phytochemical	Reference
1	Test for alkaloids • *Dragendroffs test* • *Mayers test,* • *Wagners test*	Erum etal., 2015
2	Test for flavonoids • *Shinodas test* • *$Fecl_3$ test* • *Alkaline reagent test*	Naima *et al., 2012*
3	Test for steroids and tepenes • *Salkowski test* • *Liebermann-Burchard test*	Devendra *et al.*, 2012
4	Test for cardiac glycosides • *Kellar killiani test*	Harborne, 1978
5	Test for carbohydrates • *Benedicts test,* • *biuret test*	Devendra *et al.*, 2012
6	Test for saponins	Harborne, 1993

Table 3.1: Different type of phytochemicals and their respective tests used for their detection

3.1.5. Purification of phytochemicals

The crude extract obtained, usually occurs as a combination of various types of bioactive compounds or phytochemicals. So it is very important and integral task to separate the compounds by adopting different chromatographic techniques. Phytochemicals with different polarities, different levels of chromatographic techniques are used like Paper chromatography, Thin layer chromatography (TLC), column chromatography and High performance liquid chromatography (HPLC). These chromatographic techniques are used widely because of convenience, availability of a variety of stationary phases for separation of phytochemicals. In TLC crude extracts are fractionated into different components. This technique simplifies the process of isolation and identification of bioactive compound (Sasidharan *et al.,* 2011). Another important purification technique followed after TLC is, the HPLC. It

is a versatile, robust, and widely used technique for the isolation of natural products (Cannell 1998). Currently, this technique is gaining popularity among various analytical techniques, as the main choice for fingerprinting (Fan *et al.*, 2006). In some instances, the active compound is present at minor component in the crude extract. Under these circumstances, rapid processing of multi component were carried out by following both analytical and preparative scale HPLC. Asghari *et al.* (2011); Deng, *et al.*, (2012); Nazzaro, *et al.*, (2012) isolated purified bioactive compounds from cinnamom husks, fruit wastes, chestnut and hazelnut shells by using HPLC proceedure.

There are other modern techniques in which combinations of the two techniques are involved and these techniques are employed for the detection of phytochemicals. They are diode array detector with mass spectrophotometer (Tsao and Deng 2004), Liquid chromatography and Gas chromatography coupled with mass spectrometry (LC/MS and GC/MS) (Cai *et al.*, 2002). These techniques are very advantageous, because they provide abundant information for structural elucidation of the compounds.

The combination of chromatographic techniques with mass spectrometry establishes the accurate and rapid identification of the secondary metabolites present in the plants. Jayapriya and Gricilda (2015) analyzed 13 bioactive molecules from *Justicia adhatoda (Linn.) G*. By using a GC / MS technique. Lakshmi kanth *et al.*, (2014) analyzed 31 bioactive compounds from methanolic extract of *Lactuca runcinata* by adopting GC/MS. Moldovan *et al.* (2014) analyzed 8 phenolic acids, four quercetin glycosides, and seven flavonol and flavone aglycones from five species of Mentha, cultivated in Romania by LC/MS technique. Anca *et al.*(2011) performed a comparative LC/MS analysis of salicyclic and salicin derivatives from three herbs namely *Salicis cortex, Populi gemmae* and *Ulmariae folium*. Phytochemicals in plant material appear to be multicomponent mixtures, their isolation and identification is a more cumbersome process and still creates problem. Thus the purification of bioactive compounds should be encompassed by a combination of several chromatographic techniques and various other purification techniques to isolate bioactive compounds.

3.1.6. Structural details of isolated compounds

The compounds which have been separated (purified) from several chromatographic techniques needs to be structurally characterized. This structural identification step involves several spectroscopic techniques like UV- visible spectroscopy, Infrared (IR), Nuclear Magnetic Resonance (NMR). These techniques provide a basic hints about the structural details like number of carbon and hydrogen atoms present in an isolated compound and also provides a possible data on the type of functional group attached.

Compounds from natural products are usually concerned with energy absorption spectra from three or four regions of electromagnetic spectra; they are Ultraviolet, Visible, Infrared (IR) and electron beam (Kemp, 1991a). The advantage of UV visible spectra is, it is possible to detect the nature of compound in the sample (Kemp 1991a). The functional groups can be determined using IR spectroscopic studies and this has proven to be an important tool for the identification of natural products (Eberhardt *et al.,* 2007). In IR, different chemical bond present in the compound may be detected by their vibrational frequencies. The bonds (C-C, C=C, C≡C, C-O, C=O, O-H, N-H etc.) in a natural products chemistry and in inorganic chemistry will have characteristic frequency as an absorption band in the infrared spectrum (Kemp, 1991b). The IR spectral analysis will serve as a molecular fingerprint for most of the phytocompounds. The detection of unknown compounds can be detected by comparing libraries of spectral data of known compounds.

NMR is a spectroscopic study, which is concerned with the magnetic properties of certain atomic nuclei of the compound. This type of spectroscopy facilitates to record the differences in the magnetic properties of various magnetic nuclei present in the compound and also to predict the position of these nuclei within the atom. This is also an important tool to know which type of atom present in neighboring groups and also measures how many protons are present in each of these environments (Kemp 1991c; Thitilertdecha 2010; Bouallagui *et al.*, 2012).

3.1.7. Antioxidant properties of bioactive compound

Antioxidants agents are natural plant products, having important protective role in health care system. These antioxidants play an important role in reducing the

risk of chronic diseases. These compounds possess diverse physical and chemical properties and they have the ability to trap free radicals which are implicated in several diseases. Free radicals and oxygen species are produced from a wide variety of sources, within the biological system. Antioxidants compounds like flavonoids, polyphenols and phenolic acids, scavenge free radicals and inhibit oxidative mechanism in proteins, lipids or DNA. It has been proposed that, antioxidant activities of natural compounds are mediated by the following mechanism (Cotelle, 2001).

I. Scavenging free radicals such as reactive oxygen species and reactive nitrogen species.

II. Suppression of Reactive oxygen species (ROS)/Reactive nitrojen species (RNS) formation by inhibiting enzymes or chelating trace

III. Regulating or protecting antioxidant defense.

Antioxidants, especially polyphenols and phenolic acids occur in plants with many structurally diversified functional groups. These compounds are difficult to study individually and also very difficult to separate. Moreover, study of total antioxidant nature of a complex sample is often more valuable because of the cooperative action of antioxidants. Therefore, it is desirable to standardize convenient screening methods for quick quantification of antioxidant property of natural products. For the quantification of antioxidant assay, a variety of assays such as, DPPH assay, Metal chelating assay, Hydrogen peroxide assay, Superoxide dismutase assay, oxygen radical antioxidant power, ferric reducing assay power etc. are used.

The FC reagent method was considered as a best method for determining total antioxidant power of the samples because its basic mechanism is as oxidation/reduction reaction, It is used to measure total phenolic content (Huang *et al*., 2005). In the early 80's, the search for natural antioxidants was given emphasis and several natural sources were examined. Many phytochemicals possessing antioxidant properties, have been isolated from extracts of different parts of plants, such as seeds, fruits, leaves, stems, and roots (Malecka, 2002; Schmidt *et al.*, 2003). It is reported that a purified bioactive compounds (phenolics) from plant wastes, with highest antioxidant activity having free radical inhibition activity (Shrikhande, 2000;

Muthuswamy *et al*., 2008; Tuchila *et al*., 2008). The free radical inhibition activity in the peels of oranges, lemons, pears as well as yellow and white flesh nectarines were found to contain twice the amount of total phenolic compounds than that of the pulp of these fruits (Gorinstein *et al*., 2001). Example peels of peach (Chang *et al*., 2000), pomegranate (Li *et al*., 2005), apple (Wolfe and Liu, 2003), grapes (Torres and Bobet, 2001), mango (Ajila *et al*., 2010) contain more antioxidant activity than the whole fruit. Other than peels even seeds of longans, avocados, jackfruits and tomato are reported to have higher antioxidant activity than that of the edible product (George *et al*., 2004; Soong and Barlow, 2004). Acerola fruit extract and citrus plants agro industrial waste were also evaluated for antioxidant activity (Shukla *et al*., 2009; Caetano *et al*., 2011). Antioxidant activity have also been reported from leaves of *Piper betle* (Islam *et al*., 2010).

3.1.8. Antioxidant and dental caries

Oral cavity is the formost entrance of the human body, where food, drinks and inhalents will passes. Thus this oral cavity is exposed to lot of carcinogens and is employes to form precancerous lesions which may lead to oral cancer, dental caries, oeriodontal diseases, leukoplakia, lichen planus, oral submucous fibrosis and burning mouth syndrome.

Since saliva is considered as a heterogeneous fluid rich in antioxidant compounds. It has been shown that, saliva includes many defensive mechanisms such as secretory IgA and protein– enzymatic defence system, histatin, lysosin and lactoferrin. On the contrary, nowadays, another salivary defence mechanism called as salivary antioxidant system (including uric acid, superoxide dismutase, catalase and glutathione perioxidase) is known. Uric acid is the major component of the salivary antioxidant system constituting 70% of the total antioxidant capacity. Recent development in the dentistry has hypothesized that salivary antioxidant system plays a vital role in controlling dental caries. Salivary peroxidase system which considered as a important salivary antioxidant system brings the control on oral bacteria causing dental caries. These salivary peroxidases catalyzes the SCN- ion (thiocyanite ion) to generate more stable OSCN- (hypocyanite) oxidation product. This stable product inhibits the growth and metabolism of many cariogenic bacteria resulting of controlling of dental caries (Abdulla *et al*., 2014).

Dietary substitute like plant products are known to play an important role in oral cancer and oral infectious diseases. These dietary substitutes plays an important role in the onset of dental caries and periodontal disease. This is the one area in dentistry is still unfocused and limited number of studies have undergone. The relation between antioxidant and oral disease need studies for confirmatory evidence addressing the safety issues (Bhuveneshwari, 2014).

During last 2 to 3 decades medicinal plants are utilized to treat various ailments, including numerous physiological disorders and several systematic infectious disease. Eventhough, they have been exploited to treat several diseases, only a few reports have been documented on the utility of medicinal plants and its therapeutic effect on specific diseases. Sadangi *et al.* (2005), reported the utilization of ten species of medicinal plants to treat mouth and ear diseases. It has been also reported that, the potential utilization of medicinal plants in the treatment various disorders caused by microbial origin. Several scientists are focussed their mind towards folk medicine in order to explore a new plant based antimicrobials against microbial infection. As the antimicrobial resistance has been recorded in numerous studies, to combat this resistance among the pathogens, it has been proven that the products from herbal origin are the best choice. These herbal based products, has the ability to reversal the antibiotic resistance of a drug resistant pathogens. These herbal products have been extensively studied, for their interference in developing antimicrobial resistance mechanism pathway. The utilization of plant products not only has proven positive results in the treatment of several systemic infectious diseases, but these natural phytochemicals present in them, offers an effective alternatives to antibiotics and also represents a potential loom in the prevention and therapeutic strategies for oral infections/diseases too (Singh *et al.*, 2007).

3.2. Oral health:

Oral health is considered as a mirror of the body health condition and often reflects the sign of many systemic diseases. Most of the oral diseases contribute to common risk factors with Non communicable diseases (NCDs) such as cardiovascular diseases, cancer, diabetes and some of the respiratory diseases. These risk factors include unhealthy diets (high consumption of sugar), tobacco and alcohol use. With

the global improvement in life expectancy, a life-course approach to oral health will become more important. Different ages in life, have different oral health needs, and the specific problems of older people, are often suffering from other diseases, are becoming more prevalent. Knowledge and awareness of the close associations between oral and general health are thus important for holistic health care. This bi-directional relationship between oral and general health, and its impact on an individual's health provides a fundamental concept for the assimilation of oral health care into general health care approaches.

3.2.1. Oral diseases:

Oral diseases are considered as a major health problem worldwide. The most common oral diseases are dental cavities, periodontal (gum) disease, oral cancer, oral infectious diseases, trauma from injuries, and hereditary lesions (Peterson *et al.,* 2005). Even though oral cancer and oral tissue lesions are the significant health concern, dental caries and periodontitis are the two diseases which are considered as most important global oral health problems (Peterson, 2003). Even though, concern and advancement of oral health status of the people living in industrialized countries have increased, the prevalence rate of dental caries in all the age groups is up to 90% (Peterson, *et al.,* 2005).

3.2.2. Association of oral diseases with other systemic diseases

Several reports suggest that there is a strong association between inflammation of the oral cavity and systemic inflammation, like cardiovascular disease, Diabetes and pregnancy outcome. The relationship between these two conditions may play a key role in understanding and managing the adverse effects on the multiple organ systems involved in the chronic diseases (figure 3.2).

3.2.2.1. *Cardiovascular Disease.*

Cardiovascular disease is categorized by accumulation of inflammatory plaque in the blood capillaries, resulting in thrombosis, which can lead to myocardial infarction. Atherosclerosis is a chronic inflammation, which causes dysfunction of endothelial cells, cause injuries to elastic and muscular arterial tissue. In early stages, the atherosclerotic lesions contain lymphocytes, neutrophils, and monocytes. These cells affect the lining of the vascular endothelial cells and helps in the oxidation of

low density lipoproteins (LDL). As this process proceeds the monocytes in the atherosclerotic lesions is getting induced to become macrophages and is ready to take up lipoproteins and becomes lipid filled foam cells. As the lesion further develops, there will be a degradation of vessel wall by proteolytic enzymes which results in wall ruptures. Hence, the thrombosis can occlude blood flow to the heart and brain and eventually lead to heart attack, infarction or stroke (Gurenlian *et al.*, 2006). The inflammatory nature of atherosclerosis made the utilization of inflammatory markers such as C-reactive protein (CRP), to know the relation between oral diseases with systematic disease. Several animal model studies have suggested that, clinically induced oral infection with *P. gingivalis* will increase the atheroma size and also elevates the blood CRP level (Paquette, 2004). On the other hand, several studies have shown that, treating periodontitis, will decreases the blood CRP level (Ridker *et al.*, 2000 ; Daiuto *et al.*, 2004).

3.2.2.2 *Diabetes Mellitus*.

Diabetes mellitus is another chronic disease associated with oral diseases, particularly with dental caries and periodontitis. Periodonditis is identified and considered as one of the major problems of diabetes (Nishimura *et al.*, 1998; Ryan *et al.*, 2003; Loe, 1993). When compared to people who are not suffering from periodontits, it is very difficult to maintain glycemic control in patients suffering from periodontitis with diabetes, when compared with the people without periodontitis with diabetes (Taylor *et al.*, 1996). Several studies suggest that, in diabetic patients there will be an increase level of TNF- α (A proinflammatory cytokine), which is considered as insulin resistance. This TNF – α plays a significance role and it is a primary cause of type 2 diabetes. Periodontitis, also been recognized as associated with increased levels of TNF- α which may result in killing cells that repairs damaged connective tissue or bone, leading to bone loss. The elevated production of TNF – α by periodontitis worsens the glycemic control and also intensify insulin resistance (Grossi and Genco, 1998; Salvi *et al.*, 1997; Lalla *et al.*, 2000).

3.2.2.3. Adverse pregnancy outcomes:

Adverse pregnancy outcomes like, preterm low-birth weight infants is associated with this dental caries and periodontitis. Chronic infection found in dental caries and periodontitis is able to stimulate the inflammatory process, all over the body. There will be an increased level of TNF – α, IL-1, IL-2 and elevated level of prostaglandins in placenta. These inflammatory chemicals are able to stimulate premature rupture of membranes of placenta, which results preterm labor complications and low birth weight in infants (Offenbacher *et al.,* 1996; Jeffcoat *et al.,* 2003 ; Scannapieco *et al.*, 2001).

Figure 3.2: Relation of oral diseases with other systematic disease, (plaque bacteria and their products gaining access to various sites of the body through circulatory systems and their effects in causing systematic diseases), (Gurenlian, 2007)

3.2.3. Dental caries:

Dental caries is a contagious multifactorial chronic disease, mediated by oral bacteria. This infectious disease is characterized by the slow dissolution of dental hard tissue (decalcification or demineralization) and progression of this disease may lead to

demineralization of the inorganic constituent and dissolution of organic constituents. If untreated, the disease progression speeds up, leading to a cavity formation and finally results in complete tooth loss (Nelio *et al.,* 2016). The oral cavity harbors large consortium of microflora which are responsible for causing and progression of dental caries. Selwitz *et al.* (2007) explained that, the individual who are susceptible for this disease will have this disease throughout their life time. The prevalence rate of this disease and its extent varies among population and also from individuals to individuals. In 20th century, dental caries was known as disease of economically developed countries. Over 95% of developed countries are affected as the population among those consumes a high amount of dietary sugars or carbohydrate and this has led the selective multiplication of cariogenic bacteria (Saeeda *et al.,* 2008).

The prevalence of this disease has been recorded in the history of men appearance on this earth. Several experimental and epidemiological studies have shown that, this disease is reliant on the microbes present on the plaque. It has reached epidemic proportion in modern times since a fine, consistent diet, rich in refined sugar has been consumed (Peter, 2006). The development of plaque on the surface and in between the teeth, serves as a regular irritation to the gingival. The development of dental caries requires the presence of the cariogenic bacteria, the substrate (teeth) sugar in the diet with considerable duration and frequency of exposure (figure 3.3)

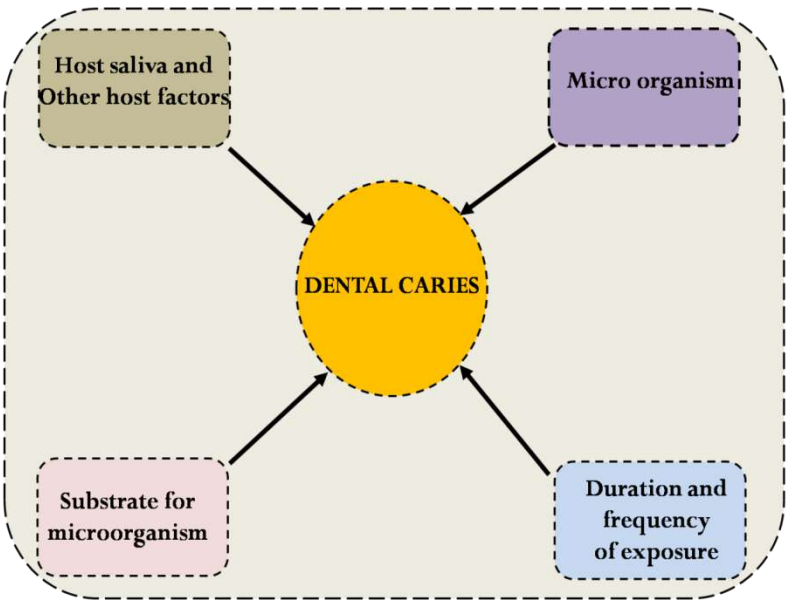


Figure 3.3: Basic requirements for the development of dental caries.

3.2.3.1 Causes of dental caries

3.2.3.1.1 Saliva

Nearly 700 to 800 ml of saliva is produced/day which serves as a cleaning effect on the teeth, and also aid in the digestion process. The quantity, composition, pH, viscosity and buffering capacity of the saliva plays a significant role in the dental caries. The function of saliva is flushing and neutralizing effects, generally referred as salivary clearance (Lagerlof and Olive, 1994). If the flow rate of the saliva is a fastest oral clearance is also faster and as well as faster buffering action (Birkhed and Heintze, 1989) and which not providing much time for the proliferation of plaque forming bacteria. Hyposecretion of saliva results in the reduced salivary flow rate and affects the oral clearance capacity, which results in the severe caries and inflammation of mucosal tissue (Daniels *et al.,* 1975; Van der Reijden *et al.,* 1996). The person having an impaired salivary flow rate often show high rates of caries incidence (Papas *et al.,* 1993; Spak *et al.,* 1994).

3.2.3.1.2. Substrate

Sugar or refined carbohydrates, particularly, disaccharide plays an important role in the etiology of dental caries. The fermentable carbohydrates get hydrolyzed by the action of salivary amylase to form mono saccharides. These disaccharides particularly sucrose affords a suitable substrate for the cariogenic bacteria. These bacteria utilize this sugar and lowers the pH of the saliva and results in the beginning of decalcification of the teeth. In addition, to sugar many factors affect the caries process, salivary flow rate, form of food, duration of exposure, eating sequence, buffering capacity of saliva and oral hygiene. Several studies have been confirmed that there is a straight relationship between dietary sugars and dental caries (Riva *et al.,* 2003).

3.2.3.1.3. Dental plaque

Dental plaque is a microbial biofilm, which is formed by the action of cariogenic organisms and dietary sugars on the surface of tooth. This dental plaque has its own significance in causing dental caries. Their variable structural entity makes them unique, dynamic and complex ecosystem where a large consortium of bacteria selectively adheres to the tooth surface forming a polymicrobial matrix.

Firstly this biofilm developes as a thin tenacious film on tooth surface, then grows by cumulative addition of aerobic and anaerobic organisms including filamentus organisms (Actinomyces sp.). This plaque is known to consists of 70% to 80% of bacteria among the solid matrix. About 1mm3 of plaque contains more than 106 with 300 different microorganism, each having unique roles in the causation and progression of the disease (Fourier *et al.,* 2000).

3.2.3.1.3.1. Mechanism of dental plaque formation

Dental plaque formation occurs in several stages, which is termed as a complex dynamic process, which includes an attachment and growth of different types of microorganism within the fluctuating environment of the oral cavity (Fourier *et al.,* 2000). Mechanism of plaque formation can be explained in following stages (figure 3.4)

a) Acquired pellicle formation

When the teeth are cleaned, salivary proteins and glycoproteins get adhered to the tooth surface, forming a thin tenacious layer called acquired pellicle. This is the first step in the plaque formation, which contains salivary proteins, glycoproteins, phosphoproteins and lipoproteins like statherin, amylase, PRP's (Proline rich proteins), glucosyltransferase and glucans. These salivary components play a critical role in the development of dental caries by giving a platform for bacterial adherence.

When these salivary component binds to the tooth surface, there will be conformational change occurs. These conformational changes lead to the development of new receptors for bacterial adherence or glucan adherence. The molecular composition and physicochemical properties of this pellicle are critical in determining the pattern of microbial colonization (Marsh and Bradshaw, 1995).

b) Transport of Microorganism and reversible attachment

Microorganisms present in the oral cavity are passively transported by the saliva at the site of the tooth surface and some of the organisms possesses locomotary organs which are located at subgingival sites.

When the bacterial cell comes in contact with acquired pellicle on the tooth surface, there will be development of weak physicochemical forces. Negatively

charged bacteria cell and proteins present in the pellicle get attracted to one another which can be explained by the DLVO theory (Derjaguin and Landau and the Verwey and Overbeek theory). This theory is used to explain the interaction between the inert particle and the substratum. The theory states that the total interactive energy (V_T) of two smooth particles is the sum of the Vander waals attractive energy (V_A) and the repulsive electrostatic energy (V_R). The components in aqueous solution can acquire a charge due to preferential adsorption of ions from the solution or ionization of certain groups attached to a particle or surface. The charge in a surface of a solution is having a balanced number of counter ion, the size of this electrical double layer is inversely proportional to the ionic strength of the environment. As a particle approaches a surface, it experience a weak Vander Waals attraction induced by the fluctuating dipoles within the molecules of the two approaching surfaces. This attraction increases as the particle moves closer to the substratum. A repulsive force is encountered if the surface continues to approach each other, due to the overlap of the electrical double layers.

A net attraction can occur at two values of h; these are refered as the primary minimum (h very small) and the secondary minimum (h= 10-20 nm) and are separated by a repulsive maximum. The reversible nature of these initial interactions suggests that, the primary minimum is not usually encountered while the high ionic strength of saliva increases the likelihood that oral bacteria could be retained near a surface by a secondary minimum area of attraction (Burton and Richard, 2000).

c. Pioneer microbial colonizers and irreversible attachment (adhesion – receptor interaction)

Within the shortest time of gap water films are removed from the interacting surfaces leading to irreversible attachment due to adhesions in the microbial cells with their complimentary receptors in the acquired pellicle. The important dehydrating effect, which is resultant of cell hydrophobicity enables the surface to get closer, so that the short range of interaction can occur.

Within a minute, the initial colonizers in the oral microflora particularly *Streptococci* appears on the surface. Once attached, these pioneer populations start to divide and form microcolonies. These microcolonies become embedded in bacterial

extracellular polysaccharides and slimes with an additional layer of adsorbed salivary protein and glycoproteins. The oral streptococci posses a range of glycosidase activities, enabling them to interact and use of salivary glycoproteins as a substrate. The irreversible attachment of cells to the tooth surface involves specific, short range and stereochemical interactions between microbial cell surface components and complimentary receptors in acquired pellicle. This specific interaction will contribute to the ecological microbial succession within the acquired pellicle to form a mature plaque. Adhesin present in the *S. gordonii* can bind to α-amylase, *S. mutans, P. gingivalis* and *P. loescheii* adheres preferentially to proline rich proteins (PRP's). Colonization by *S. sorbinus* is more dependent on the sucrose mediated mechanism in which glucan is synthesized by the action of GTase enzyme which was produced by *S. mutans* (Jenkinson and Demuth, 1997).

Some organisms shows adhesion mechanism involving lectin like bacterial proteins interacting with carbohydrates and glycproteins adsorbed to the tooth surface. *S. sanguis* can bind to the terminal sialic acid residues in adsorbed salivary proteins , while *S. oralis* expresses their lectin which interacts with trisaccharide containing sialic acid , galactose and N-acetylgalactosamine.

Actinomyces species contains two antigenically and functionally distinct type of fimbrias. Type I fimbriae mediates bacterial adherence to PRP and to statherin (protein, protein interaction) and type II fimbriae are associated with lactose sensitive mechanism involving the adherence of cells to already attach bacteria resulting in co-adhesion and also to buccal epithelial cells (Klier *et al.*, 1997).

A number of proteins in the cell wall of *S. mutans* (antigen I/II, B, P_1 or Pac) have been identified as adhesions, which interacts with salivary components. Some organisms possess a large sized cell wall protein, which will interact with one or more adhesive receptors. These will interacts with salivary proteins and also with *A. naeslundii* (co-aggregation), *S. sanguis* can use multiple adhesion to bind to saliva coated surfaces via lectin like hydrophobic and or specific protein interaction (Jenkinson, 1994).

A critical factor in plaque formation concerns, the site at which the specific interactions between bacterial adhesions, an host receptors takes place. The host

derived receptor, residues in molecules that are not only adsorbed on the tooth surface, but also present in the solution in saliva. Sometimes, these molecules are designed to aggregate bacteria in solution, thereby facilitating their removal from the mouth by swallowing. It is known that plaque forming process, not all bacteria is involved in aggregation in free solution of saliva before they reach the tooth surface. It was found that *A. naeslundii* could bind to acidic PRP's, when they are found in bounded with the surface rather than in solution. It has also been proposed that, hidden molecules segments of PRP's become exposed only when the proteins are adsorbed to the surface of such hidden receptors for bacterial adhesions called Cryptitopes. Like this, there will be a selective mechanism for facilitating natural plaque formation has evolved by which the host can promote the attachment of specific bacteria without compromising this process in the planktonic phase (Jenkinson and Lamount, 1997).

Adhesions, which recognizes the cryptitopes in the surface associated molecules would provide a strong selective advantage for any microorganism which can colonize a mucosal or tooth surfaces.

Different types of cryptitopes involve the recognition of galactosyl binding lectins by oral bacteria. Epithelial cells and acquired pellicle have mucins and terminal sialic acid. Few bacteria, such as *A. naeslundii* synthesize neuraminidase enzyme which cleaves sialic acid exposing the galactosyl sugar residue. Many oral bacteria posses galactosyl binding lectins which will take the benefit of the exposed cryptotopes

d. Co-aggregation/adhesion or microbial succession

Over the time the plaque bacteria become more diverse. There is a shift from the initial Streptococci in a biofilm with an increasing proportion of Actinomyces and gram positive bacilli. Some organism which is unable to colonize the pellicle are able to colonize with pioneer species by further adhesion receptor interaction (coaggregation and co-adhesion).

Addition to this, the metabolism of initial colonizers alters the environment of the oral cavity and influences the condition, which makes more suited for the growth of fastidious late colonizers. The metabolism of pioneer species generates nutrients

(peptides) and fermentation products (lactate, butyrate, acetate) that can be used by another organism as a primary nutrient source. Thus, the composition of plaque bacteria changes and over time due to a series of complex interaction these changes are termed as microbial succession (Whittaker and Klier, 1996).

Co-aggregation and co-adhesion in the interaction between adhesive interactions of cells on the surface rather than cell to cell recognition of genetically distinct partner cell types and is a key process in microbial succession and biofilm formation (London and Allen, 1990).

Early plaque accumulation is facilitated by intra and intergeneric coaggregation among *Streptococci* and *Actinomyces*. The subsequent development of dental plaque will involve the further intergeneric congregation between other genera and the primary colonizers. It involves lectins, which binds to the complimentary carbohydrate containing receptor on another cell. *Fusobacterium* species have been found to co-aggregate with the widest range of bacterial genera, but do not aggregate with each other. Early colonizer of plaque coaggregates extensively with *F. nucleatum,* while later colonizers such as *Eubacterium* species and *Selenomonas* species do not coaggregate with early colonizers by coaggregation with *F. nucleatum*.

Co-aggregation may also be an important mechanism in the functional organization of microbial communities. The persistence and survival of obligately anaerobic bacteria in an aerobic habitat is enhanced, if they are physically close to oxygen consuming species such as *Neisseria species*. Such interaction can be mediated by coaggregation. Co aggregation could be a mechanism to increase the probability species, that need to interact and collaborate (in order to survive) during the early stages of plaque development. Cell to cell signaling can occur between bacteria, such as *Streptococci* and *Veiollnella* species to facilitate their involvement in a food chain (Cisar *et al.,* 1991).

e. Mature biofilm formation

Due to successive waves of microbial succession and growth of plaque microorganism, the microbial diversity also increases. The growth rate of individual bacteria within the plaque slows down.

Some of the bacteria can synthesis extracellular polymers (soluble and insoluble glucans, fractions and heteropolymers) which will make a major contribution to the plaque matrix. Glucans are synthesized by glucosyltransferases helps in absorption of other bacteria or on the tooth surface to form acquired pellicle, where, they can remain functional and contribute further to matrix formation (Banas *et al.,* 1990).

In mature plaque, the microflora displays maximum diversity which may contain 12-27 distinct species. A matrix is common feature of all biofilms, which makes a significant contribution to the structurally integrity and general tolerance of biofilms to adverse environmental factors and antimicrobial agents. This matrix is biologically active retains water, nutrients and enzymes within the biofilm. The chemistry of this matrix can also restrict the penetration of other types of molecules including antimicrobial agents.

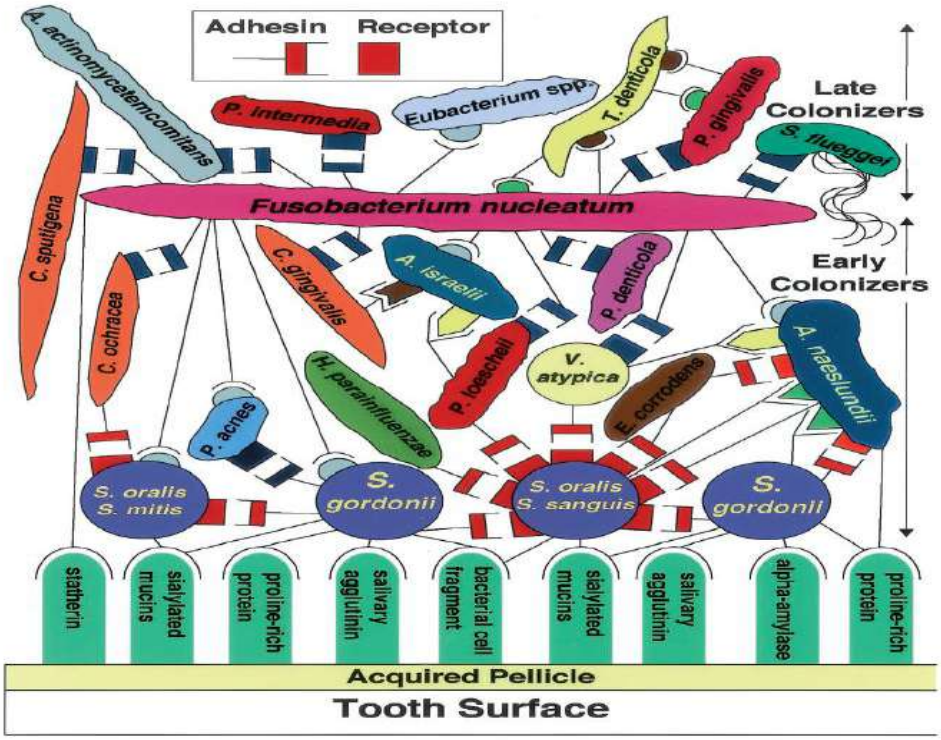


Figure 3.4 : A model of development of dental plaque (initial attachment of salivary pellicle to tooth surface, further aggregation of bacteria to salivary components and coaggregation with other group of bacteria with the help cell surface adhesions forming a complex consortium of bacteria with in the dental plaque).

3.2.4. Pathogenesis of Dental caries

In early 20 th century, Robert Koch and his co-workers developed simple methods for the isolation of pure microbial cultures from the infected samples. By the development of pure culture techniques, oral microbiology was influenced greatly and further advances in the identification of infectious pathogens lead by Koch and Pasteur experiment have linked to searches for specific etiological agents. Current knowledge of oral microbiology is based on the knowledge of such pure cultures, based on studies over the past 100 years. The fundamentals of understanding the pathogenesis of dental caries dates back to these studies. In 1890, WD Miller put forward his chemo-parasitic theory of tooth decay. This theory suggested that bacteria accumulated on the teeth and fermentable carbohydrates present in the oral cavity are responsible for the production of acids which destroyed the tooth tissues. He realized that several potentially key pathogens were uncultivable and these are involved in the acid production and also tolerated pH levels at which dental caries could occur. At that time it was believed that *Lactobacillus* could be the causative agent for dental caries as it is known to survive at lower level of pH. By the discovery of *S. mutans* by Clarke in 1924, has favoured the involvement of this organism in tooth decay. In 1950, Orland and his co-workers began to study caries using animal model systems. They used gnotobiotic hamsters infected with *S. mutans* and fed with diet rich in refined carbohydrates. This study, suggested that *S. mutans* has been established in the development of caries. In the same work lactobacilli were not able to initiate dental caries, as they failed to attach to the enamel, but could effectively decalcify tooth tissue, that had already become partly decayed. From this study onwards, *S. mutans* was considered as a chief bacterium to initiate dental caries in several animal models. These studies also suggested that the ability of *S. mutans* to adhere tooth surface and ferment sucrose, which will induces caries in animals that are fed with sucrose rich diet. Later, these studies concluded that lactobacilli are involved in further decaying the enamel.

As mentioned above, caries is caused by a combination of one or more organisms present in the mouth. The role of dental plaque bacteria in causing dental caries can be explained in three hypotheses like

a) Specific plaque hypothesis,

b) Non Specific hypothesis and

c) Ecological plaque hypothesis. (Theilade, 1986).

a. Specific Plaque hypothesis and dental caries

In 1924, Clarke isolated *streptococcus sp.* from the human carious lesion with distinctive characteristics and named it as *S. mutans*. In the year 1960 Keyes becomes the first person to state the direct relation to the involvement of specific organisms in the etiology of dental caries. In his studies, he observed that albino hamsters only developed caries when caged with caries active hamsters. He also observed that caries active hamsters became caries inactive when treated with penicillin and erythromycin. He concluded that, even though the albino hamsters harbored complex bacterial population, it did not develop caries, because they did not harbor cariogenic bacteria. However, they did develop caries when they acquired with cariogenic organisms from caries active hamsters. This observation made by Keyes is now evident that specific organisms were involved in dental caries and also disease was transmissible. Recent studies have shown that *S. mutans* isolated from dental caries lesions are now subcatagorised into seven distinct species and are collectively referred as *mutans streptococci* (MS). These MS shares common properties pertinent to caries-inducing ability. In addition to this, as they are frequently encountered in dental lesions and other sites of the mouth, they are much needed, to refer to them as a cluster rather than as individual species. Thus, it is now agreed that a particular organism is involved in the development of dental caries, which is referred as a specific plaque hypothesis (Clarke, JK. 1924).

b. Nonspecific plaque hypothesis and dental caries

In the 16th century, the discovery of microorganism in the mouth by Antonie van Leeuwenhoek has lead the suggestion that microorganism are involved in the tooth decay. Further earlier studies, explained the involvement of microorganism with this disease. In late 1800, chemoparasitic theory of Miller suggested that the microorganisms present in the mouth are involved in the enzymatic breakdown of dietary carbohydrates. Due to this action acid is produced and demineralization of the enamel occurs.

According to the Miller, all the microorganisms present in the oral cavity are considered as potential carriages and involved in demineralization process and this concept is known as 'non specific plaque hypothesis'. There have been a number of documents have been published on the correlation of dental caries with the presence of MS and oral Lactobacilli species. The presence of these organisms has been demonstrated as an accurate predictor of caries activity. However, further advancement of culture independent techniques suggested that the dental caries causing organism are also persists in the tooth surface without causing lesion development and also caries can develop in the absence of these species. In these conditions, it is suggested that other acidogenic bacteria like *non mutans streptococci* and *Actinomyces* species are involved in the pathogenesis of dental caries. To support these facts, the recent molecular approaching studies of identifying the bacteria suggested that, the involvement of other novel phylotypes and species including *A. naeslundii*, *A. israeli* and a wide range of *non-mutans streptococci* species and *Veillonella sp* (Balakrishnan, 2005)

c. Ecological plaque hypothesis

The new investigations in ecology of dental caries has lead the several investigators to think over it. Marsh and his co-workers, hypothesized another hypothesis called an ecological plaque hypothesis. According to this hypothesis, the organisms in the complex biomatrix can work together or in conflict depending on the environmental condition within the oral cavity. The important feature of this hypothesis is the selection of pathogenic bacteria, is directly coupled to changes in environment and disease need not to have specific etiology. Any species with relevant trait can contribute to the disease progress. MS are the groups of bacteria are best adapted for cariogenic environment with low pH and high sugar, but such traits are not unique to this bacteria only. Briefly, biofilm would respond to a diet rich in fermentable carbohydrate by allowing the acid producing and acid tolerating organisms (eg. MS, lactobacillus sp. and other species). Bacteria such as *Veillonella sp.* utilizes the acid produced by acid producers in dental plaque and thus preventing the MS inhibition. The net result would be such that, the biofilm would develop in the direction of acid formation and low pH tolerance, while at the same time, dextran production by biofilm bacteria would adhere better to the teeth. Further dextran layer

become much thicker, inhibiting acid buffering effect of saliva on the tooth surface. As a result the decalcification of dental caries takes place by acid attack and finally results in dental caries (Marsh, 1989).

3.2.5. Organisms involved in dental caries:

In oral cavity harbors more than 750 species of bacteria, among these many of them are implicated in oral disease. Among oral disease, dental caries and periodontitis are the infection caused by bacterial origin. Dental caries being a bacterial mediated disease, a number of bacteria are involved in initiation and progression by forming a dental plaque around the hard and soft tissues in the oral cavity (Sharon and Ofek, 2002). This dental plaque is a complex polymicrobial matrix that contains a number of bacterial species. A number of studies have conducted in the enumeration of bacterial communities responsible for tooth decay. Among the bacterial communities, *Streptococcus* sp. (*S. mutans, S. sobrinus, S. sanguis* and others) ranks first for its ability to initiate the dental caries process and further progression of the disease is mediated by other groups of microorganisms (*non mutans streptococci, lactobacilli, Actionmyces sp and Candida sp.*)

3.2.5.1 Mutans streptococci and dental caries

Mutans group of bacteria are well known group of bacteria for the initiation of dental caries. These groups are also called viridans streptococci (ability of an organism to destruct red blood cells partially). These viridians group of streptococci are not only involved in dental caries, they are also known to cause dental sepsis, endocarditis and other systemic diseases. In oral microbiology, there is a considerable interest about this group because they show variable reactions with Lancefield's antisera and relatively low virulence in man. The frequently isolated in association with dental caries among *mutans streptococci* are *Streptococcus salivarius, Streptococcus sanguis, Streptococus mites, and Streptococcus sobrinus*. The other species within this group like *Streptococcus cricetus* and *Streptococcus rattus* are isolated from animals and less frequently isolated from humans (French *et al.,* 1989; Amoroso *et al.,* 2003).

3.2.5.2. Streptococcus mutans and dental caries

Streptococcus mutans was first isolated from caries lesions by Clarke in 1924, but subsequently this species almost disappeared from the literature for more than three decades (Hardie and Whiley, 1999). Interest in *S. mutans* flared up in the 1960s when it was used to demonstrate the infectious and transmissible nature of dental caries in experimental animal models (Keyes, 1960). Since then, *S. mutans* has become the primary focus of caries microbiology. Hundreds of cross-sectional, longitudinal, case control, and interventional clinical studies have been conducted, providing strong evidence for the central role of *S. mutans* in the initiation of dental caries (Tanzer *et al.*, 2001). They have the ability to ferment mannitol and sorbitol and have the ability to synthesize dextran with the help of Glucosyltransferase enzyme (Coykendall, 1974). When *S. mutans* were Gram stained,they appeared as oval rather than round and Clarke concluded these as mutant form of streptococcus (Clarke, 1924). When *S. mutans* were isolated from diverse sources, it became evident that the significant existence of genetic and serological heterogeneity (Dunny *et al.*, 1973). Even though at this heterogeneity, they shared similarities in pathogenesis. This similarity has made several investigators to call them by specific epithet *S. mutans*. On the basis of carbohydrate antigens and deoxyribonucleic acid (DNA) hybridization studies exposed the existence of four genetic and eight serotypes. *S. mutans*, which possess c, e and f antigens, 70 to 80% of the human isolates of mutans streptococci contains serotype 'c' and therefore *S. mutans* is considered as specific epithet for the human type of MS. Other human isolates of MS posseses d, g, h antigens and they were assigned as *S. sobrinus, S. rattus* (serotype b), *S. cricetus* and *S. ferus* were the epithets assigned to *Mutans Streptococci* isolated from laboratory - bred rats, laboratory - bred hamsters and wild rats respectively (Masuda, 1979).

The most important character of *S. mutans* is to colonize the tooth's surface by sucrose dependent and sucrose independent mechanisms. The former involves extracellular glucosyltransferases (GTFs) for synthesis of glucans (glucose polymers) that mediate bacterial adhesion and contribute to biofilm formation (Loesche, 1986). *S. mutans* has three enzymes namely GTFs: GTF B, GTF C, and GTF D. The former two synthesize primarily water-insoluble glucans, while the latter synthesizes only water soluble glucans; the activity of all three enzymes is required for optimal

adherence of *S. mutans* (Ooshima *et al.,* 2001). *S. sobrinus* also possesses GTFs: GTF I, GTF T, GTF S, and GTF U; only GTF I synthesizes insoluble glucans (Nanbu *et al.,* 2000). Sucrose independent colonization of *S. mutans* involves interaction between cell surface polymers, such as the cell surface protein antigen PAc (also called antigen I/II, B, IF, P1 or MSL-1), and the acquired dental pellicle (Yu *et al.,* 1997)

3.2.5.3. Virulence factors of *S. mutans*

3.2.5.3.1. Initial and polysaccharide-mediated adherence

Adherence of bacteria to the host tissue is the first and important step for infection. *S. mutans* contains proteins (180- 210 kDa) like adhesins on their surface, which mediate the initial adherence to the tooth surface (Russell and Lehner 1978). These proteins are designated as Pac, antigen I/II, P1 and Spa P1, these adhesins not only mediate the attachment to the tooth surface but also provides the sites for attachment. Recent studies suggested that, the suppression of these proteins would reduce the virulence of *S. mutans* (Nakano *et al.,* 2006). *S. mutans* also synthesis water insoluble and /or insoluble glucan by the utilization of sucrose. This reaction is catalyzed by three types of isozymes of glucosyltransferases (GTF-B, GTF-C and GTF- D). These enzymes catalyze the transfer and addition of a glucosyl moiety to the terminal site of a primer or elongating glucan (Monchois *et al.,* 1999) according to the reaction scheme:

Sucrose + (1,6-α-D-glucosy))$_n$ ⟶ D-fructose+ (1,6-α-D-glucosy))$_{n+1}$

The glucans consist of a α -(1-6) -linked glucose polymer with α -(1-3) branch linkages (Long and Edwards, 1972). The sticky nature of glucan facilitates the adherence of bacteria to the tooth and resists its detachment by normal mechanical forces, such as mastication, swallowing, and chewing in the mouth. Also, *S. mutans* produces three glucan-binding proteins Gbps (GbpA, GbpB, and GbpC). The role of these proteins in mediating sucrose-dependent adherence has been established (Matsumura *et al.,* 2003).

In addition to glucan synthesis, fructans are also produced by the action of fructosyltransferases to synthesis repeating units of 8 to 27 sugar residues of β- (2-1)-

D-fructo-furanosidic. These fractions acts as an extracellular storage reservoir (Burne *et al.,* 1996) and recent studies have been reported that, these fructans are also have important roles in plaque formation by utilization of same substrate sucrose as glucosyltransferases utilizes (Loo *et al.,* 2003).

3.2.5.3.2. Adaptation

S. mutans has the ability to use ammonia as a nitrogen source, which helps in ecological advantage. In deep caries lesion this bacterium can adapt and grow itself, where there will be deficient for diffused amino acids. This bacterium utilizes the ammonia in the anaerobic environment survives without exogenous amino acids (Berkowitz *et al.,* 1981)

3.2.5.3.3. Intracellular polysaccharides

S. mutans is known to produce intracellular glucogen like polysaccharides (IPSs) in presence of extracellular glucose and sucrose (Van houte *et al.,* 1970). The metabolism of IPS may promote the development of caries by increasing the exposure time to organic acids, when the bacterium is devoid of an external food source. Several studies have been reported that, IPS will add the cariogenic character to *S. mutans* along with acidogenecity and acid tolerance (Harris *et al.,* 1992)

3.2.5.3.4. Aciduricity and acidogenicity

S. mutans is a homofermentative lactic acid bacterium (Drucker, 1968), which produces acid and also tolerates the acid environment within the oral cavity (Tanzer *et al.,* 1969). In limited supply of carbohydrate, this bacterium also known to produce formate, acetate and ethanol. This result that, this bacterium is acidogenic and this reaction is mediated by high efficient phosphotransferase system for sucrose and glucose (Nascimento *et al.,* 2004). These characteristics add *S. mutans* as a potential cariogenic bacterium. Overall, this is achieved by upregulation of a proton translocating process by F_1-F_0 ATPase that extrudes H^+ from the Cell (Bender *et al.,* 1968) which helps the bacterial cell to overcome stress, such as salt, heat, starvation and oxidative stress (Svensater *et al.,* 2000).

3.2.5.4. *S. sanguis* and dental caries

S. sanguis is another predominant organism which colonizes the teeth and helps in the causation of dental caries. When compared to *S. mutans*, it is less cariogenic and certain strains within this group show minimal cariogenic effect on animals but most are not. Several investigators have identified *S. sanguis* in plaque samples and also on some tongue a by using immunofloroscent techniques (Naveen e*t al.*, 2009).

3.2.5.5. *S. salivarius* and dental caries

S. salivarius is a heterogenous group of bacteria which is found in plaque, nasal pharynx and dorsal region of the tongue. Erin *et al.* (2012) reported that *S. salivarius* was noticeably associated with caries. When compared to *S. mutans* the cariogenic potential of this species is low, but in some plaque samples they are the primary pathogens associated with caries. *S. salivarius* is known produce urease, which hydrolyses urea to ammonia and this ammonia may protect tooth from demineralization (Burne and Chen, 1996). Some of the studies also suggest that these species produces bacteriocins, which have the ability of inhibiting growth of mutans streptococci within the cariogenic environment (Wescombe *et al.*, 2009). These bacteriocin produced by *S. salivarius* strains are ribosomally synthesized narrow spectrum antimicrobials that are directed to show action against closely related bacterial species (Wescombe *et al.*, 2009). In other studies, it has been used as a bacteriocin producing probiotic targeting MS, as a replacement therapy to prevent or to control tooth destruction (Hillman *et al.*, 1987).

3.2.5.6. *Streptococcus mitis* and dental caries

The proportion of this group varies among subjects, however, it is found most regularly on the non-keratinized mucosa, particularly the cheek, lip and ventral surface of the tongue. This group of bacteria is facultative anaerobes, and some studies shows they are also involved in colonization by cell surface adhesions with other bacteria followed by intermicrobial signalling. This intermicrobial signalling leads the formation of extracellular polymeric substance (EPS) and the nutritional adaption leading to a society produced by cell division and multiplication (Nobbs *et al.*, 2009).

3.2.5.7. *Streptococcus sobrinus* and dental caries

S. sobrinus is a group of MS which are frequently isolated in association with dental caries. This group of species is much relevant to *S. mutans* in caries development and progression. Several *in vitro* and epidemiological studies suggest that, *S. sorbinus* may be more cariogenic than *S. mutans* under some circumstances (De Soet *et al.,* 1991). Some studies also suggest that, higher incidence of dental caries is seen among pre-school and 15 year school children, when they harbor both *S. sobrinus* and *S. mutans* than those with *S. mutans* alone. *S. sorbinus* is also known to produce an enzymatic production of glucan, which is involved in the dental plaque formation and also for acid tolerance within the biofilm and thus sharing a similar character with *S. mutans* (Okada *et al.,* 2005).

3.2.5.8. *Lactobacilli* and dental caries

Lactobacillus sp. are also called lactic acid bacteria as they are involved in acid production. These bacteria play an important role in progression of dental caries infection, as they are acid producers and aciduric. Lactobacilli represent 1% of total oral microflora *L. acidophilus, L casei* and *L. rhamnoses* are frequently encountered with dental caries (Edwardson, 1974). They are present in high concentration in saliva and appear during the first year of a child's life. When mutans streptococci start to colonize by initiation of plaque around the teeth, the number of lactobacilli starts to colonize in the oral cavity leading to the progression of disease by acid production. Lactobacillus produces acid by two metabolic pathways, a homolactic, Embden Meyerhof pathway, result in the production of only acid, and heterofermentive Phosphoketolase pathway resulting in the production of acid along with Co_2 and ethanol. The mentioned pathways results in the acidification of the oral environment and results in the cavitation of the dentin (Caufield *et al.,* 2007).

3.2.5.9. *Actinomyces* and dental caries

Actinomyces are the group of bacteria which plays an important role in caries process, as that of *mutans streptococci* and *lactobacilli.* Several studies have focused that, *S. mutans* and lactobacillus are prominent group of bacteria at the initial stages of tooth decay. However, several culture based and PCR based studies suggest that Actinomyces are also involved in the earlier stages of dental caries and are also

involved in progression of dental caries. They are Gram-positive, non motile, non sporing organism occurring as a rods and filaments. Actually five species have been frequently encountered in relation with dental caries, they are, Anaerobic: *A. israelli, A. bovis,* Facultative anaerobic: *A. viscosus, A. naeslundii, A. odontolyticus* (Ellen 1976*).* Several studies suggest that though they are having role in the initial stages of dental caries and will become prominent on the stage of biofilm maturation. At this stage, population changes in the biofilm may affect the growth rates and/or they will get differences in nutritional profiles. The relationship of this bacteria with other groups within the biofilm has not been disclosed in a culture based techniques, but DNA amplification and PCR studies have shown that, they possess adhesion proteins on their surface which will help in co-aggregation with other groups of bacteria. These Actinomyces sp. are involved in inter and intrageneric coaggregation with viridans streptococci species and lactobacilli sp. playing an important role in the maturation of dental plaque and also involved in every step of caries progression. The important character of *Actinomyces sp*. is, they are as versatile to adapt to the to the complex biofilm environment by possessing a unique glycolytic system (Takahashi *et al.,* 1995). In this system, hexokinase and phosphofructokinase are synthesized by the utilization of high energy polyphosphate and pyrophosphate. Thus, they act as a phosphoryl donars instead of ATP. They will exploit ATP molecule to synthesize polyphosphate and recover energy from pyrophosphate. This pyrophosphate is a high energy molecule containing phosphoryl bond formed by the metabolism of nucleic acid and glycogens. In addition, they are ureolytic and are able to utilize lactate as a carbon source for the growth within the complex biofilm environment (Takahashi *et al.,* 1996). These adaptive characters of Actinomyces species are very advantageous to survive and dominate at dental plaque maturation by playing a pivotal role in caries progression leading to tooth destruction (Takahashi and Yamada, 1999).

3.2.5.10. *Candida albicans* and dental caries

Candida albicans is the only fungal species that have the ability to colonize to the mucosal surface of the oral cavity (Calderone, 2012). They become opportunistic pathogens and are involved in causing mucosal and disseminated infections including dental caries. The important character of *C. albicans* is that, they can switch its morphology between yeast and hyphal forms contributing to it cariogenic potency.

Several studies suggest that, they have the ability to colonize with *S. mutans* in the pathogenesis of dental caries. A mutual relationship is observed between *C. albicans* and *S. mutans*.

It is observed that, lactate which is excreted by *S. mutans* is utilized as a sole carbon source for the growth of *C. albicans* and inturn, *C. albicans* reduces the oxygen tension levels required by bacteria and also provides different growth stimulatory factors for the bacterial growth (Brogden, 2008).

Several studies suggest that the adherence of *S. mutans* is enhanced by the presence of *C. albicans*, which describes the possible facilitation mechanism. Scanning electron microscopic analysis of dental plaque formed on hydroxyapetite substrate revealed that there is a strong co-adherence between these two organisms and also to the substrata. The cariogenic potential of *C. albicans* is attributed to its acid production and acid tolerance capacity (Klinke *et al.*, 2011). *In vivo* experimental studies also suggests that *C. albicans* is capable of inducing caries in rats at higher rate. Thus, indicating experimental evidence suggesting that the involvement of this species in the pathogenesis of dental caries (Raja, *et al.*, 2010).

3.2.6. Identification of dental caries pathogens

Clinical microbiology deals with isolation of pathogens from the source of infection and their identification by morphological characters on growth media and biochemical characters. This historic method of isolation and identification depend upon the accurate method of performing morphologic and phenotypic description of type of strains to be identified. Perfect identification and enumeration of complex mixtures of bacteria present in the oral cavity are difficult to process.

Primarily sampling procedure must ensure that, the number and quantity of microorganism in the sample are not altered during the collection, storage and processing. Sampling procedure should ensure that, there should be no chance of contamination from foreign microorganisms during every step of isolation and identification of pathogens. Several sampling strategies for oral microbes have been developed to access bacteria from various sites in the mouth. Saliva is easily sampled, but it contains a mixture of bacteria from many ecosystems. It is best to isolate the

cariogenic bacteria from the plaque samples, or within the cavity with the help of swabs or by collecting the infected teeth samples upon uprooting (Marsh, 2009).

Several studies have shown that, the importance of various selective and non selective media for the isolation of dental caries pathogens. Before inoculation of infected samples to various media, the collected samples should show viability. It is very difficult and a challenging task to maintain the viability of the bacterial within the transport media. Viability can be achieved by selection of specific transport media, so it should maintain the viability of the cell until it is inoculated into specific media. Several transport media have been suggested from several researchers. One of the widely used media used as transport media are reduced transport media and VMG II media for the isolation of dental caries pathogens (Syed and Loesche, 1972).

The detection of pathogens from the infected clinical samples requires the data related to phenotypic characters of the required organism that has to be detected. The classical approach of detecting microorganism is to place viable cells from transport media onto solid or liquid medium containing all the nutrients that favours the growth of target microbe, so that its morphological character is seen. Blood agar is used as a broad spectrum non selective media for the cultivation of oral species (Kushbu and Prakash, 2015). This medium is used to cultivate fastidious organisms based on the haemolytic characters. In oral microbiology, particularly mutans group of streptococci is involved in the pathogenesis.

As described in earlier section mutans streptococci are also known as viridians streptococci which show a characteristic alpha hemolysis on blood agar. This observation on blood agar plays an important role in identification of oral streptococci involved in dental caries. Infected dental caries sample produces a typically diverse array of colony character on solid media. It is very difficult to sort out from a mixed culture, and species may be developed as a small percentage of total bacteria may not even be seen. It requires further complete identification of phenotypical character by using a set of selective media such that complete identification of the phenotypes would be possible.

Several investigators used special media for the isolation, Brain heart infusion agar, Pikes Streptococcus agar (Maripandi *et al.,* 2011), Tryptic soya agar (Shuvho *et*

al., 2015), de Mann rogosa agar for the isolation of *Lactobacillus* species (Rogosa *et al.*, 1951), Saborauds dextrose agar for the isolation of *C. albicans* (Girija *et al.*, 2012) and Starch casein agar for the isolation of actinomyces species. Some investigators also utilized differential medium for the isolation of pathogens, Mutans sanguis agar for the differentiation of *S. mutans* and *S. sanguis* (Nada *et al.*, 2008), Mitis salivarius agar for the differentiation of *S. mitis* and *S. salivarius* (Maripandi *et al.*, 2011: Patricia *et al.*, 2003).

After preliminary isolation of bacterial pathogens based on phenotypic characters, further conformation and identification of dental caries pathogens is considered to be an important step for the understanding of dental caries. Several methods have been proposed to identify and differentiate oral pathogens: microbiologic methods, biochemical tests, immunologic and genetic methods with DNA probes, polymerase chain reaction (PCR). The PCR method is faster, more sensitive and specific, than the current microbiologic methods (da Silva *et al.*, 2008). Mutans Streptococci were primarily identified on the basis of their characteristics colonial morphology, colonies in blood agar, Gram-stained smears, and catalase test. Biochemical tests were also performed (Shklair and Keene, 1974). Biochemical tests have been employed in the identification of viridans streptococci Table 3.2 shows the different biochemical characters playing a vital role in the identification of mutans group/viridians group of streptocci involved in dental caries.

Group	Species	Arginine	Esculin	VP	Mannitol	Sorbitol	Urea	Origin
Mutans group	*S. mutans*	-	+	+	+	+	-	Human
	S. sorbinus	-	+	+	+	+	-	human, rats
	S. cricetus	-	+	+	+	+	-	human, rats
	S. downei	-	-	+	+	+	-	Monkey
	S. ratti	+	+	+	+	+	-	Rat
	S. macaccae	-	+	+	+	+	-	Monkey
Salivarius group	*S. salivarius*	-	+	+	-	-	V	Human
	S. vestibularis	-	V	V	-	-	+	Human
	S. thermophilus	-	-	+	-	-	-	dairy products
Anginosus group	*S. anginosus*	+	+	+	-	-	-	Human
	S. constellatus	+	+	+	-	-	-	Human
	S. sinensis	+	+	-	-	-	-	Human
Sanguinis group	*S. sanguis*	+	+	-	-	-	-	Human
	S. parasanguinis	+	V	-	-	-	-	Human
Mitis group	*S. mitis*	-	-	-	-	V	-	Human
	S. oralis	-	V	-	-	-	-	Human
	S. cristatus	-	-	-	-	-	-	Human
	S. infantis	-	-	-	-	-	-	Human
	S. orisratti	-	+	-	-	-	-	Rat

Table 3.2: Different biochemical characters of mutans group of streptococci or viridians group of streptocci

Beighton *et al.* (1981) isolated *S. mutans* serotype A from dental plaque of monkey and characterized based on the production of acid from mannitol, glucans from sucrose, no hydrolysis of arginine or starch, production of acetylmethylcarbinol from glucose.

Yoo *et al.* (2007) conducted typing of 95 strains of mutans streptococci into various biotypes. Biochemical characterization also performed by tests such as

arginine hydrolysis, fermentation of mannitol, sorbitol, raffinose, melibiose etc. Of two strains of variant biotype, one strain (ChDC YM16) was similar to a biotype IV except that, it was positive to the arginine hydrolysis test. *S. mutans* ChDC YM217 could not ferment mannitol, sorbitol, raffinose, and melibiose and could not hydrolyze arginine.

Antony *et al*. (2010) characterized *S. mutans,* from patients undergoing orthodontic treatment, based on biochemical reactions along with hemolytic and other properties. The isolated bacteria were found to be positive for mannitol, esculin and sorbitol hydrolysis, VP test. Arginase and urease activity were not detected.

Girija *et al.* (2012), isolated Lactobacillus, alpha hemolytic streptococci and *C. albicans* from 75 cases. Identification of these compounds is based on morphological characters and biochemical characters. In their study, it has been found that, caries were found to be very predominant among 25 to 35 years of age group. Lactobacillus was found to be dominant in dentinal caries followed by *C. albicans* (11.9%) and 9% of *S. mutans,* 27% of *lactobacillus species* were found. Among smooth surface caries the predominant microorganism was found to be *Actinomyces* 50%, *Lactobacillus sp.,* followed by *C. albicans* and *S. mutans*.

In a study conducted by Nada *et al.* (2008), 45 bacterial isolates were isolated from 50 plaque samples based on the morphological characters on mitis salivarius agar. Further, biochemical and Lancefield grouping identification system revealed that the presence of mutans streptococci of various group like *S. sobrinus* (serotype D, G), *S. mutans* (serotype C, F), *S. cricetus* (serotype A) and *S. rattus* (serotype B) with percentages of (39.29%), (30.30%), (18.18%) and (3.03%). Biochemical tests like mannitol, sorbitol and inulin fermentation test were performed for the differentiation of mutans streptococci.

Maripandi *et al*. (2011) screened 70 infected adult out patients between the age group 20 to 60. They isolated 87 bacterial strains by inoculating them into Pikes streptococcus agar, n Brain heart infusion agar (5% defibrinated sheep blood) and Mitis salivarius agar. Streptococcal colonies were isolated on the basis of colony morphology on solid media and preliminarily identified by Gram staining and catalase test. The colonies showing Gram positive cocci and catalase negative isolates were

further identified by performing a set of biochemical characteristics, like glucan production, sugar fermentation tests, amino acid and esculin hydrolysis tests, etc. In their study , among 87 bacterial isolates, 63 clinical isolates were facultative anaerobes and 24 were anaerobe, among facultative anaerobe, *S. mutans (*22.98%) was dominant in infected samples, followed by *S. salivarius* (13.79%) and *Candida* spp (14.97%). In anaerobes, *Fusobacterium* spp and *Prevotella* spp with isolation rate of 5.74% and 12.64% respectively.

In a study, Streptococci species were isolated and identified from 262 saliva samples, from 131 children and adolescents from public school nursery. The entire 262 clinical specimen were pre-processed in a laboratory and inoculated on mitis salivarius agar supplemented with 5% sheep blood. *Streptococci* strains were primarily recognized on the basis of their haemolytic pattern on blood agar, Gram-stained smears, and catalase test. Biochemical tests were also performed by fermentation of sugars like adonitol, starch, arabinose, cellobiose, dextrin, dextrose, dulcitol, galactose, glucose, inulin, lactose, maltose, mannose, manitol, mellibiose, raffinose, sucrose, sorbitol, sorbose, xylose, and amino acid arginine hydrolysis. Isolated strains were subjected to Gram staining showed characteristic chain-linked cocci morphology. Among the isolated bacteria, 77.86% were *Streptococcus salivarius* which was found to be most prevalent, followed by 62.59% (*Streptococcus mutans*), 22.90% (*Streptococcus sobrinus*), 16.79% were *Streptococcus* of the *mitis* group, 1.52% of *Streptococcus rattus*, and 25.95% belonged to other species. When the standard technique were followed, the species *S. salivarius* again was the most predominant with isolation rate 89.31%, followed by *S. mutans* (73.28%). Seventeen *Streptococcus sobrinus* strains (13.74%), (22.90%) *Streptococcus* strains of the *mitis* group, 1 (0.76%) *Streptococcus ratti* strains, and 13 (9.92%) strains belonging to other species were also identified (Amoroso *et al*., 2003).

Kushbu and Prakash (2015) in a cross sectional study, isolated Gram positive and Gram negative organisms. In their study, they used blood agar and Mc conkey agar for the isolation and identification was done by using different biochemical tests. For Gram positive isolates catalase, oxidase, optochin sensitivity, indole, urease, bile, coagulase and carbohydrate fermentation tests weredone and for Gram negative isolates, catalase test, O/F test, SIM test, citrate test, urease test, TSI test was done,

their study reveals that, 297 isolates were Gram positive (43.77% were *S. mutans*, 31.64% were *S. aureus*, 10.77% were *S. mitis*, 8.08% were *S. albus*, 5.72% were *S. vestibularis*.) and 28 isolates were Gram negative (39.28% were *Pseudomonas spp.*, 32.14% were *K. pneumoniae*, 17.85% were *P. vulgaris* and 10.71% were Enterobacter spp.)

In a study, *Streptococcus species* involved in dental caries was isolated from dental caries patients of various age. They have isolated pathogens based on their colonial character on tryptose soya agar, nutrient agar and Macconkey agar. Biochemical tests like voges-proskauer (VP), glucose, fructose, sorbitol, trehalose, melibiose, mannitol, inulin, salicin, lactose, raffinose, arginin, esculin hydrolysis pyrrolidonearylamidase, β galactosidase, β glucuronidase and alkaline phosphatise was done for the identification of caries pathogens. Based on biochemical characters they have characterised 184 clinical isolates among them *S. mutans* (28.80%), *S. mitis* (23.91%), *S. aureus* (28.26%) and *S. salivarius* (19.02%). The study also revealed that, the age group between 26 to 35 years are haveing highest prevalence rate of 32.60% of infection and lowest prevalence rate (10.86%) was found to be in the age group of 15 years (Sreeja *et al* ., 2013).

Shyla *et al.* (2011) isolated dental caries bacterial pathogens from infected sample by using Brain heart infusion agar (5% defibrinated blood), Nutrient agar and Mac conkey agar and they are further identified by biochemical characters like catalase, oxidase and sugar fermentation tests. Fungal pathogen *C. albicans* associated with dental caries were isolated by inoculating, the infected samples on SDA agar and the presence of *C. albicans* was further confirmed by its colonial characteristics and by performing germ tube test.

Gamal and Sherbiny (2014) isolated cariogenic organisms from a total of 70 saliva and plaque samples. The infected samples were processed and inoculated on the Brain Heart Infusion agar**,** Gold's medium and azide blood agar medium. The identification of bacteria was done on the basis of colonial morphology, Gram staining characters and standard biochemical tests. Out of 70 infected samples, they have recovered 100 isolates. They were identified as *Streptococcus* sp. which represented 56% from total isolates followed by *Lactobacillus* sp. 15%,

Staphylococcus sp. 12 %, *Fusobacterium* sp.10% and *Corynebacterium* sp. 7%. Among Streptococcus sp. *S. mutans (*40%) and *Streptococcus sobrinus(*16%) were isolated.

Enweani *et al.* (1999) studied on the prevalence of dental caries pathogens in different groups. The samples collected includes dentine samples from incisors, canine and premolars of primary and permanent dentition. The samples collected were processed to disperse the organism within the sample and inoculated onto chocolate agar and mitis-salivarius agar. Based on the colony characteristics, following biochemical tests were employed in this study, which includes catalase test, optochin sensitivity, bacitracin sensitivity, hemolysis, arginine and esculin hydrolysis, hippurate , glucan production test, inulin, raffinose, sucrose, lactose and mannitol fermentation tests. Lactobacillus identification was done by observing growth in 5% Nacl. It was found that the most prevalent pathogen associated with dental caries is *S. mutans* (72%) followed by *S. salivarius* (14%) and *Lactobacillus acidophilus* (10%).

3.2.7. Traditional plant based medicine to control dental caries:

Medicinal plants have been used in the treatment of several systemic diseases, however these plants based products are also converted as a potential therapeutic agents for the treatment of oral infections like dental caries and periodontitis. Number of plant derived medicine are exploited and investigated against oral microbial pathogens (Kalemba and Kunicka, 2003; Cowan, 1999).

The use of traditional plants based products is considered as primary and major source to treat oral diseases. These plant based medicine offers a diverse range of structurally distinctive bioactive molecules (Newman and Cragg, 2007) and numerous studies have been conducted in the field of usage of traditional plant products in the treatment of oral disease like dental caries and periodontitis. Many plant based medicaments have been recorded in many pharmacopeias as agents to treat various infections, and some recent investigation also suggests that these products can be utilized for their potentiality against dental caries (Kalemba and Kunicka, 2003; Cowan, 1999). The rise in the dental caries incidence particularly in developing countries has made the treatment options and products for oral diseases that are safe, economic and as well as effective also. In many developing countries,

dentist's offers diverse chemically synthesised antimicrobial agents (penicillins and cephalosporins, erythromycin, tetracycline and derivatives and metronidazole cetylpyridinium chloride, chlorhexidine, amine fluorides) for the treatment of dental caries. These agent are known to cause undesirable side effects, like antibiotic associated diaarrhea, vomiting, tooth staining and alteration of oral and intestinal microflora etc. Some of the reports also focused in the bacterial resistance among the oral pathogens due to increased use of these chemically synthesised antibacterial agents. Some antibacterial agents used in mouth washes also contains ethanol as the additives is known to cause oral cancer. Thus to combat these side effects, there is a need for the exploration of alternative medicines for the treatment of dental caries. Natural products isolated from the plants are considered as best alternatives to synthetic chemicals which are found to be effective and good alternative lead for drug discovery.

3.2.7.1. Influence of natural products in the pathophysiology of dental caries

As explained in the earlier sections, dental caries results in the interactions of specific group of bacteria. Within the oral microbial environment, the virulence properties of bacteria and their metabolic constituents, dietary carbhohydrates with salivary constituents are continuously interacted on the susceptible tooth surface, as a result dental caries occurs. The plant products used to control the pathogenesis should have the potency to interfere with all the factors associated with dental caries. Generally the most targeted organisms in the pathohophysiology of dental caries are MS and Lactobacilli and *C.albicans*. Several antibacterial studies have shown that, the plants products have been used to inhibit dental caries pathogens (figure 3.5). Several secondary metabolites such as alkaloids, phenols, tannins, terpenoids and essential oils are known to interference with growth and multiplication of microorganism involved in the etiology of dental caries (Wen *et al.*, 2004). Natural compounds inhibiting the viability of the pathogens may involves two or more mechanism, i) distruption of cell wall synthesis ii) Complexing with cell adsorbed components iii) inhibition of protein synthesis, DNA synthesis and also enzyme synthesis (Cowan, 1999).

Several Studies have also shown the natural products can also acts as inhibitors of acid produced by the cariogenic streptococci and lactobacillus species.

However, a little is known on the inhibition of glycolytic activity /acidurity of cariogenic organism, the inhibitory effects may be due to the reduction in bacterial growth. Some possible mechanism of natural product that affects the acidogenicity of cariogenns includes i) distruption of membrane proton motive force, ii) inhibition of enzyme activity and expression of specific enzymes related to sugar transport. 7-epiclusianone from *Rheedia gardneriana*, tt-farnesol, flavonoids and phenols are involved in increase of proton permeability among the *S. mutans* cells which leads to cytoplasmic acidification and distrupting the accumulation of intracellular iodiophilic polysaccharides (IPS). These IPS also contribute in the development of dental caries, and it acts as a endogenous storage source of carbohydrates for *S. mutans* and other cariogenic microorganisms, when there is a depletion of exogenous sugar within the oral cavity (Koo *et al.,* 2003; Jeon *et al.,* 2009).

One of the novel approach for the reduction of dental caries incidence is by inhibition of exopolysaccharide synthesis (EPS) in oral streptococci. As explained in earlier section these exopolysaccharide are the glucans synthesized by glucosyltransferase enzyme which uses sucrose as a substrate.

As *S. mutan*s being a chief bacterium, to cause dental caries. It is known to produces different isozymic glucosyltransferase enzymes (Gtfs, GtfB, A and D). These enzymes play an important role in the pathogenesis dental caries by synthesizing glucan providing a suitable substrate for early and also late colonizers. Thus Glucosyltransferase acts an ideal target to control dental caries. Some of the studies have shown that polyphenolic compounds have the ability to interfere with glucan production by nonspecific glucan binding domain and by precipitation of protein within the solution. Other than polyphenol, the phytochemical like condensed tannins and hydrolyzable tannin, proanthocyanidans, has also ability to inhibit the activity of glucosyltransferases (Yamanaka *et al.,* 2004).

In the process of dental caries there will be continous interaction between teeth and oral flora. Bacterial adherence to tooth surface and coaggregation of them with other species is an important task in the etiology of dental caries. Thus for several investigators, this has become target point to inhibit the bacterial adherence. Some natural products like high molecular weight polyphenols, chitosan and 1-

deoxynojirimycin can interfere with bacterial adherence, which inturn interfere with ecological sucession resulting in the control of dental plaque (Saeki *et al.*, 1996; Wolinsky *et al.*, 1996). The following table 3.3 represents the list of of plant products interfereing in the pathphysiology of dental caries.

Table 3.3: List of plants interfering in the pathophysiology of dental caries

Sl No	Name of the plant	Plant parts used	Biological activity	Conclusion	Reference
Antibacterial activity					
1	*Bixa orellana L.*	Leafs and seed	Inhibitory activity against *S. mutans* and *S. sanguis*	Leaves extract showed highest activity when compared to seed extract	Dyanne, *et al.*, (2016).
2	*Myrciaria dubia*	Seed and pulp	Inhibitory activity *against S. mutans* and *S. sanguis*	Seed extract showed highest activity when compared to pulp extract	Rosella, *et al.*, (2016)
3	*Calotropis gigantia (L.) R. Br.*	Latex	Inhibitory activity against *Actinomyces viscosus, Lactobacillus acidophilus, Lactobacillus casei, Streptococcus mitis*and *Streptococcus mutans.*	The chloroform extracted fraction of latex showed inhibitory effect against *S. mutans* and *L. acidophilus* with MIC value of 0.032 and 0.52 mg/mL, respectively. Qualitative investigation on structure elucidation of bioactive compound using IR, NMR and GC–MS techniques revealed the presence of methyl nonanoate, a saturated fatty acid.	Kalpesh *et al.*, (2012)
4	*Eucalyptus globules Labill.*	leaves	*Lactobacillus acidophilus, Lactobacillus casei, Staphylococcus aureus*and *Streptococcus mutans.*	The ethyl acetate extracted fraction of plant leaves showed good inhibitory effects against all selected bacteria. In *Eucalyptus globules*, hexane and ethyl acetate extracts found highly effective against, *Lactobacillus acidophilus* with MIC value of 0.031 and 0.062 mg/mL, respectively.	Kalpesh *et al.*, (2013)
5	*Hibiscus sabdariffa L*	Calyx	*Streptococcus mutans, Streptococcus sanguinis, Lactobacillus casei, Actinomyces naeslundii, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Porphyromonas gingivalis* and *Prevotella intermedia*	Antimicrobial activity against all the microorganism used in this study. A significant activity was observed *in Fusobacterium nucleatum, Prevotella intermedia and Porphyromonas gingivalis.* It also showed antibiofilm activity.	Herastuti, *et al.*, (2016).

6	*Embelia ribes*	Leaves	*S. mutans*	Antibacterial and Antibiofilm activity against *S. mutans*	Deepak and Vinod, (2016)
7	*Piper longum,*	Leaves	*S. mutans*	Antibacterial and Antibiofilm activity against *S. mutans*	Deepak and Vinod, (2016)
8	*Camellia sinensis* *Spilanthus calva* *Azadiractha indica* *Acacia nilotica (Linn.)* *Ocimum basilicum Linn* *Spilanthus calva DC* *Syzygium aromaticum* *Embelica officinalis Gaerth* *Hemidesmus indicus R.Br* *Terminalia bellirica roxh* *Syzygium cuminii (Linn.) Skeels*	Leaves	*S. mutans, S. mitis, S. sanguis* and *A. actinomycetocomitans*	All the plant extracts showed a significant antibacterial activity against pathogens of dental caries.	Anupama, *et al.,* (2012)
9	*Mentha cordifolia Opiz.,* *Ocimum africanum L.,* *Ocimum basilicum Lour., .* *Ocimum sanctum L.* *Piper betle L.* *Piper chaba Hunter,* *Piper nigrum L.,* *Piper sarmentosum Roxb.,* *Alpinia galanga (L.) Willd.* *Curcuma longa L.* *Curcuma zedoaria Roscoe,* *Zingiber officinale Roscoe*	Leaf	*Enterobacter faecalis* ATCC 19433, *Lactobacillus fermentum* ATCC 14931, *Lactobacillus salivarius* ATCC 11741, *Streptococcus sobrinus* ATCC 33478 and *Streptococcus mutans* ATCC 25175, and 2 Gram negative periodontopathogenic bacteria, *Aggregatibacter actinomycetemcomitans* ATCC 33384 and *Fusobacterium nucleatum* ATCC 25586.	The different plant extracts exhibited varied inhibited zone ranged from 1 to 20 mm. *P. betle* leaves showed a good result of antibacterial activity against all tested microorganisms using agar well diffusion assay. The MIC of *P. betle* extract ranged from 1.04 to 5.21 mg/mL and MBC ranged from 2.08 to 8.33 mg/mL, respectively	Rawee, *et al.,* (2016)
Antiglucosyltransferase activity/Antibiofilm activity					
1	*Camellia sinensis*	Leaves	*S. mutans*	Inhibitory effects on Gtf activity in biofilm formation	Hattori *et al.,* 1990; Touyz and Amsel, 2001; Linke and LeGeros,

					2003
2	*Vaccinium macrocarpon*	Leaves	*S. mutans*	Antimicrobial activity against biofilm cells of *mutans streptococci* Reduction of formation of *S. mutans* biofilms and EPS content	Steinberg *et al.*, 2004; Yamanaka *et al.*, 2004;
3	*Mentha piperita*	Leaves	*S. mutans*	Inhibitory effects on human plaque formation	Takarada *et al.*, 2004;
4	*Rosmarinus officinalis*	Leaves	*S. mutans*	Inhibitory effects on human plaque formation	Rasooli *et al.*, 2008
5	*Azadirachta Indica*	Leaves	Antiglucosyl transferase activity, *S. mutans.*	Inhibitory effects on water-insoluble glucan synthesis by mutans streptococci	Wolinsky *et al.*, 1996;
6	*Melaphis Chinensis*	Leaves	*S, mutans*	Inhibitory effects on water-insoluble glucan synthesis by mutans streptococci	Pai *et al.*, 2004;
7	*Lentinus edodes*	Leaves	Anti-adherence, Antiglucosyl transferase activity and against *S. mutans.*	Reduction in biofilm formation and waterinsoluble glucan synthesis by mutans streptococci	Hirasawa *et al.*, 1999; Shouji *et al.*, 2000
8	*Mentha cordifolia Opiz.*, *Ocimum africanum L.*, *Ocimum basilicum Lour.*, . *Ocimum sanctum L.* *Piper betle L.* *Piper chaba Hunter*, *Piper nigrum L.*, *Piper sarmentosum Roxb.*, *Alpinia galanga (L.) Willd.* *Curcuma longa L.* *Curcuma zedoaria Roscoe*, *Zingiber officinale Roscoe*	Leaves	*Streptococcus mutans* ATCC 25175 and *Aggregatibacter actinomycetemcomitans* ATCC 33384	Antibiofilm and antibiofilm eradication assays	Rawee, *et al.*, (2016)
9	*Myrisica fragrans* Houtt.) Macelignan	seeds		macelignan at 10 µg/mL for a 30 min exposure time could remove more than half of each single oral biofilm formed *by S. mutans, S. sanguinis and A. viscosus*.	Nikitina, *et al.*,(2007)
10	*Psidium guajava L.*	Leaves	Antiplaque activity	Demonstrated high potential antiplaque agent by	Osawa, *et*

	Guaijaverin-(-3-O-α-L-arabino-pyranoside)			inhibiting the growth of the *S. mutans.*	*al*.,(1992)
11	*Magnolia officinalis* Magnolol	bark	Anti –Gtase activity	reduced by 87.3% GTF activity 0.32 mg/mL	Osbourn, (1996)
12	*Magnolia officinalis* Magnolol ,Honokiol	bark	Anti –Gtase activity	reduced by 58.1% GTF activity	Osbourn, (1996)
Inhibition of acid production					
1	*Vaccinium macrocarpon*	Leaves	Inhibition of acid production by *S. mutans*	Disruption of acidogenic/aciduric properties	Duarte *et al.*, 2006a; Gregoire *et al.*, 2007; Koo *et al.*, 2010a
2	*Alcea longipedicellata* (Malvaceae) malvidin-3,5-diglucoside (malvin)	Leaves	Inhibition of acid production by *S. mutans*	0.1% malvin could inhibit strongly acid producing ability of *S. mutans*.	Ooshima,*et al.*, 1993
Inhibition of bacterial adherence					
1	*Gloiopeltis furcata*	Leaves	Anti adherence activity against *S. mutans*	Inhibition of mutans streptococci adherence	Saeki *et al.*, 1996;
2	*Azadirachta Indica*	Leaves	Anti adherence activity against *S. mutans*	Leaf extract inhibited bacterial aggregation of various oral streptococci	Wolinsky *et al.*, 1996;
3	*Melaphis Chinensis*	Leaves		Leaf extract inhibited bacterial aggregation of various oral streptococci	Pai *et al.*, 2004;
4	*Vaccinium macrocarpon* Quercetin-3- arabinofuranoside, Myricetin and Procyanidin A2	Leaves	Anti-GTase activity	Inhibition of glucosyltransferase, blocking of bacterial adhesion mediated by surface glucans and reduction of insoluble glucan content Quercetin-3- arabinofuranoside 21–41% Inhibition of GTF activity at 500 mmol L−1 500 mmol L−1 GTF activity at 500 mmol L−1 21–41%.	Vercauteren, (2009)

5	*Helichrysum italicum* (Compositae)	Leaves	Anti adhesion activity against *S. mutals*	Plant extract exhibited reduction in cell surface hydrophobicity, adherence to glass and cellular aggregation of *S. mutans* in the presence of dextran.	Ooshima, *et al.*, (1993)
6	*Syzygium aromaticum* (Myrtceae)	Leaves	Anti adhesion activity against *S. mutals*	inhibit adhesion of the bacteria to glass, reduce cell surface hydrophobicity and inhibit the production of glucosyl transferase	Johnson-White 2006
7	*Piper betle*	flower	Anti adhesion activity against *S. mutals*	inhibit the growth, adherence and glucan production of *S.mutans*	Rahim and Khan 2006: Nalina and Rahim 2007
8	*Alcea longipedicellata* (Malvaceae) malvidin-3,5-diglucoside (malvin)	flower	Anti adhesion activity against *S. mutals*	60% effective in inhibiting bacterial adherence	Ooshima, *et al.*, (1993)
9	*Britton var. japonica* Hara. Luteolin	seeds	Anti adhesion activity against *S. mutals*	Inhibition of adhesive	Sato, *et al.*, (2003)

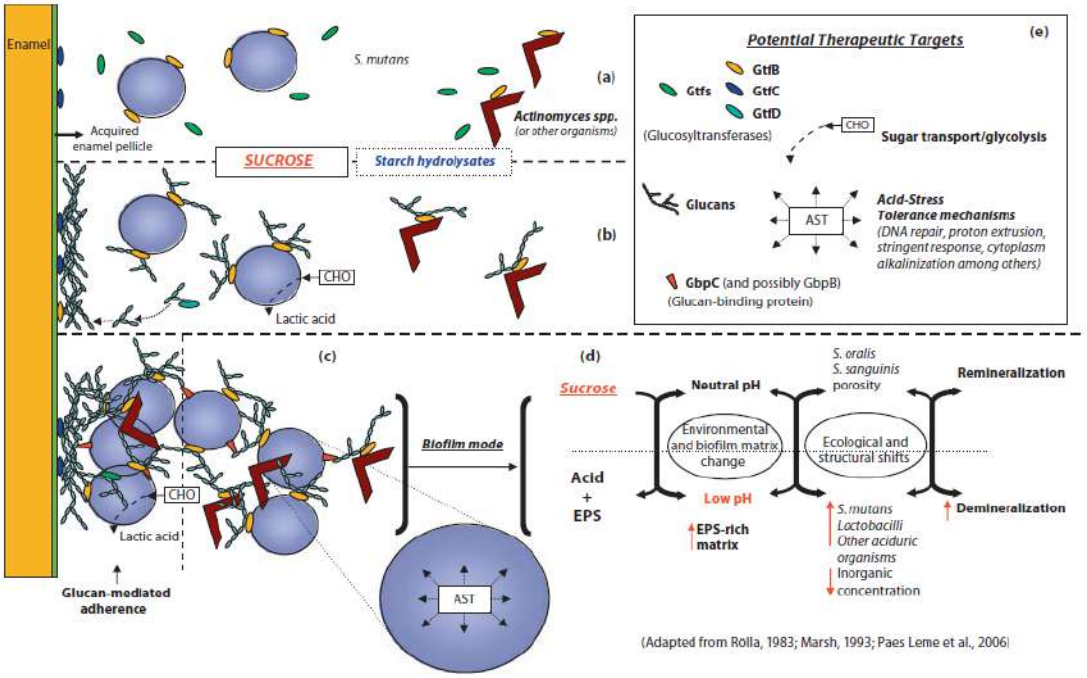


Figure 3.5: A model showing the possible target of natural compounds in pathophysiology of dental caries.

3.2.8. Plants selected for the study

Several plants have been investigated against various microbial infections, the secondary metabolites present in the plants have an important role in antioxidant, antibacterial, antifungal activities and are used in the curing of various physiological disorders. The following are the plant reviews selected for this study,

Anisomeles indica (L.) Kuntze (Labiatae) is a specific woody shrub, and has been commonly used in traditional Chinese medicines for various disorders, such as gastrointestinal disorders, liver disease and inflammatory skin diseases (Yadava RN and Deepak B., 1998). It is also reported that *A. indica* extracts and isolated constituents inhibit inflammatory mediators and tumor cell proliferation. Furthermore, the aqueous extract of *A. indica* has been shown the anti-histaminerigic and analgesic activities. Recently, the ethanol extract of *A. indica* exhibited anti-bacterial activity has been reported (Vivek KB, *et al.*, 2012).

Annona muricata. Linn. commonly known as graviola or soursop, belongs to the family of Annonaceae. It is a typical tropical tree with heart shaped edible fruits and widely distributed in most of tropical countries. The leaves are lanceolate with

glossy and dark green in color had been traditionally used to treat, headaches, hypertension, cough, asthma and used as antispasmodic, sedative and nervine for a heart condition. Earlier reports over the years have demonstrated that the leaf, bark, root, stem, and fruit, seed extracts of *Annona muricata* are anti-bacterial, antifungal and anti-malarial properties. Its leaf extract was also found to possess antioxidant and molluscicidal properties (Vijayameena C, *et al.*, 2013).

Azadirachta indica. A.Juss., (Meliaceae), is known for its therapeutic values and has been in use as a traditional medicine to treat a wide range of human disorders since ancient times. It is an evergreen tree indigenous to south Asia and in most parts of the Indian subcontinent. Antimicrobial activities of neem have widely been reported. The antimicrobial properties of neem can be attributed to several bioactive compounds found in different parts of this tree, which are categorized into two major classes, isoprenoids and non- isoprenoids. Although every part of the neem tree is known to have medicinal properties, extracts from neem leaf, bark, twigs, fruits and neem oils are most commonly documented in literature for their antibacterial effects. (Amal kumar G, *et al.*, 2009)

Chrysophyllum roxburghii G. Don belongs to the family Sapotaceae. It is commonly called 'Indian star apple' and it is a tree growing up to 15 meters height. It is one of the canopy trees in evergreen forests. It is commonly found in Western Ghats of India and Sri Lanka. Seed is a berry. The seeds are used in a formulation to treat pneumonia at the Sivasagar district of Assam state. The ripe fruits are considered edible for the tribal people of Anamalais of Western Ghats, India consumes them as fruits. The fruit is shown to contain an appreciable quantity of minerals and amino acids. In recent studies the methanol extracts of leaves are known to show antibacterial activity. (Ramaswamy *et al.*, 2001; Chandrika *et al.*, 2005)

The genus *Hypericum mysorense*. B. Heyne belongs to the family Hypericaceae and consists of approximately 20 species exclusively of tropical origin. *Hypericum mysorense* is a plant native to the Nilgiri Hills in India. It is closely related to *Hypericum perforatum* (Shanmugam and Shanmugasundaram, 2009). *Hypericum mysorense* is mentioned in Ayurvedic texts, as having anti-viral and nerve calming properties. *Hypericum mysorense* have been used to treat wounds as part of the

Ayurvedic system of traditional medicine. Gopinath, *et al.,* (2013) carried out antiherpetic work using *Hypericum mysorense* extracts.

Malpighia glabra. L. or Barbados cherry (Acerola) is a shrub or small tree growing 10 to 15 feet tall, native in southern Texas, the West Indies, Northern South America, Central America and Mexico. It has leaves which are oval with pointed ends, neither glossy nor leathery, very dark green and small in size. Branches are flexible, the flower is rose-pink and the fruit is edible, a rich source of Vitamin C. The fruits of acerola are known to its nutritional capacity and vitamin contents. It is a rich source of Vitamin C, flavonoids, Carotenoids, precursors of vitamin A, lycopene and also contains traceable elements such as thiamin, riboflavin, niacin, pantothenic acid, calcium, iron and magnesium. In recent years, many studies have been conducted on fruit extracts and their role of reactive oxygen species (ROS) in the etiology of various diseases and less information have been reported on biological activities of leaves, bark and root (Roberta da silva 2011).

Mangiferra indica . L. belongs to the family Anacardiaceae Mango contains various classes of polyphenols, carotenoids, and ascorbic acid, which demonstrate different health promoting properties, mainly from their antioxidant activities. The aqueous leaf extract of *M. indica* has been reported to be rich in polyphenols amongst which mangiferin has been extensively studied by several authors and proposed as the bioactive principle (Shah KA, *et al,* 2010). *M. indica* L. Stem bark and leaf extracts possess several pharmacological activities including antioxidant, analgesic, antidiabetic, anti-inflammatory, antitumor, immunomodulatory, anti-HIV, and antimalarial effects, especially against *P. falciparum*. Various parts of the plant are used as an antimicrobial agent, dentifrice, antiseptic, astringent, diaphoretic, stomachic, vermifuge, tonic, laxative and diuretic and to treat diarrhea, dysentery, anemia, asthma, bronchitis, cough, hypertension, insomnia, rheumatism, toothache, leucorrhoea, hemorrhage and piles (Nathalie, *et al.,* 2007).

Scleropyrum pentandrum (Denn.) Mabb belongs to the family Santalaceae and grows along the margin of evergreen to semi-evergreen forests between 600 and 1600 m. *S. Pentandrun* is distributed in Cambodia, China, Thailand, Sri Lanka and Laos. In India, it is distributed in Peninsular India, Western Ghats, South and Central

Sahyadris and generally found on sandy soil. The whole plant parts are applied externally to treat skin irritation in Kani tribal settlement, the roots are boiled and the decoction is taken as a contraceptive by Semalai people. It is believed that women will become barren after consuming the decoction. Paste prepared from stem bark and leaf is also applied externally to treat skin diseases. Methanol extracts of leaves are also known for showing antibacterial and antifungal activities (Ajithbabu, *et al.,* 2013)

Toddalia asiatica (L.) Lam belongs to family Rutaceae, a spiny woody vine with sharp curved prickles. Leaves are shiny green citrus-scented, trifoliate, leaflets are sessile and ovate. Flowers are yellow-green and fruits are small, nearly spherical, taste like orange peel. This plant is used to treat stomach problems, fever, cough, malaria, indigestion, rheumatic arthritis, sprains, malaria, bronchitis, nausea, diarrhea, and chest pain. The root contains coumarins that have antiplasmodial activity. The extracts from the leaves of the plant have been used for its antibacterial, anti-inflammatory and anti-diabetic activities (Rachael, *et al.,* 2013)

Wrightia tinctoria (Roxb.) R.Br. (Sans: Asita-Kutaja) belonging to family Apocynaceae, a small deciduous tree with pale grey with smooth bark, distributed in tropical Africa and Asia. Different parts of this plant are used in Ayurveda, Siddha and other traditional systems of medicine for curing various ailments such as psoriasis, astringent, stomach, tonic febrifuge and seeds are used for kudal vriddhi and pittavayu diseases. The reported constituents are alkaloids, terpenes, wrightia, Tryptanthrin, Indole and flavonoids. The crushed fresh leaves when filled in the cavity of decayed tooth relieve toothache. Bark and seeds are used to cure bilious infections, psoriasis, leprosy, asthma and various skin diseases (Vedhanarayan, *et al.,* 2013).

4.

Materials and Methods

4.1. Collection and extraction of bioactive compounds from traditional medicinal plants.

4.1.1. Collection of plant material

The plant material (Leaf) was collected from, in and around Shivamogga and Chikmagalore Districts, Karnataka state, India. The collected leaf materials were thoroughly washed, shade dried. Further, leaf material was powdered mechanically and stored for future work (Patra *et al*., 2014). Below mentioned table 4.1 represents the different plants selected for the present study.

Sl No	Name of the plant	Part taken for the study	Place of collection
1	*Anisomeles indicus(L.)Kuntze*	Leaf	Shivamogga
2	*Annona muricata . Linn*	Leaf	Shivamogga
3	*Azadiractha indica. A. Juss.,*	Leaf	Shivamogga
4	*Crysophyllum roxburghii G. Don*	Leaf	Shivamogga
5	*Hypericum mysoresence. B. Heyne*	Leaf	Chikmagalore
6	*Malphigia glabra L.*	Leaf	Shivamogga
7	*Mangiferra indica. L.*	Leaf	Shivamogga
8	*Scleropyrum pentandrum(Denn.) Mabberley*	Leaf	Shivamogga
9	*Toddalia asiatica (L.) Lam*	Leaf	Chikmagalore
10	*Wrightia tinctoria (Roxb.)R.Br.,*	Leaf	Shivamogga

Table 4.1: List of plants selected for the study

4.1.2. Solvent Extraction

The powdered leaf material of all plants (500g) was soaked in methanol to yield a methanol extract for preliminary screening. The extracts were filtered through Whatman No.1 filter paper and the filtrates were concentrated under reduced pressure to get a pasty mass. Each plant extract obtained was stored at 4^{o}C in refrigerator separately for further studies such as, phytochemical screening, *in vitro* antioxidant activities and for bioassay against selected dental caries pathogens (Al Hebshi *et al.,* 2005).

4.1.3. Phytochemical analysis of methanol extracts

Phytohemical analysis was carried out in methanol extracts of ten different plants by following standard methodologies (Naima *et al.,* 2012;Lakshman, 2012).

4.1.3.1 Qualitative analysis of phytochemical constituents

Plant extracts (methanol) were screened for the presence of Flavonoids, Alkaloids, Tannins, Steroids, Terpenoids, Saponins, Glycosides, and Carbohydrates. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytoconstituents.

4.1.3.1.1 Qualitative test for Carbohydrate:

a. **Benedicts test:** One ml. of extract is mixed with Benedicts reagent and boiled for 5 mins. Formation of a brick red coloured precipitate indicates the presence of carbohydrate.

4.1.3.1.2 Qualitative test for alkaloids:

About 15 mg of each extract was stirred with 1% HCl (6 ml) on a water bath for 5 min and filtered. These filtrates were divided into two equal parts.

a. **Dragendorff's test:** Add 5 ml of distilled water, 2 ml of concentrated HCl for 2ml of extract and kept for some time, then add 1 ml of Dragendorff's reagent. Formation of orange red precipitate indicates the presence of alkaloids.

b. **Mayer's test:** Two ml of extract was mixed with the Mayers reagent (Potassium mercuric iodide solution). Formation of white or pale yellow precipitate indicates the presence of alkaloids.

4.1.3.1.3. Qualitative test for flavonoids:

a. **Shinodas test:** About 0.5 g of each plant extract was dissolved in ethanol, and it was warmed and then filtered. Three pieces of magnesium chips were then added to the filtrate followed by a few drops of concentrated HCl. Formation of pink, reddish or brown color indicate the presence of flavonoids.

b. Ferric chloride test: About 0.5 g of each plant extract was boiled with distilled water and then filtered. Take 2 ml of the filtrate and add few drops of 10 % ferric chloride solution. A green-blue or violet coloration indicates the presence of a phenolic hydroxyl group.

c. Alkaline reagent test: Test solution when treated with sodium hydroxide solution, shows increase in the intensity of yellow colour which becomes colourless on addition of few drops of dilute acid, indicates the presence of flavonoids.

4.1.3.1.4. Qualitative tests for steroids and terpenoids

a. Salkowski test: The crude extract (about 100 mg) was separately shaken with chloroform (2 ml) followed by the addition of concentrated H_2SO_4 (2 ml) along the side of the test tube, a reddish brown coloration at the interface indicates the presence of terpenoid.

b. Liebermann-Burchard test: Extract (100 mg) was shaken with chloroform in a test tube; few drops of acetic anhydride were added to the test tube and boiled in a water bath and rapidly cooled in chilled water. Concentrated H_2SO_4 (2 ml) was added along side of the test tube. Formation of a brown ring at the junction of two layers and turning the upper layer to green shows the presence of steroids while formation of deep red color indicates the presence of triterpenoids.

4.1.3.1.5. Qualitative test for tannins:

Extract was stirred with distilled water (10 ml) and then filtered. A few drops of 5% ferric chloride were added. Black or blue-green coloration or precipitate was taken as a positive result for the presence of tannins.

4.1.3.1.6. Qualitative test for Saponins:

Five ml of plant extract was treated with sodium bicarbonate with vigorous shaking for 3 to 4 mins, formation of honey comb like froth shows the presence of saponins.

4.1.3.1.7. Qualitative test for Glycosides

Cardiac glycoside (Keller-Killiani test): Extract (0.5 g) was shaken with distilled water (5 ml). To this, glacial acetic acid (2 ml) containing a few drops of ferric chloride was added, followed by H_2SO_4 (1 ml) along the side of the test tube. Formation of the brown ring at the interface gives positive indication for cardiac glycoside and a violet ring may appear below the brown ring.

4.1.3.2. Quantitative estimation of phytochemicals:

4.1.3.2.1. Determination of total phenol content:

Folin-Ciocalteu colorimetric method was used to determine the phenolic content in the methanolic extracts. Briefly, all the extracts and the standard (gallic acid) were serially diluted to get the concentration of 200, 400, 600, 800 and 1000µg/ml separately in a test tube. To each test tube containing 1ml of plant extract with different concentration was reduced with 1 ml of FC reagent and the reactions were neutralized by adding 2 ml of sodium carbonate (7.5% w/v). The blue colored complex is formed after incubating the reaction mixture at dark for 90 mins at 23^{o}C. After incubation the absorbance of the reaction mixture was read at 760 nm. The phenol content of extracts was evaluated from a gallic acid calibration curve and was expressed as milligrams of gallic acid equivalents (GAE) per gram of dried sample. The experiments were performed in triplicates. (Adriana *et al.*, 2012; Mahfusa *et al.*, 2013).

4.1.3.2.2 Determination of total flavonoid content.

The aluminium chloride method was employed for the determination of total flavonoid content in the plant extract. Briefly, all the extracts and the standard (catechol) were serially diluted to get the concentration of 200, 400, 600, 800 and 1000µg/ml separately in a test tube. To each test tube, 4 ml of distilled water and 0.3 ml 5% sodium nitrite was added separately. After 5 mins, 0.3 ml of 10% aluminium

chloride was added and further the reaction mixture is accompanied by the addition of 2ml of 1M sodium hydroxide solution. The volume of the reaction mixture was made up to 10 ml by adding distilled water. The absorbance of the reaction mixture was measured spectrophotometrically at 510 nm against a blank. Results were expressed as catechol equivalents and the experiment were performed in triplicates (Nimmi *et al*., 2012).

4.1.5. Determination of *in vitro* anti oxidant activity of methanol extract

4.1.5.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay:

The ability of plant extract to scavenging DPPH free radicals was assessed by the method of Murali *et al*. (2013) with minor modification. The DPPH stock solution was prepared by dissolving 24 mg DPPH in 100 ml methanol and stored at 4°C until it is used. The working solution was obtained by diluting the DPPH solution with methanol to attain an absorbance of about 0.98±0.02 at 517 nm using the spectrophotometer. The stock solution of methanolic extract and ascorbic acid was prepared in methanol to obtain the concentration of 1000 µg/ml separately. Further, both the mixtures were diluted to get the concentration 200, 400, 600, 800 and 1000 µg/ml respectively. 2 ml of DPPH in methanol solution was added to each test tube separately. All the test tubes were kept in the dark for 30 mins. Further the absorbance of the control and the test samples were taken at the 517 nm. Radical scavenging activity was calculated using the formula: % of inhibition = $[(A_{control}-A_{test}/A_{control}]$ x 100. The half maximum inhibitory concentration of DPPH radicals (IC_{50}) was calculated according to the standard curve and the experiment were conducted in triplicates and results were expressed in mg/ml.

4.1.5.2. Ferric reducing assay:

Ferric reducing assay was carried according to the methodology described by (Fejes *et al.*, 2000). Different concentration of plant extracts (200 - 1000µg/ml) were taken in a separate test tubes, to this 2.5 ml of 1% Potassium ferricyanide solution is added individually and solution is kept at 50^{o}C in a water bath for 20 min. After incubation period, the reaction was stopped by adding 2.5 ml of 10% trichloroacetic acid solution after cooling. The reaction mixture was centrifuged at 3000 rpm for 10 min and the supernatant was taken. Then 0.1% of ferric chloride is added to a mixture

of 2.5 ml of supernatant and 2.5 ml of distilled water. The mixture is kept for 10 min and the absorbance was read at 700 nm. Control was prepared in a similar way excluding samples and ascorbic acid samples were used as a positive control.

4.1.5.3. Metal chelating activity

Metal chelating capacity of methanol extracts was measured according to the method described by Suerialoasan *et al*. (2015). 1 ml of different concentration (100-1000µg/ml) of extracts was added to 0.05 ml of 2mM ferric chloride solution. The reaction was initiated by the addition of 0.2 ml of 5 mM Ferrozine and the mixture was shaken vigorously. After 10 min, the absorbance of the test solution was measured at 562 nm against blank. All readings were taken in triplicate and EDTA was used as the standard. The percentage of metal chelating activity as calculated by following equation.

% of inhibition (Inhibition of ferrozine-Fe^{2+} complex) = [($A_{control}$-A_{test}/$A_{control}$] x 100

IC_{50} values, which represented the concentration of the extract that caused 50% of Fe^{2+} ion chelation, were calculated from the plot of chelating percentage against concentration.

4.1.6. Extraction of bioactive compounds from *Hypericum mysoresence*

Based on the results of antioxidant and antimicrobial activities of methanolic extracts of ten plants, *Hypericum mysoresence* was selected for further studies like sequential extraction of phytochemicals, phyochemical analysis, *in vitro* antioxidant activities, and antimicrobial activites.

4.1.6.1 Solvent extraction:

The leaf material of *Hypericum mysoresence* was collected in Mulayyanagiri hills of Chikmagalore district, Karnataka, India. The collected leaves was thoroughly washed in a running tap water, shade dried and pulverized mechanically using a blender. The powdered leaf material of 1 kg was packed in a soxhlet apparatus and refluxed successively with the solvents in the following order, first by petroleum ether (2.5 l, 60° – 80° C, Himedia, Mumbai, India), chloroform (2.5 l, 61° C, Himedia, Mumbai, India), methanol (2.5 l, 65° C, Himedia, Mumbai, India) and at last by

double distilled water for minimum of 48 hours to get Petroleum ether extract (PEH), Chloroform extract (CEH), Methanol extract (MEH) and aqueous extract (AEH) respectively. The extracts were filtered through Whatmann filter paper No.1, (Himedia, Mumbai, India), the filtrates were concentrated in vacuum under reduced pressure and then the extracts were kept in a water bath for complete evaporation of solvent. The dried extracts were preserved in desiccators and further stored in screw capped bottle at 4^{o} C in a refrigerator for further work.

4.1.6.2.Phytochemical analysis of crude extracts of *Hypericum mysoresence*

4.1.6.2.1 Qualitative extimation of phytochemical of crude extracts of *H. mysoresence*

Phytochemical analysis of crude extracts of *Hypericum mysoresence* like PEH, CEH, MEH, and AEH was carried using standard procedure as described earlier in the methodology section 4.1.3. Crude extracts was screened for the presence or absence of Flavonoids, Alkaloids, Tannins, Steroids, Terpenoids, Saponins, Glycosides, and Carbohydrates

4.1.6.2.2. Quantitative estimation of phytochemical of crude extracts of *H. mysoresence*

Crude extracts of *Hypericum mysoresence* like PEH, CEH, MEH, and AEH was subjected for quantitative estimation of phytochemicals. Determination of total phenol content (TPC) and total flavonoid content (TFC) was done by following standard procedure as described in the methodology section 4.1.4.

4.1.6.3 Determination of *in vitro* Anti oxidant activity of crude extracts of *Hypericum mysoresence.*

In vitro antioxidant activity such as DPPH assay, Ferric reducing assay and metal chelating assay was done for PEH, CEH, MEH, and AEH by following the standard methodology as described earlier in the methodology section 4.1.5.

4.2 Purification and identification of potent purified compound.

4.2.1. Isolation and purification of pure compound

Based on the results of inhibitory activity of crude extracts of *Hypericum mysoresence*, PEH was selected for further study and separation is done by column chromatography.

4.2.1.1.Standardization of mobile phase

In order to select the best mobile phase for eluting the individual fraction, 5µl of a 100mg/ml solution of crude extract was spotted on TLC and run with different combination of solvents with increasing polarity to get a single spot. The active compounds can be detected by using UV chamber and non UV visible compounds were detected by iodine chamber. In this way the solvent system which is exhibiting the most favorable were used for the separation of compounds.

4.2.1.2.Column packing

Glass column with (50cm) long with a diameter of (4 cm) was used for separation of constituents from the active extracts. A small plug of cotton was placed at the bottom of the column and approximately 40 gm of silica (Hi media, 60-120 mesh size) gel activated for 24h at 80^0C in an oven. The slurry was prepared using chloroform and was transferred into the column using a long stirring glass rod. The $^3/_4{}^{th}$ of column was filled with eluting solvent. Conical flask was placed under the column while adding the mixture to the column and allowed the solvent to flow freely for the rest of the experiment.

4.2.1.3.Loading the plant extract to the column

The eluting solvent was drained to approximately 1cm above the top of the silica bed. Using a long pipette, the extract was transferred to the top of the silica bed making sure not to squirt the mixture onto the inner sides of the column. Later, the mixture was allowed to adsorb on the top of the silica gel before adding more elution solvent. Whatmann filter paper was cut to the size of column diameter and inserted at the top end of the stationary phase to prevent disruption during addition of solvent. Once the addition of solvent to the column was started, the solvent level was maintained not to go below the top of the silica bed. The eluting solvent, instead of

rising by capillary action up a thin layer, flows down through the column filled with the adsorbent. Just as in TLC, there is an equilibrium established between the solute adsorbed on the silica gel or alumina and the eluting solvent flowing down through the column.

4.2.1.4. Monitoring the column with TLC

The eluted fraction was monitored by comparative TLC of extract and column eluted fraction continuously. Using capillary tube, approximately 5µl eluted fraction was loaded on TLC plates (Himedia) just 3mm above from the bottom. Fractions with the similar TLC pattern were pooled together and concentrated at reduced pressure and temperature. The concentrated components were further dried in vacuum desiccators. Completely dried components were weighed to calculate the total mass extracted and the extracted compound was numbered tentatively with a suffix F_1, F_2, F_3 and so on. The obtained pure fractions were subjected for antimicrobial activities against pathogens of dental caries.

4.2.2. Purification and identification of potent purified compound.

Based on the results of antimicrobial activity, five compounds were selected out of 19 purified compounds. These compounds were sent to sophisticated test and instrumentation centre (STIC), Cochin University, Kochi for spectral studies like HPLC, LCMS and proton nuclear magnetic resonance spectroscopy etc. Obtained spectral data were incorporated in the result section, so as to deduce the structure of each compound.

4.3. Collection and screening of clinical samples for dental caries isolates

4.3.1. Collection of infected teeth samples

Clinical samples were collected from dental caries patients visiting to District Mc- Gann hospital, Shivamogga, Karnataka, India. Teeth samples with dental lesions were collected from patients after being uprooted by the dentists in a reduced transport medium (0.4% agar, 0.15% thioglycollate/phosphate buffered saline 0.001M). After collection, samples were brought to the laboratory and further processed for the isolation of pathogens (Druw *et al*., 2012).

4.3.2. Isolation of pathogens of dental caries.

Blood agar (supplemented with 5% Sheep blood), Mutans Sanguis agar (MS), Mitis Salivarious agar (MIS), deMan, Rogosa and Sharpe agar (MRS) (HiMedia, Mumbai) were used as selective and differential media for the isolation of bacterial pathogens. Sabouraud dextrose agar media (SDA) (HiMedia, Mumbai) was used for the isolation of fungal pathogens. Clinical samples were vortexed, streak inoculated on the above said medium and plates were kept for incubation at 37^0C for 24 to 48 hours for bacteria and at 30^oC for 24 to 72 hours for fungal isolation. After the incubation, cultural characteristics of clinical isolates such as color, size, shape, elevation and margin of colony on BA, MSA, MIS, MRS and SDA media were recorded based on the manufacturer's instructions (Table 4.2). These cultures were further sub cultured on slants containing Brain Heart Infusion agar (BHIA) and nutrient agar for bacterial cultures and fungal cultures were sub cultured on SDA. The obtained pure cultures were stored at 4^0C in a refrigerator for further study.

Isolation media	Characteristic	Organism
Blood Agar media (BA)	Pin point colonies with alpha hemolysis	Viridans Group of Streptococci (Streptococcus species)
Mutans Sanguis agar (MS)	Heaped irregular colonies, white color, producing a drop of liquid on top of the colony with 0.5 to 2 mm in diameter	*Streptococcus mutans*
	Smooth or rough colonies which grow by adhering to the surface of the agar media with 1-3 mm in diameter	*Streptococcus sanguis*
Mitis Salivarious agar (MIS)	Small pin point blue color colonies	*Streptococcus mitis*
	Small smooth or rough gum drop colonies with 1-5mm in diameter	*Streptococcus salivarius*
deMan, Rogosa and Sharpe agar (MRS)	Large or small white colonies	Lactobacillus spp.
Saborauds Dextrose agar (SDA)	Round, oval shaped cells of about 4 to 8 mm in diameter.	Candida species

Table 4.2: Colony characteristics considered for the isolation of pathogens from different media.

4.4 Biochemical characterization and determination of antibiotic sensitivity pattern of dental caries isolates against standard antibiotics.

4.4.1. Biochemical Identification of clinical isolates

4.4.1.1. Gram's staining

A clean glass slide was taken and a circle was drawn at the centre of the slide with the help of a glass marker. On the opposite side, a loopful of culture from the slant was taken with the help of sterile inoculation loop and smeared uniformly inside the marked circle. The smear was air dried and then heat fixed by passing it over the flame few times in order to fix the cells firmly to slide surface. The slide was cooled and covered with crystal violet (primary stain) and allowed to stand for a minute. The slide was washed under running water to remove excess of crystal violet. The slide was covered with Gram's iodine (mordant), left for 30 sec and washed. Decolorization of the smear was done by treating the smear with 95% ethanol until no more trace of violet color goes out of the slide. The slide was gently washed, counter stained with safranine (counter stain) for 30 seconds to one minute and washed to remove excess safranine. The slide was blot dried and observed under oil immersion objective of the microscope (Sundararaj, 2003).

4.4.1.2 KOH solubility test

This test was conducted as a supporting test for Gram's staining. A drop of 3% KOH (Appendix) was placed at the centre of the glass slide. A loopful of the culture from slant was taken with the help of sterile inoculation loop, placed in the drop and mixed for 10 seconds. The inoculation loop was gently lifted and observed for mucoid thread formation if any (Sundararaj, 2003).

4.4.1.3 Optochin sensitivity test

This test was conducted to differentiate *Streptococcus pneumoniae* and other viridans streptococci. The BHIA slant cultures of isolated bacteria were sub-cultured in BHI broth and incubated at 37^{o}C for 24 hrs. The fresh broth cultures obtained were swabbed on sterile sheep blood agar plates uniformly using L-shaped glass rod. Optochin discs (HiMedia, Mumbai) were placed at the centre of inoculated plates,

gently pressed and the plates were incubated at 37ºC for 24 hours in inverted position. After incubation, the plates were observed for the zone of inhibition around the disc if any. Absence of inhibition zone around the disc confirms the presence of viridans streptococci in the specimen (Murray *et al*., 2003).

4.4.1.4 Catalase test (slide test)

A drop of 3% H_2O_2 (Appendix) was taken on a clean glass slide. A loopful of the culture from the slant was taken with the help of sterile inoculation loop and mixed with the drop of H_2O_2. Observation was made for the formation of bubbles or effervescence. Appearance of bubbles was taken as positive for catalase production by the isolate (Sundararaj, 2003).

4.4.1.5 Arginine hydrolysis

In this test, the ability of the isolate to hydrolyze arginine was detected. Mueller's decarboxylase broth supplemented with arginine (Appendix) tubes were inoculated with a loopful of slant cultures and immediately overlaid with sterile mineral oil. The tubes were incubated aerobically at 37ºC for up to 7 days. The observation was made for deep purple color development in the broth which was taken as positive result. The development of yellow color or no color change was considered as negative result (Murray *et al*., 2003).

4.4.1.6 Esculin hydrolysis

The ability of bacteria isolated from dental caries patients to hydrolyze esculin was tested in esculin agar (Appendix). The sterile esculin agar (HiMedia, Mumbai) slants were inoculated with loopful cultures and incubated aerobically at 37ºC for 48 hours. Formation of black color over one-half or more of the slant was taken as positive result (Sundararaj, 2003).

4.4.1.7 Urea hydrolysis

The ability of the isolated bacteria to hydrolyze urea was detected using urea as the substrate. A loopful of cultures from slants was aseptically inoculated into test tubes containing sterile urea broth using sterile inoculation loop. The tubes were incubated aerobically at 37ºC for up to five days. The observation was made for

development of pink color in the broth which was taken as positive for urease production by the isolate (Sundararaj, 2003).

4.4.1.8 Voges-Proskauer (VP) test

This test specifically detects the production of neutral end products from the aerobic metabolism of glucose. Briefly, the sterile MR-VP broth (Appendix) tubes were inoculated with loopful cultures from slants and incubated at 37°C for 48 hours. After incubation, 12 drops of alpha naphthol and 4 drops of KOH was added to the test tubes, shaken vigorously and left for 30 minutes with plug open. Observation was made for color change to pink or red which was considered positive (Sundararaj, 2003).

4.4.1.9 Mannitol fermentation

This test is used to detect the ability of isolate to ferment mannitol with the production of acid. Sterile mannitol broth (Appendix) tubes were inoculated with fresh cultures from BHA slants and incubated at 37°C for 48 hours. A positive reaction was recorded in case of color change to yellow (due to acid production) (Sundararaj, 2003).

4.4.1.10. Sorbitol fermentation

The ability of the viridans streptococci to utilize sorbitol and produce acid was tested using sorbitol broth. A loopful fresh culture from BHI agar slant was aseptically inoculated into sterile sorbitol broth tubes and incubated at 37°C for 48 hrs. A positive reaction was indicated by color change to yellow (Sundararaj, 2003).

4.4.1.11 Carbhohydrate Fermentation

This test is performed to know the ability of bacteria ferment the glucose to produce acid and gas. Clinical isolates were inoculated into glucose broth containing Durhams tube and incubated for 24 to 48 hrs. After incubation observation were made for color change in the media and gas production in the Durhams tube (Sundararaj, 2003 and Murray *et al*., 2003).

4.4.1.12 Germ Tube Test

Clinical isolates recovered from SDA media, were allowed to grow again on SDA media for 24 hrs. at 37^{O}C individually. Then the cultures were diluted to 15X 10^{9} cells/ml in

sterile physiological solution. About 100 µl of culture suspension was taken and added to 0.5 ml of human serum, and indubated at 37OC for 2 to 3 hrs. and observed for germtube formation under light microscope (Sandra AM, *et al.*,2010).

4.4.2. Determination of antibiotic susceptibility pattern of clinical isolates using standard antibiotics.

4.4.2.1. Antibacterial susceptibility test of clinical isolates against standard antibiotics.

A total of 20 clinical isolates were selected randomly from each species *i.e.* 20 clinical isolates from *Streptococcus mutans, Streptococcus sanguis, Streptococcus mitis, Streptococcus salivarious* and *Lactobacillus acidophilus* were selected for the study. Antibacterial susceptibility test was also done for standard strains namely *Streptococcus mutans* (MTCC 497) and *Streptococcus mutans* (MTCC 897). Antibiotic susceptibility test to clinical isolates using standard antibiotics was assessed by following the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2012). Briefly, Bacterial strains were first grown in Mueller Hinton broth (MHB) at 37℃ for 24 hour and after the incubation period the turbidity of the culture was maintained to 0.5 McFarland turbidity to yield 10^8 CFU/ml of bacteria. From this 100 µl of the test inoculum were spread evenly with a sterile glass spreader on Mueller Hinton Agar (Hi Media) plates and allowed to dry for 10 min. Discs of different standard antibiotics (Table 4.3) were placed on top of the inoculated media with the help of sterile forceps. The plates were left for 30min at room temperature for compound diffusion and then incubated in upright position for 24 hrs. at 37^0C. The inhibition zones were measured in mm and scored as sensitive, intermediately susceptible and resistant according to the CLSI recommendations.

Sl No	Antibiotic
1	Ampicillin (A)
2	Chloramphenicol (C)
3	Erythromycin (E)
4	Gentamicin (G)
5	Kannamycin (K)
6	Norfloxacin (Nx)
7	Ofloxacin (Ox)
8	Penicillin (P)
9	Polymixin (Pb)
10	Streptomycin (S)
11	Tetracycline (T)
12	Trimethoprim (Tr)

Table 4.3: List of standard antibiotic selected for the study.

4.5. Bioassay of Extracted Compounds on Selected Dental Caries Isolates

4.5.1. Antimicrobial activity of methanol extracts of medicinal plants against pathogens of dental caries.

4.5.1.1. Antibacterial activity of methanol extracts against pathogens of dental caries

4.5.1.1.1. Preparation of bacterial inoculum

All the selected bacterial isolates and standard bacterial strains were subjected for antibacterial activity. Bacterial strains were initially grown in Mueller Hinton Broth (MHB) at 37°C for 24 hours. After incubation period the turbidity of the culture was maintained to 0.5 McFarland turbidity to yield $1x10^8$ CFU/ml and kept for further work (Jain *et al.,* 2015).

4.5.1.1.2. Antibacterial activity

A total of 20 clinical isolates were selected randomly from each species i.e. 20 clinical isolates from *Streptococcus mutans, Streptococcus sanguis, Streptococcus mitis, Streptococcus salivarious* and *Lactobacillus acidophilus* respectively. Antibacterial activity was also done for standard strains of *Streptococcus mutans* MTCC no: 497 and *Streptococcus mutans* MTCC no: 897. Antibacterial activity of leaf extracts of all plants (methanol extracts) was determined by the agar well diffusion method by following CLSI 2012 standards. Bacterial strains were first grown in Mueller Hinton broth (MHB) at 37℃ for 24 hours and after the incubation period the turbidity of the culture was maintained to 0.5 McFarland turbidity to yield $1x10^8$ CFU/ml of bacteria, from this 100 µl of the test inoculum were spread evenly with a sterile L shaped glass spreader on Mueller Hinton Agar (MHA) plates. The seeded plates were allowed to dry, wells were made using sterile 6mm cork borer in the plate. The extracts were prepared by dissolving the extract with 10% DMSO to get the concentration of 10 mg/ml, 25mg/ml and 50 mg/ml respectively and 100 µl of extract was loaded into the labelled wells. The standard (Tetracycline, 10 µg/ml) and control (10% DMSO) were added into the labelled wells. The plates were incubated at 37℃ for 24 hours. The plates were observed for the presence or absence of inhibition of bacterial growth that was indicated by a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity was expressed as average diameter of the zone of inhibition in millimeters (Gamboa *et al.,* 2004).

4.5.1.2. Antifungal activity of methanol extracts against *Candida albicans*.

4.5.1.2.1. Preparation of Fungal inoculum

Fungal inoculums of *Candida albicans* was first grown in Sabarauds Dextrose Broth (SDB) at 37℃ for 24 hours and after the incubation period the turbidity of the culture was maintained to 0.5 McFarland turbidity to yield 2.5×10^6 CFU/ml, which was optically measured at 530 nm wavelength (OD value 0.12) and used for further work (Ghaleb *et al.,* 2012).

4.5.1.2.2. Antifungal activity

A total of 20 clinical isolates of *Candida albicans* were selected for antifungal activity against ten methanolic plant extracts by following agar well diffusion method.

Briefly, a suspension of *C. albicans* (24 hours) was made by physiological saline. Briefly, *C. albicans* was first grown in Sabarauds dextrose broth (SDB) at 37°C for 24 hours and after the incubation period the turbidity of the culture was maintained to 0.5 McFarland turbidity to yield 2.5 x 10^6 CFU/ml which was optically measured at 530 nm wavelength (OD value 0.12). Next, 10 µl of suspension was transferred to SDA medium and swabbed on the surface with the help of glass spreader. Further wells of 6 mm diameter were made on the surface of inoculated media and wells were labelled. The crude methanol extracts of ten selected plants was dissolved in 10% DMSO to get the concentration of 10mg/ml, 25mg/ml and 50 mg/ml was prepared and 100 µl of these extracts loaded into labeled wells with the help of micropipette. Fluconozole (10 µg/ml) was taken as positive control and 10 % DMSO was kept as negative control. Further the plates were kept for incubation at 30^0 C for 24 to 48 hours. After incubation the plates were observed for the inhibition zone (Madhumitha and Saral, 2011).

4.6. Bioassay of crude and purified compounds of *H.mysoresence* on selected dental caries isolates.

4.6.1 Determination of antimicrobial activity

The antimicrobial activity of PEH, CEH, MEH, AEH and purified compounds was evaluated by standard agar well diffusion method with slight modification. 0.1ml of the test inoculum (both bacteria and fungi) were spreaded evenly with a sterile glass spreader on Mueller Hinton agar (Hi Media) plates for bacteria and Saborauds agar (Hi Media) plates for fungi. The seeded plates were allowed to dry, wells were made using sterile 6mm cork borer. 100 µl of PEH, CEH, MEH and AEH extracts with different concentration was loaded to the labelled wells (10 mg, 25mg and 50 mg/ml was dissolved in 10 % DMSO). For purified compounds (19 compounds) obtained by petroleum ether crude extract was dissolved in 10% DMSO to get 1mg/ml concentration was loaded. Tetracycline (10 µg/ml) was taken as positive control for antibacterial activity and Fluconozole (10 µg/ml) was taken as positive control for antifungal activity. 10% DMSO was taken as negative control. Further, the plates were incubated at 37°C for 18 to 24 hours for bacteria and 30°C for 24 hours

for fungi. After incubation the plates were observed for zone of inhibition and results were recorded as average value in millimeter (Jain *et al.*, 2015;Ghaleb *et al.*, 2012).

4.6.2. Determination of Minimum inhibitory concentration (MIC), Minimum Bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC) of crude extracts and pure compounds of *Hypericum mysoresence*.

MIC is defined as the lowest inhibitory concentration of the compound, at which visual growth of microorganism is inhibited. MIC was determined by broth micro-dilution method by following CLSI guidelines. In this study, the minimum inhibitory concentration was performed for PEH, CEH, MEH, AEH and purified compounds (F3, F4, F5, F9 and F10). For crude extracts the initial concentration was taken as 10 mg/ml. The crude extract of PEH, CEH, MEH and AEH extracts were dissolved in 2% dimethyl sulfoxide (DMSO) and was serially diluted up to two folds to get the concentration of 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, and 0.625 mg/ml respectively. For purified compounds, the initial concentration was taken as 1mg/ml**.** The pure compounds were dissolved in 2% dimethyl sulfoxide (DMSO) and were serially diluted up to two folds to get the concentration of 1mg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.50 µg/ml, 31.25 µg/ml, 15.62 µg/ml, 7.81 µg/ml and 3.90 µg/ml respectively. Each tube containing Muller Hinton Broth for bacteria and Saborauds dextrose broth for fungus were inoculated with 0.5 ml of cell suspension containing 10^8 CFU/ml of bacteria, 2.5 X 10^6 CFU/ml of fungi and 1 ml of diluted crude and pure compound respectively. The tubes with bacteria were incubated for 24 hrs at 37°C and for fungal tubes were incubated at 30° C for 48 hrs. The lowest concentration of the crude extracts and pure compounds that produced no visible growth (no turbidity) on compared with control was regarded as MIC (Katsura *et al.*, 2001).

A loop full of inoculums from respective MIC tubes was streaked on sterile plates containing Mueller Hinton agar for bacteria and Saborauds agar for fungus to determine Minimum bactericidal concentration and Minimum fungicidal concentration respectively. Plates were incubated for 24 hours at 37°C and observed for the growth of bacteria. The highest dilution that yielded no bacterial growth was

taken as MBC and that showing no fungal growth was taken as MFC (Katsura, *et al.*, 2001).

4.6.3 Statistical analysis

Data are expressed as mean ± SD from three separate observations. All analyses were carried out in triplicates. Statistical analysis was conducted by using SPSS software version 12.0. Pearson correlation analysis was used to evaluate the relationships among the variable of interest. A P- value less than 0.05 were considered statistically significant. Microsoft Excel 2007 (Roselle, IL, USA) was also used for some of the statistical and graphical evaluations.

5. Results

5.1. Collection, extraction of bioactive compounds from traditional medicinal plants

5.1.1. Collection of plant materials

A total of ten plants were selected by regular field visit to Chikkamagalore and Shivamogga districts of Karnataka state, India. Plants were identified with the help of a taxonomist by observing their morphological characters like stem, leaves and flowers. Depending on these characters the collected plants were identified and assigned to their respective families (Table 5.1 and Plate 1 and 2). Herbarium samples of the respective plants with accession number were kept in the Department of Microbiology, Sahyadri Science College (A), Shivamogga.

Sl. No.	Name of the plant	Classification				Accession no
		Kingdom	Division	Order	Family	
1	*Anisomeles indicus*	Plantae	Angiosperms	Lamiales	Lamiaceae	SSCMBH1
2	*Annona muricata*	Plantae	Angiosperms	Magnoliales	Annonaceae	SSCMBH2
3	*Azadiractha indica*	Plantae	Angiosperms	Sapindales	Meliaceae	SSCMBH3
4	*Crysophyllum roxburghii*	Plantae	Angiosperms	Ericales	Sapotaceae	SSCMBH4
5	*Hypericum mysoresence*	Plantae	Angiosperms	Malpighiales	Hypericeaceae	SSCMBH5
6	*Malphigia glabra*	Plantae	Angiosperms	Malpighiales	Malpighiaceae	SSCMBH6
7	*Mangiferra indica*	Plantae	Angiosperms	Sapindales	Anacardiaceae	SSCMBH7
8	*Scleropyrum pentandrum*	Plantae	Angiosperms	Santalales	Santalaceae	SSCMBH8
9	*Toddalia asiatica*	Plantae	Angiosperms	Sapindales	Rutaceae	SSCMBH9
10	*Wrightia tinctoria*	Plantae	Angiosperms	Gentianales	Apocynaceae	SSCMBH10

Table 5.1: Details of selected plants with their respective taxonomic ranks.

5.1.2. Solvent extraction

The leaf materials (500gm) of ten plants individually soaked with methanol to yield respective methanol extract. Methanolic extracts of all plants yielded a large quantum of crude material which varies from green to dark green in color with sticky solid mass. Methanolic extracts thus obtained from leaf materials were stored in refrigerator at 4^0C for further studies like phytochemical screening, antioxidant and antibacterial activities against clinically isolated pathogens of dental caries and standard strains of *S. mutans*.

5.1.3. Phytochemical screening of methanol extract.

5.1.3.1. Qualitative analysis of methanol extracts

The qualitative analysis of all methanolic extracts showed the presence of phytochemicals and the results were represented in the table 5.2. Flavonoid was found in all methanolic extracts and Carbohydrate was also found in all the plants except *A. indicus, C. roxburghii, H. mysoresence and W. tinctoria*. Alkaloids were noticed in all plants except *W.tinctoria*. Tannins was absent in *A. muricata, M. glabra, S. pentandrum, T. asiatica,* and *W. tinctoria*. Steroids are present in *A. indicus, A. muricata, H. mysoresence, M. glabra, T. asiatica and W. tinctoria*. Terpenoids was found in all plants except *A. muricata, M. glabra* and *W. tinctoria*. Saponins was absent in *A. indicus, A. indica and H. mysoresence*. Cardiac glycoside was found in all plants except *A. indica, C. roxburghii, H. mysoresence and W. tinctoria* (Table 5.2).

5.1.4. Quantitative estimation of phytochemicals:

5.1.4.1 Determination of total phenol content

The total phenolic content (TPC) was determined by using Folin-Ciocalteu's reagent, total phenolic content of the methanolic extracts of different plants are expressed as milligrams of gallic acid equivalents. The obtained results were tabulated in the table 5.3. Briefly, highest TPC was found in *H. mysoresence* (71.32±0.16 µg /mg) followed by *W. tinctoria* (68.35±0.16 µg /mg), *T. asiatica* (65.10±0.21 µg /mg), *M. indica* (59.23±0.25 µg /mg), *S. pentandrum* (58.37±0.36 µg /mg), *A. muricata* (58.2±0.72 µg /mg). Moderate phenol content was observed in *A. indica* (56.38±0.52

µg /mg), *C. roxburghii* (56.23±0.23 µg /mg) and *A. indicus* (51.30±0.68 µg /mg). Least phenol content was observed in *M. glabra* (49.62±0.81 µg /mg).

5.1.4.2. Determination of total flavonoid content

Total flavonoid content in methanolic extracts of selected plants was determined using spectrophotometric method using aluminum chloride. The total content of flavonoids was expressed as milligrams of catechol equivalent. The obtained results were represented in the table 5.3. In brief, highest flavonoid content was found in *W. tinctoria* (63.16±0.25 µg/mg) followed by *H. mysoresence* (61.23±0.32 µg/mg), *S. pentandrum* (53.66±0.45 µg/mg), *T. asiatica* (52.23±0.36 µg/mg), *M. indica* (51.25±0.25 µg/mg), *M. glabra* (51.25±0.26 µg/mg). Moderate phenol content was observed in *A. muricata* and *A. indica with* 49.62±0.23 µg/mg and 49.56±0.26 µg/mg respectively. Least phenol content was found to be in *A. indicus* (30.65±0.72 µg/mg).

5.1.5. Determination of *in vitro* anti oxidant activity of methanol extract

5.1.5.1. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay:

The results of conversion of $DPPH^+$ (free radical) into DPPHH by methanolic extracts of all plants exhibited promising antioxidant activity in dose dependent manner (Table 5.4). The free radical scavenging activity of standard (ascorbic acid) was greater than that of methanolic extracts. The IC_{50} values of all methanolic extracts were calculated and represented in the table 5.5, the highest radical scavenging activity was observed in *H. mysoresence* (261.90 µg/ml) followed by, *S. pentandrum* (335.0 µg/ml), *A. muricata* (335.0 µg/ml), *A. indica* (356.23 µg/ml), *W. tinctoria* (365.80 µg/ml), *T. asiatica* (457.77 µg/ml), *M. indica* (479.15 µg/ml), *C. roxburghii* (495.28 µg/ml), *A. indicus* (624.65 µg/ml) and least activity was observed in *M. glabra* (1090 µg/ml).Pearson correlation data between TPC and DPPH assay reveals that, a positive linear correlation was observed which indicates that phenol content present in each plant extract are responsible for their respective antioxidant activity (Table 5.6). Highest correlation was observed in the *T. asiatica* (Correlation coefficient 0.999, $P<0.01$), followed by *W. tinctoria* (Correlation coefficient 0.990, $P<0.01$) and *S. pentandrum* (Correlation coefficient 0.986, $P<0.01$).

5.1.5.2. Ferric reducing assay:

The Ferric reducing assay was carried to estimate the antioxidant capacity of the samples to transform Fe^{3+} to Fe^{2+} *in vitro*. The reductive power of sample was confirmed by change of yellow color of the test solution to various shades of green and blue depending on the concentration of the methanol extracts. The reducing power of the extracts and standard ascorbic acid increased with increase in concentration. In this test, the OD ranges from 0.272 to 1.151 for ascorbic acid(Table 5.4). At 1000 µg/ml the highest reducing power was observed in *H. mysoresence* (0.894± 0.002) and *S. pentandrum* (0.894 ± 0.004) followed by *C. roxburghii* (0.827 ± 0.004), and *M. glabra* (0.818 ± 0.001). Moderate reducing power was observed in case of methanol extracts of *A. indica* (0.795 ± 0.006), *A. muricata* (0.795±0.006) and *W. tinctoria* (0.712± 0.00). Least activity was observed in methanol extract of *M. indica* (0.692 ± 0.001).

A. indicus (0.647±0.016) and *T. asiatica* (0.640 ± 0.017). In this present study, positive linear correlation was observed between TPC and ferric reducing assay. The best relation between TPC and ferric reducing assay was best explained by the methanol extract of *H. mysoresence* (0.979, P<0.0) followed by *A. muricata*(0.974, P<0.01), *W. tinctoria* (0.970, P<0.01), *A. indica* and *C. roxburghii* with identical correlation value of 0.953, P<0.01 (Table 5.6). A linear correlation is also observed in all extracts between TFC and ferric reducing, but non-significant correlation is also observed in the extract of *A. indicus* with correlation value of 0.675

.5.1.5.3. Metal chelating activity

The metal chelating activity was employed to know the role of antioxidants in the extract which may cause the reduction of the Fe^{3+}ferricyanide complex to the ferrous form. Hence, measuring the formation of blue color at 700 nm can monitor the Fe^{2+} concentration. The results of metal chelating activity revealed that the methanol extracts showed dose dependent activity (Table 5.4). The IC_{50} value was calculated and highest metal chelating activity was observed in *S. pentandrum* (429.43 ± 0.12 µg/ml) followed by, *H. mysoresence* (446± 0.60 µg/ml), *M. indica* (451.23 ± 0.12 µg/ml), *A. muricata* (455.28 ± 0.12 µg/ml), *A. indica* (512.08 ± 0.12 µg/ml) and *W. tinctoria* (523.58 ± 0.12 µg/ml).

Name of the plant extract (Methanol)	Carbohydrate	Alkaloids,	Tanins,	Steroids,	Terpenoids,	Saponins,	Glycosides,	Flavonoids
Anisomeles indicus	-	+	+	+	+	-	-	+
Annona muricata	+	+	-	+	-	+	+	+
Azadiractha indica	+	+	+	-	+	-	-	+
Crysophyllum roxburghii	-	+	+	-	+	+	-	+
Hypericum mysoresence	-	+	+	+	+	-	-	+
Malphigia glabra	+	+	-	+	-	+	+	+
Mangiferra indica	+	+	+	-	+	+	+	+
Scleropyrum pentandrum	+	+	-	-	+	+	+	+
Toddalia asiatica	+	+	-	+	+	+	-	+
Wrightia tinctoria	-	-	-	+	-	+	-	+

'+' presence of phytochemicals, '-'the absence of phytochemicals

Table 5.2: Results of qualitative analysis of phytochemicals present in the methanol extract of medicinal plants selected for the study.

Name of the Plant extract (Methanol)/Tests	Total Phenol Content (mg of GAE/g)	Total flavonoid Content (mg of CE/g)
Anisomeles indicus	51.30±0.68	30.65±0.72
Annona muricata	58.2±0.72	49.62±0.23
Azadiractha indica	56.38±0.52	49.56±0.26
Crysophyllum roxburghii	56.23±0.23	43.25±0.56
Hypericum mysoresence	71.32±0.16	61.23±0.32
Malphigia glabra	49.62±0.81	51.25±0.26
Mangiferra indica	59.23±0.25	51.25±0.25
Scleropyrum pentandrum	58.37±0.36	53.66±0.45
Toddalia asiatica	65.10±0.21	52.23±0.36
Wrightia tinctoria	68.35±0.16	63.16±0.25

GAE= Gallic acid equivalent, CE= Catechol equivalent

Table 5.3: Total phenolic, total flavonoid contents in the methanol extract of medicinal plants selected for the study

Moderate activity was observed in *C. roxburghii* (590.16 ± 1.0 µg/ml), *T. asiatica* (594.06 ± 0.13 µg/ml), and *A. indicus* (640.67 ± 0.11 µg/ml). Least activity was observed in *M. glabra* (715 ± 0.11 µg/ml). Whereas, the IC_{50} value of standard, EDTA was found to be 414.59 ± 0.13 µg/ml indicating that methanol extracts showed less metal chelating activity when compared to standard (Table 5.5).The correlation between the TPC and Metal chelating assay showed varied results, the best linear correlation was observed in *H. mysoresence* (0.999, P<0.01 l) followed by *S. pentandrum* (0.996, P<0.01) and *M. indica* (0.994). In our study, positive correlation was observed between TFC and metal chelating activity, best correlation was observed in *M. indica* with r value 0.998, P<0.01 and least r value was obtained in *A. indicus* (0.928, P<0.01) (Table 5.6).

5.1.6. Extraction of bioactive compounds from *Hypericummysoresence*

5.1.6.1. Solvent extraction

The leaf material was refluxed successively, with solvents like petroleum ether, chloroform, methanol and double distilled water, in a soxhalet extractor for 48 hours (Plate 3a). The extract was filtered through Whatmann filter paper No. 1 and the filtrates were concentrated under reduced pressure. Among the four solvent extracts, petroleum ether extract has given a maximum yield of 23g/Kg with dark green color followed by methanol 16 g/kg. Chloroform extract yields a thick sticky mass of 5g/kg and lowest yield was obtained in water with brown powdery mass of 3g/kg**.**

All the above extract was further checked for phytochemical analysis, *in vitro* antioxidant activities, and antimicrobial activities against selected clinical isolates.

5.1.6.2.Phytochemical analysis of crude extract of *H. mysoresence*

5.1.6.2.1 Qualitative phytochemical analysis of crude extracts of *H.mysoresence*

Phytochemical analysis was done for petroleum ether extract (PEH), chloroform extract (CEH), methanol extract (MEH) and aqueous extract (AEH). The results revealed that, alkaloids and flavonoids were noticed in MEH and AEH. Steroids were present only in PEH and presence of triterpenoids was observed only in PEH and MEH. Tannins especially pseudotannins and gallotannins was found to be present only in PEH and MEH respectively.Saponins was absent in all the extracts (Table 5.7).

5.1.6.2..2 Quantitative phytochemical analysis of crude extracts of *H.mysoresence*

5.1.6.2.2.1 Total phenol content:

The total phenol content (TPC) was determined by using Folin-Ciocalteu's reagent, total phenol content of PEH, CEH, MEH and AEH of *H. mysoresence* are solvent dependent and values are expressed as milligrams of gallic acid equivalents (Table 5.8). Highest TPC was found in AEH (165.3 ± 2.51), followed by MEH (127-±1.52 µg /mg) and CEH (64.0 ±0.5 µg /mg). Least phenol content was observed in PEH (50.3 ±1.25 µg /mg).

5.1.6.2.2.2 Total flavonoid content:

Total flavonoid content in crude extract of *H. mysoresence* was determined using spectrophotometric method with aluminum chloride. The total content of flavonoids was expressed as milligrams (mg) of catechol equivalent. Highest flavonoid content was found in MEH (126.3±1.15 µg/mg) followed by CEH (91.3 ±2.08 µg/mg). Least flavonoid content was observed in AEH (79.0 ±1.00 µg/mg). Flavonoids were found to be absent in PEH, as this extract does not produced pink color (Table 5.8).

Activity/ Name of the plant		*A. muricata*	*A. indicus*	*A. indica*	*C. roxburghii*	*H. mysoresence*	*M. glabra*	*M. indica*	*S. pentandrum*	*T. asiatica*	*W. tinctoria*
DPPH assay	**200**	63.75[a]	24.67 [a]	59.11 [a]	42.57 [a]	56.66 [a]	24.98 [a]	51.89 [a]	64.70 [a]	52.70 [a]	60.86 [a]
	400	67.80 [a]	38.75 [a]	66.33 [a]	52.64 [a]	84.158 [a]	26.38 [a]	55.62 [a]	65.87 [a]	54.45 [a]	65.87 [a]
	600	75.97 [a]	57.34 [a]	73.09 [a]	54.39 [a]	93.418 [a]	34.56 [a]	59.69 [a]	72.74 [a]	63.01 [a]	71.34 [a]
	800	77.95 [a]	62.36 [a]	77.75 [a]	74.83 [a]	93.535 [a]	41.32 [a]	68.6 [a]	81.82 [a]	70.10 [a]	74.02 [a]
	1000	79.5 [a]	68.08 [a]	84.74 [a]	78.91 [a]	94.816 [a]	49.35 [a]	71.92 [a]	84.74 [a]	76.35 [a]	77.75 [a]
Metal chelating assay	**200**	42.80 [a]	32.54 [a]	42.21 [a]	36.46 [a]	41.13 [a]	33.61 [a]	51.55 [a]	48.28 [a]	38.13 [a]	41.40 [a]
	400	57.25 [a]	42.48 [a]	47.20 [a]	45.54 [a]	56.33 [a]	37.05 [a]	56.39 [a]	57.08 [a]	46.34 [a]	48.33 [a]
	600	68.60 [a]	52.09 [a]	61.92 [a]	55.31 [a]	69.44 [a]	51.02 [a]	63.58 [a]	68.52 [a]	54.24 [a]	57.46 [a]
	800	73.52 [a]	60.63 [a]	72.44 [a]	63.90 [a]	78.03 [a]	52.57 [a]	71.21 [a]	76.58 [a]	62.29 [a]	67.99 [a]
	1000	77.87 [a]	64.98 [a]	73.89 [a]	67.99 [a]	81.09 [a]	58.59 [a]	79.05 [a]	80.55 [a]	67..23 [a]	76.58 [a]
Ferric reducing assay	**200**	0.403[b]	0.067 [b]	0.376 [b]	0.337 [b]	0.386 [b]	0.256 [b]	0.270 [b]	0.343 [b]	0.248 [b]	0.362 [b]
	400	0.485 [b]	0.183 [b]	0.463 [b]	0.555 [b]	0.485 [b]	0.380 [b]	0.346 [b]	0.487 [b]	0.296 [b]	0.396 [b]
	600	0.571 [b]	0.364 [b]	0.562 [b]	0.613 [b]	0.572 [b]	0.564 [b]	0.397 [b]	0.562 [b]	0.348 [b]	0.457 [b]
	800	0.785 [b]	0.526 [b]	0.661 [b]	0.780 [b]	0.784 [b]	0.687 [b]	0.482 [b]	0.791 [b]	0.455 [b]	0.508 [b]
	1000	0.894 [b]	0.647 [b]	0.795 [b]	0.827 [b]	0.894 [b]	0.818 [b]	0.692 [b]	0.894 [b]	0.640 [b]	0.712 [b]

'a' represents the % inhibition values for DPPH assay and metal chelating assay, 'b' represents the absorption values in ferric reducing assay

Table 5.4: Antioxidant activities of methanol extracts of medicinal plants selected for the study

Name of the Plant extract	DPPH assay (µg/ml)	Metal chelating assay (µg/ml)
Anisomeles indicus	624.65	640.67
Annona muricata	335.00	455.28
Azadiractha indica	356.23	512.08
Crysophyllum roxburghii	495.28	590.16
Hypericum mysoresence	261.90	446.00
Malphigia glabra	1090.0	715.00
Mangiferra indica	479.15	451.23
Scleropyrum pentandrum	335.00	429.43
Toddalia asiatica	457.77	594.06
Wrightia tinctoria	365.80	523.58

Table 5.5: IC_{50} values of methanol extract of selected plants in the present study

Name of the plant/Total phnolic and Total flavonoid content		DPPH	Metal chelating activity	Ferric reducing activity
A. muricata	**TPC**	0.976**	0.988**	0.974**
	TFC	0.997**	0.980**	0.933**
A. indicus	**TPC**	0.895*	0.953**	0.725
	TFC	0.863*	0.928**	0.675
A. indica	**TPC**	0.586	0.535	0.576
	TFC	0.999**	0.975**	0.953**
C. roxburghii	**TPC**	0.930**	0.939**	0.891*
	TFC	0.971**	0.975**	0.953**
H. mysoresence	**TPC**	0.975**	0.99**	0.979**
	TFC	0.978**	0.968**	0.924**
M. glabra	**TPC**	0.957**	0.975**	0.891*
	TFC	0.962**	0.981**	0.902*
M. indica	**TPC**	0.990**	0.994**	0.931**
	TFC	0.999**	0.998**	0.920**
S. pentandrum	**TPC**	0.986**	0.996**	0.950**
	TFC	0.999**	0.985**	0.915**
T. asiatica	**TPC**	0.999**	0.990**	0.911**
	TFC	0.994**	0.978**	0.877*
W. tinctoria	**TPC**	0.990**	0.988**	0.964**
	TFC	0.982**	0.995**	0.970**

'*'Significance at the 0.05 level, '' Significance at the level 0.01 level**

Table 5.6: Pearson correlation coefficient obtained between total phenolic, flavonoid composition and different antioxidant activities

5.1.6.3 Determination of *in vitro* antioxidant activities of crude extracts of *H. mysoresence*

5.1.6.3.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay:

The results of conversion of $DPPH^+$ (free radical) into DPPHH by PEH, CEH, MEH and AEH showed potent antioxidant activity in dose dependent manner. The IC_{50} values of all the extracts were calculated and represented in table 5.9, the highest radical scavenging activity was observed in AEH (IC_{50} 126.98 ± 0.29 µg/ml) followed by PEH (IC_{50} 305.02± 0.67 µg/ml) and MEH (IC_{50} 344.46±0.62 µg/ml).Least scavenging activity was found in CEH (IC_{50} 365.67± 0.23 µg/ml).A positive correlation is also observed in all extracts between TPC and DPPH and the correlation values (r) were represented in Table 5.10. The highest correlation was observed in PEH (r=0.997, P<0.01) and least co-relation was observed in MEH (r=0.840, P<0.01).

In our results, it has been observed that, no correlation was observed in PEH between TFC and DPPH activity. Highest correlation was observed in CEH (r=0.990, P<0.01) and least correlation was observed in AEH (r=0.711, P<0.01) between TFC and DPPH radical scavenging activity.

5.1.6.3.2. Ferric reducing assay:

The Ferric reducing assay was employed to estimate the antioxidant capacity of the samples to transform Fe^{3+} to Fe^{2+} *in vitro*. The reductive power of sample was confirmed by change of yellow color of the test solution to various shades of green to blue depending on the concentration of the extracts. The reducing power of the extracts and standard ascorbic acid increased with increase in concentration. In this test, the OD ranges from 0.272 to 1.151 for ascorbic acid. At 1000 µg/ml the highest reducing power was observed in PEH (0.912 ± 0.003) followed by MEH (0.739 ± 0.006). Moderate activity was observed by (0.644 ± 0.005) and least activity was observed in AEH (0.426 ± 0.006). In this study, the absorbance was found to increase with the dose of plant extracts and standard which is suggestive of reducing power. A positive correlation is also observed in TPC and ferric reducing assay with highest correlation in MEH (r=0.961, P<0.01) and least was observed in CEH (r=0.916, P<0.01). The correlation between TFC and ferric reducing assay was found to be

highest in MEH (r=0.978, P<0.01) and least was observed in WEH with r value 0.546, P<0.01 (Table 5.10).

5.1.6.3.3. Metal chelating activity

Excess of free irons have been implicated in the induction and formation of free radicals in biological systems. Free radical reactions can be catalyzed by Fe^{2+} which may lead to oxidative damage. The metal chelating of crude PEH, CEH, MEH and AEH increased with increasing concentration and $IC_{50}$was calculated and expressed as microgram/millilitre (Table 5.9). Highest activity was obtained at 1000 µg/ml concentration but at the same concentration EDTA (Standard) gave 95.11 % inhibitory activity. Highest activity was found to be in PEH (IC_{50}, 463.78 µg/ml) followed by PEH (IC_{50}, 481.85 µg/ml). CEH exhibited moderate chelating activity with IC_{50}value of 625.24 µg/ml and least was found to be in AEH ($IC_{50,}$ 649.19 µg/ml). The IC_{50} values have been represented in the table 5.8.In this study, there was a positive correlation was observed between TPC and metal chelating activity and r value were represented in Table 5.10. The correlation between TPC and metal chelating activity was found to be highest in PEH (r=0.952, P<0.01) and least was observed in CEH (r= 0.879, P<0.01). The correlation between TFC and metal chelating was found to be highest in CEH (r= 0.943, P<0.01) and least correlation was observed in AEH with r value 0.497, P<0.01.

Name of the Plant extract	Carbohydrate	Alkaloids	Tanins	Steroids	Terpenoids	Saponins	Glycosides	Flavonoids
PEH	+	-	+	+	+	-	-	+
CEH	-	-		-	-	-	+	-
MEH	+	+	+	-	+	-	+	+
AEH	+	+		-	-	-	-	+

Table 5.7: Results of qualitative analysis of phytochemicals present in the *H. mysoresence*leaf extract.

Name of the Plant extract (Methanol)/Tests	Total Phenol Content(mg of GAE/g)	Total flavonoid Content (mg of CE/g)
PEH	50.3 ±1.25	-
CEH	64.0 ±0.50	91.3 ±2.08
MEH	127.0 ±1.52	126.3±1.15
AEH	165.3 ± 2.51	79.0 ±1.00

GAE= Gallic acid equivalent, CE= Catechol equivalent

Table 5.8: Total phenolic, total flavonoid contents in the *H. mysoresence*leaf extract.

Sl no	Extract	DPPH ACTIVITY (µg/ml)	METAL CHELATING ACTIVITY (µg/ml)
1	*Petroleum ether extract*	305.02	481.85
2	*Chloroform extract*	365.67	625.24
3	*Methanol extract*	344.46	463.78
4	*Aqueous extract*	126.98	649.19

Table 5.9: IC 50 value of different crude extracts of *H. mysoresence* plants

Name of the plant/Total phenolic and Total flavonoid content		DPPH	Metal chelating activity	Ferric reducing activity
Petroleum ether extract	**TPC**	0.997**	0.952**	0.960**
	TFC	-	-	-
Chloroform extract	**TPC**	0.980**	0.879**	0.916**
	TFC	0.990**	0.943**	0.967**
Methanol extract	**TPC**	0.840**	0.902**	0.961**
	TFC	0.989**	0.923**	0.978**
Aqueous extract	**TPC**	0.967**	0.925**	0.951**
	TFC	0.711	0.497	0.546

**** Correlation is significant at the 0.01 level,**

Table 5.10: Pearson correlation coefficient (r) obtained between Total phenolic, flavonoid composition and antioxidant activities of crude extracts of *H. mysoresence*

5.2. Purification and identification of potent purified compound.

5.2.1 Isolation of pure compound

Based on the antibacterial activity result, crude petroleum ether extract *H. mysoresence* was subjected for column chromatography for the separation of bioactive compounds. Totally 19 compounds have been preliminarily separated and named as F_1 F_2, F_3, F_4, $F_5$to ……F_{19}, respectively. These compounds were further screened for antibacterial activity usingknown dental caries isolates (Plate 3b).

5.2.2. Identification of potent purified compound.

The pure compounds isolated through column chromatography were further subjected to FT-IR, ^{1}H-NMR and MASS spectral studies. Based on the corresponding data, the compounds were characterized. Finally, the structure of the compound was drawn by using Chem Draw software. Five compounds showing potent antibmicrobial activity were selected for the spectral analysis. Based on the spectral data, the compoundswere further characterized.

Based on the data obtained by spectral studies the compounds identified as

F_3: 1-((E)-7-(3-(5-chloronon-7-yn-2-yl)-5-hydroxyphenyl)-2-propylhept-4-enylamino)butan-2-one.

F_4: (2E,9E)-7-bromo-N-((E)-3-chlorohept-4-enylidene)-13-cyclopropyl-4-hydroxytridec-6-enamide

F_5: 6-chloro-1-(5-(4-chlorobutyl)-4-hydroxy-2-((E)-4-hydroxybut-2-enyl)piperidin-3-yl)-3-methyldec-8-yn-4-one.

F_9: N-((6E)-13-(3-(2-bromoethyl)-5-((Z)-7,16-dichloroheptacos-22-enyl)cyclopent-1-enyl)-8-chlorotridec-6-enyl)acetamide

F_{10}: 6-chloro-8-(2-(3-chloro-7-(2-methylcyclopropyl)hept-6-ynyl)-4,5-dihydro-4-(hydroxymethyl)oxazol-4-yl)octan-2-one

The details of scientific procedure followed in the isolation and characterizatio of the above said compounds are described below.

5.2.2.1 Spectral characterization of compound

F_3:1-((E)-7-(3-(5-chloronon-7-yn-2-yl)-5-hydroxyphenyl)-2-propylhept-4-enylamino) butan-2-one (figures 5.1 to 2.20).

Physical state: Yellow

Melting point: 230-235^{0} C

Solubility: DMSO and Chloroform

Stability: Hygroscopic, stable at room temperature

IR Spectra: Intense broad band near 2950 cm^{-1}due to (-OH) group, stretching vibration at1680 cm^{-1} due to C=O stretching vibration, and 1200 cm^{-1} to 1600 cm^{-1} peaks are aromatic ring stretching vibration.

HNMR spectra (δ ppm): 7.8(s, 1H), 7.1(s, 1H), 6.8(s, 1H), 6.6 (s, 1H), 5.1 (s, 2H), 5.7 (t,-2H) and 3.6 (s, 2H).

Mass: From the mass spectra molecule base peak m/z 475.25 corresponds to molecular mass of compound F-3 (Mass= 473). The mass spectra represents the chlorine patron (m+2 peak) in spectra.

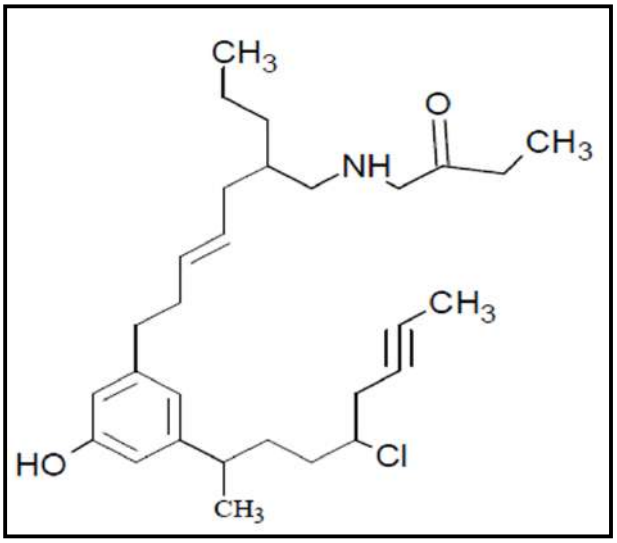


Figure 5.1: Structure of 1-((E)-7-(3-(5-chloronon-7-yn-2-yl)-5-hydroxyphenyl)-2-propylhept-4-enylamino) butan-2-one).

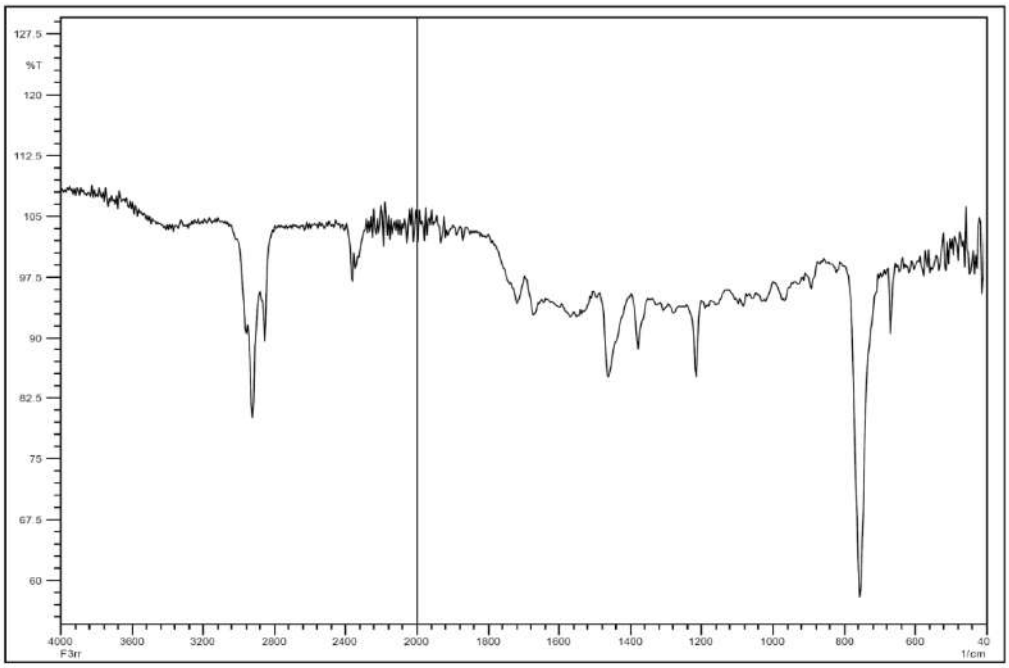


Fig 5.2: FT-IR spectra of pure compound F_3

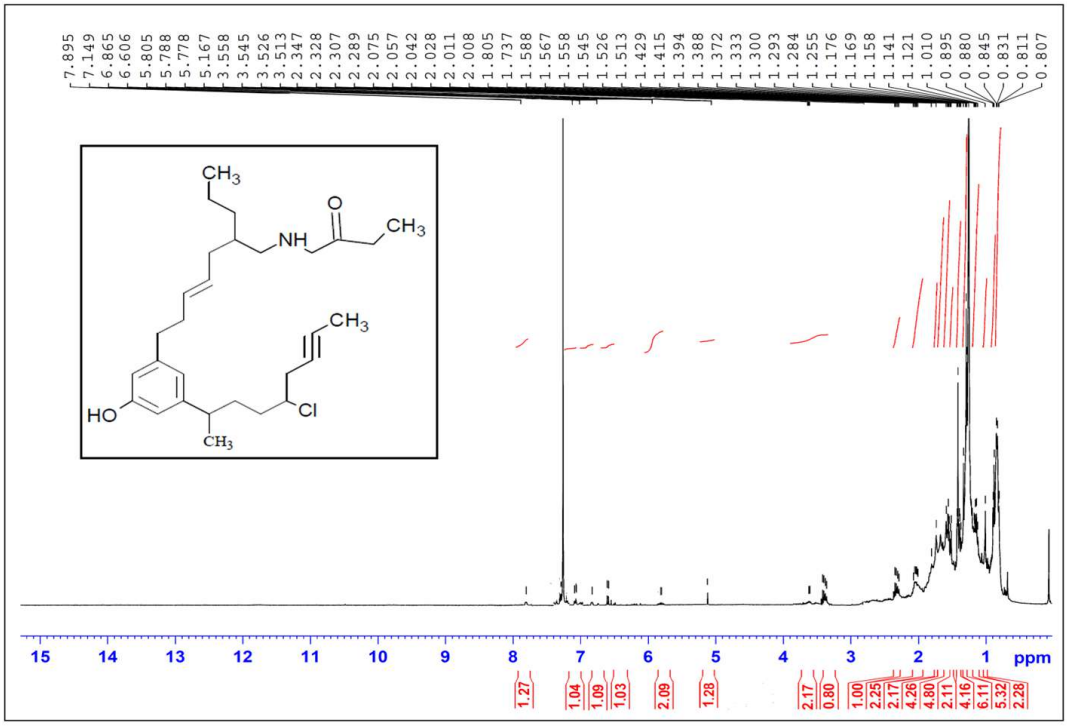


Figure 5.3: ^{1}H-NMR spectra of pure compound F$_3$

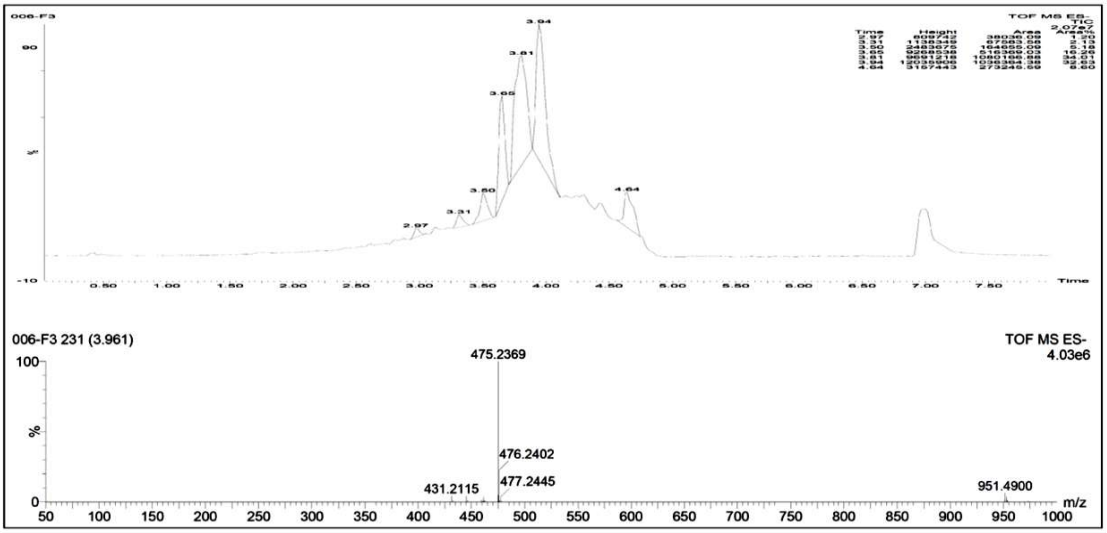


Figure 5.4: Mass spectra of pure compound F$_3$

5.2.2.2. F$_4$ Sample: Spectral characterization of compound F$_4$: (2E, 9 E)-7-bromo-N-((E)-3-chlorohept-4-enylidene)-13-cyclopropyl-4-hydroxytridec-6-enamide.

Physical state: Yellow

Melting point: 230-235ºC

Solubility: DMSO, DMF and Chloroform

Stability: Hygroscopic, stable at room temperature

IR Spectra: Intense broad band near 2950 cm^{-1}due to -OH groups, stretching vibration at 1970 cm^{-1} is due to C=O and 2915 cm^{-1} stretching vibration is due to N=C. The 1490cm^{-1} peaks indicating aliphatic $–CH_2$ groups.

HNMR spectra (δ ppm): 7.81(t, 1 p), 5.8 (t, 2p), 5.5(t, 2p), 4.2 (q,1p), 3.1 (p,1p), 3.4 (p,1p) and 2.3 (s, 1p).

Mass: From the mass spectra molecule base peak m/z 461.22 corresponds to molecular mass of compound F-4 (Mass= 460). The mass spectra represents the chlorine patron (m+2 peak) and bromo patron (m+3) in spectra.

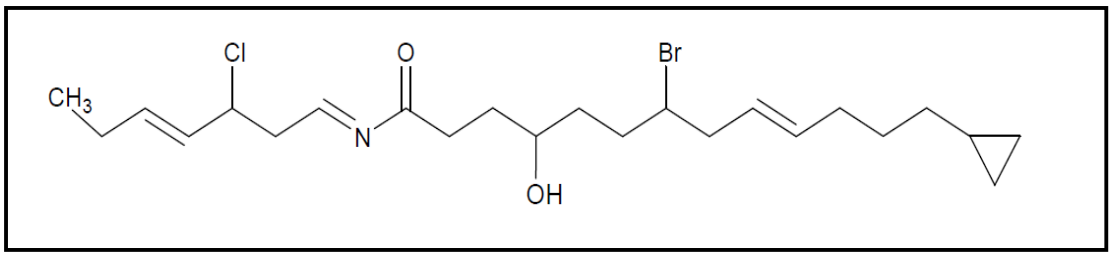


Figure 5.5: Structure of pure compound F_4

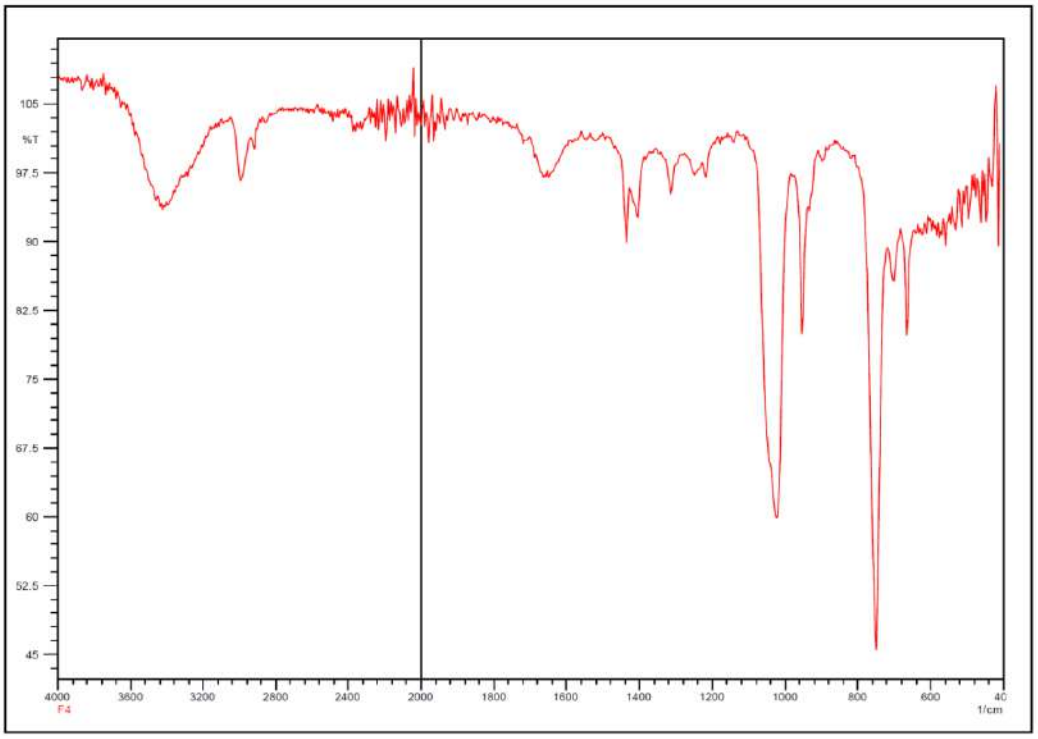


Fig 5.6: FT-IR spectra of pure compound F_4

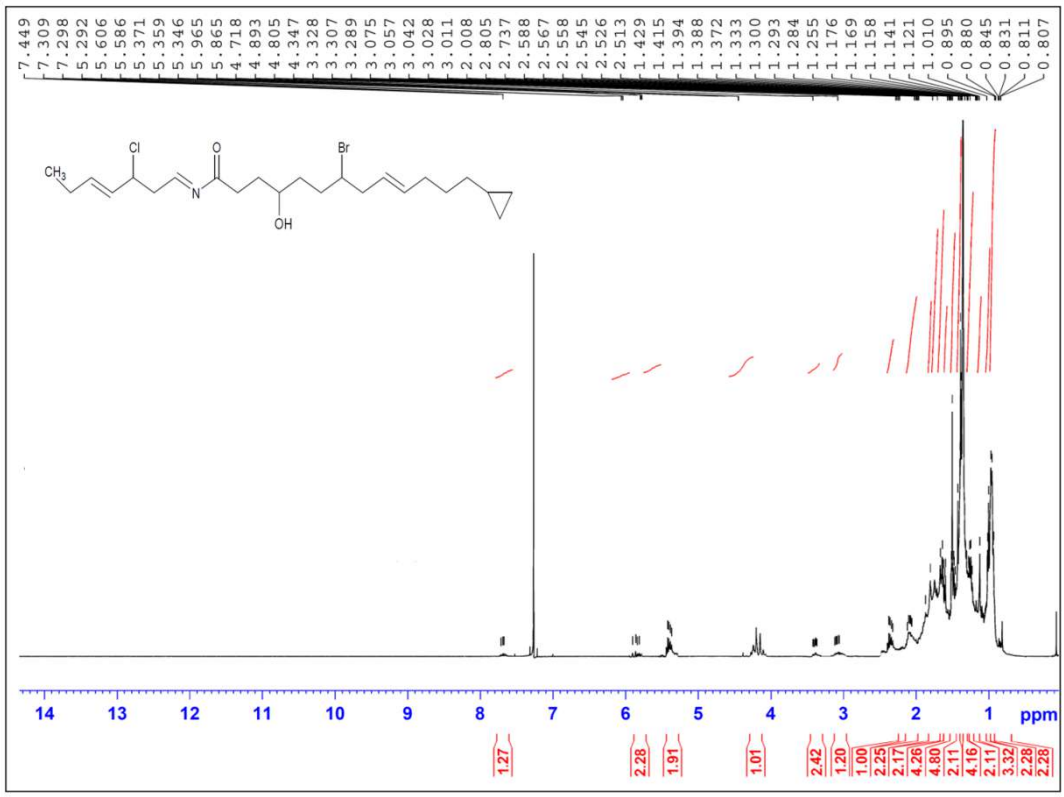


Figure 5.7: ^{1}H-NMR spectra of pure compound F4

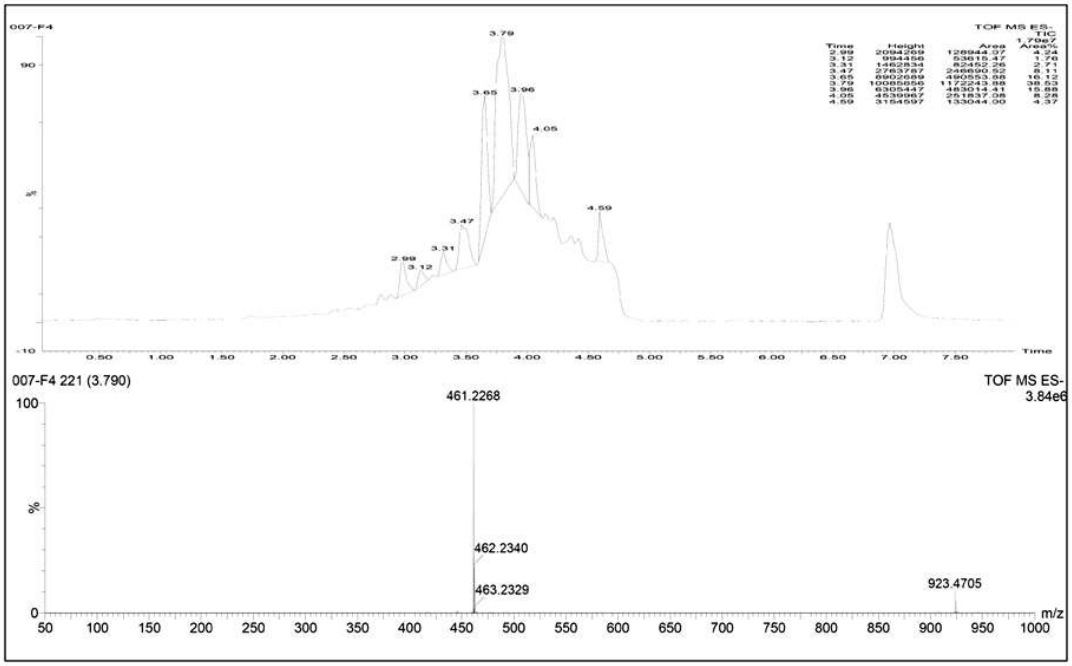


Figure 5.8: Mass spectra of pure compound F4

5.2.2.3. F_5 Sample:

Spectral characterization of compound F_5: 6-chloro-1-(5-(4-chlorobutyl)-4-hydroxy-2-((E)-4-hydroxybut-2-enyl) piperidin-3-yl)-3-methyldec-8-yn-4-one

Physical state: Black

Melting point: 231-235

Solubility: DMSO, DMF and Chloroform

Stability: Hygroscopic, stable at room temperature

IR Spectra: Intense broad band near 3200 cm^{-1}due to (-OH) groups, stretching vibration 800 to 1400 cm^{-1} is due to aliphatic chain, and 1700 cm^{-1} due to C=O stretching vibration and 600 cm^{-1} is due to C≡C stretching vibration.

HNMR spectra (δ ppm): 5.6(t, 2p), 5.3(s, 1p), 4.8(s, 1p), 4.4 (d, 2p), 3.8 (p, 1p), 3.4 (t, 1p) and 3.2 (S, 1p) and 3.1 (s, 1p).

Mass: From the mass spectra molecule base peak m/z 461.23 corresponds to molecular mass of compound F-5 (Mass= 460). The mass spectra represents the chlorine patron (m+2 peak) in spectra.

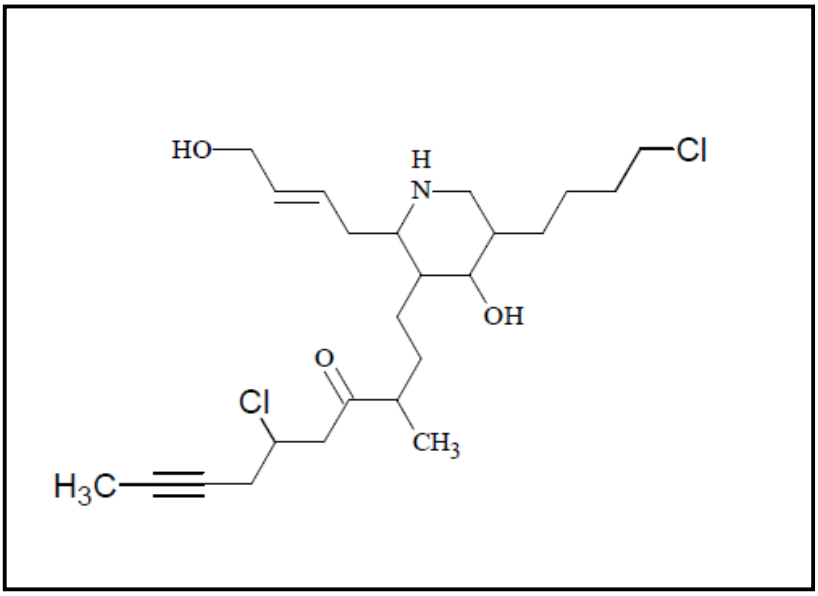


Figure 5.9:Structure of pure compound F_5

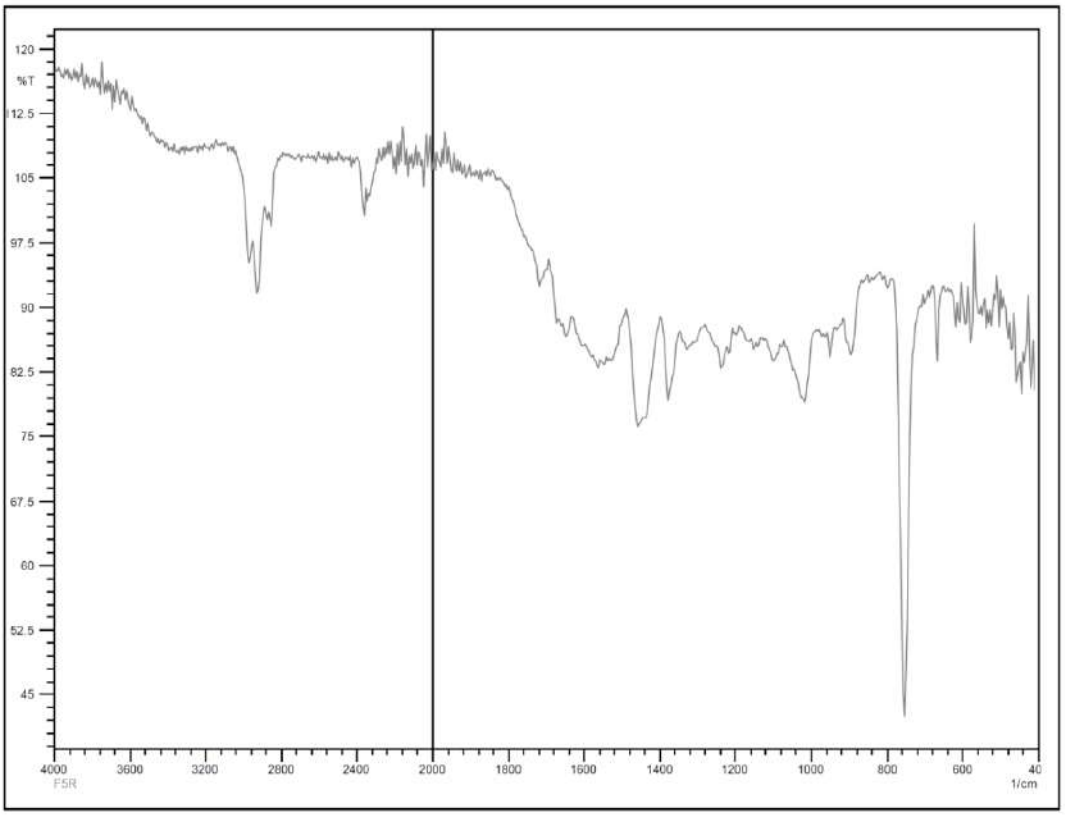


Fig 5.10: FT-IR spectra of pure compound F_5

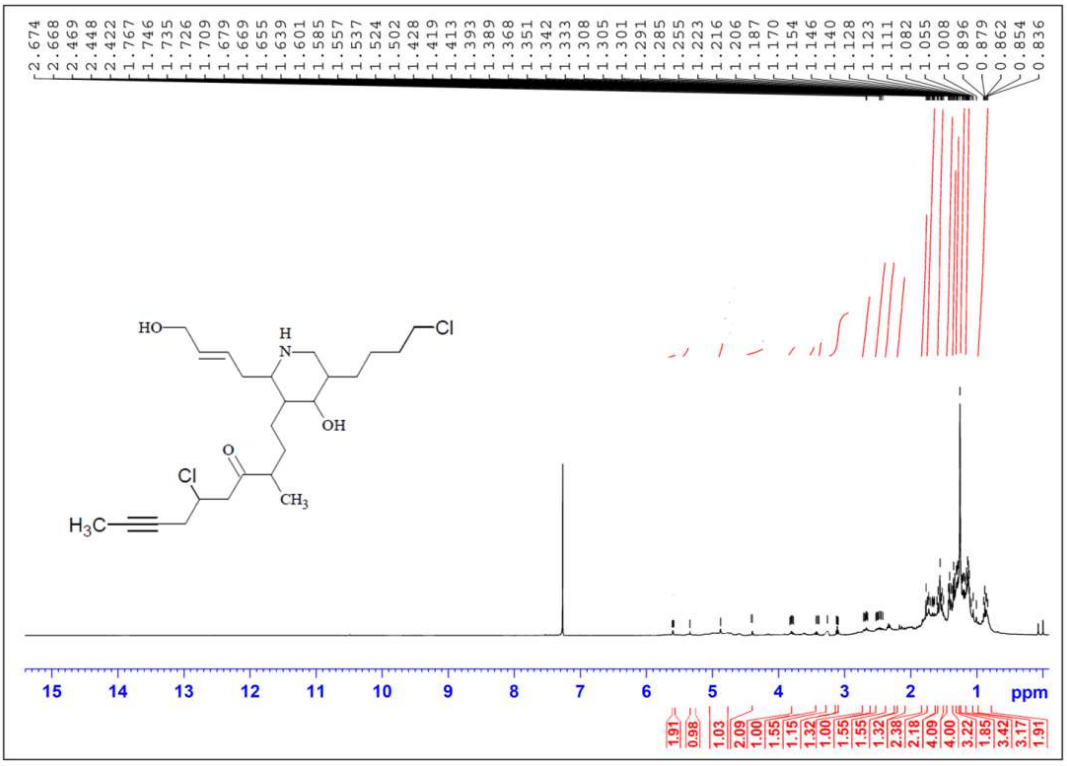


Figure 5.11: ^{1}H-NMR spectra of pure compound F_5

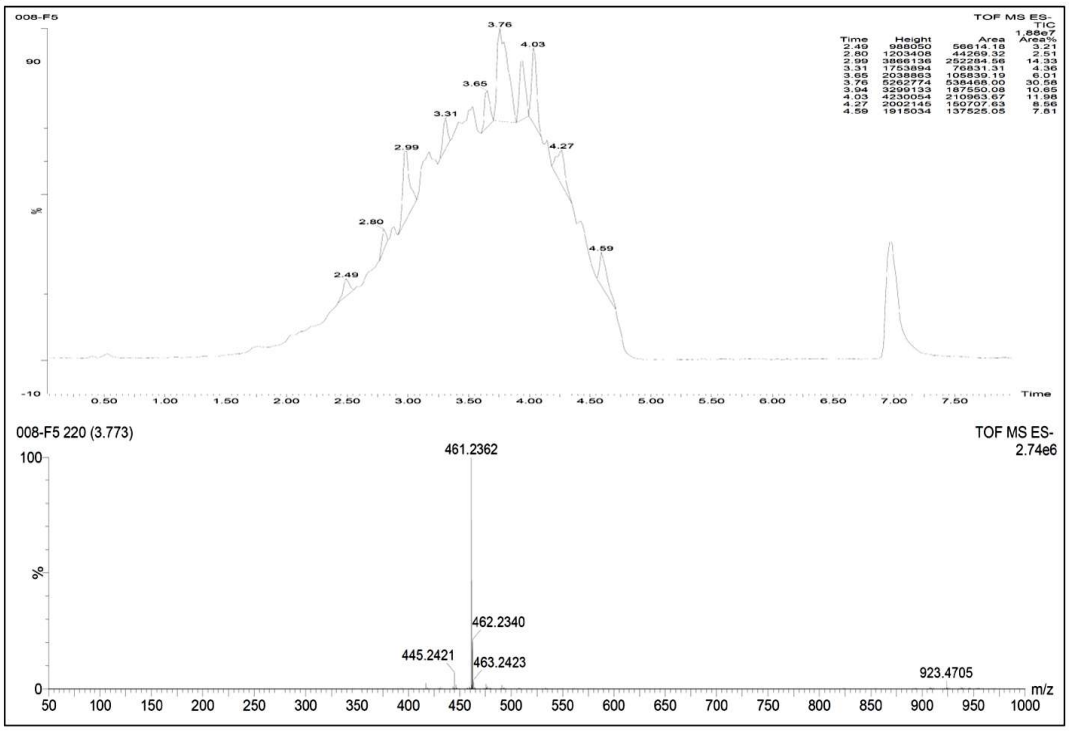


Figure 5.12: Mass spectra of pure compound F_5

5.2.2.4. F_9 Sample:

Spectral characterization of compound F_9: N-((6E)-13-(3-(2-bromoethyl)-5-((Z)-7, 16-dichloroheptacos-22-enyl) cyclopent-1-enyl)-8-chlorotridec-6-enyl) acetamide

Physical state: Brown

Melting point:236-237

Soluble: Chloroform

Stability: Hygroscopic, stable at room temperature

IR Spectra: Intense broad band near 3400 cm^{-1}due to (-NH) amide stretching vibration, 800-1200 peaks indicating presence of long chain alkane and 2900 cm^{-1} due to C=C stretching vibration.

HNMR spectra (δ ppm): 8.1 (s, 1H), 5.8(t, 2H), 5.1(d, 1H), 3.9 (p, 1H), 3.7 (t, 2H), 3.0 (t, 2H) and 2.4(s, 3H).

Mass: From the mass spectra molecule base peak m/z 445.24 corresponds to molecular mass of compound F-9 (Mass= 444). The mass spectra represents the chlorine patron (m+2 peak) and also shows it was in dimeric form.

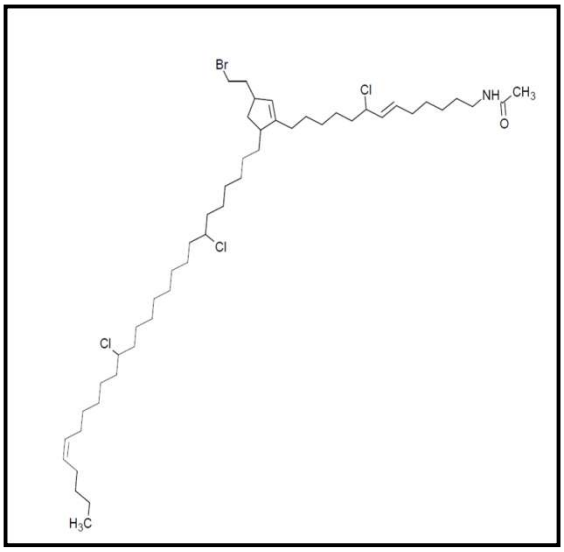


Figure 5.13: Structure of Compound F_9

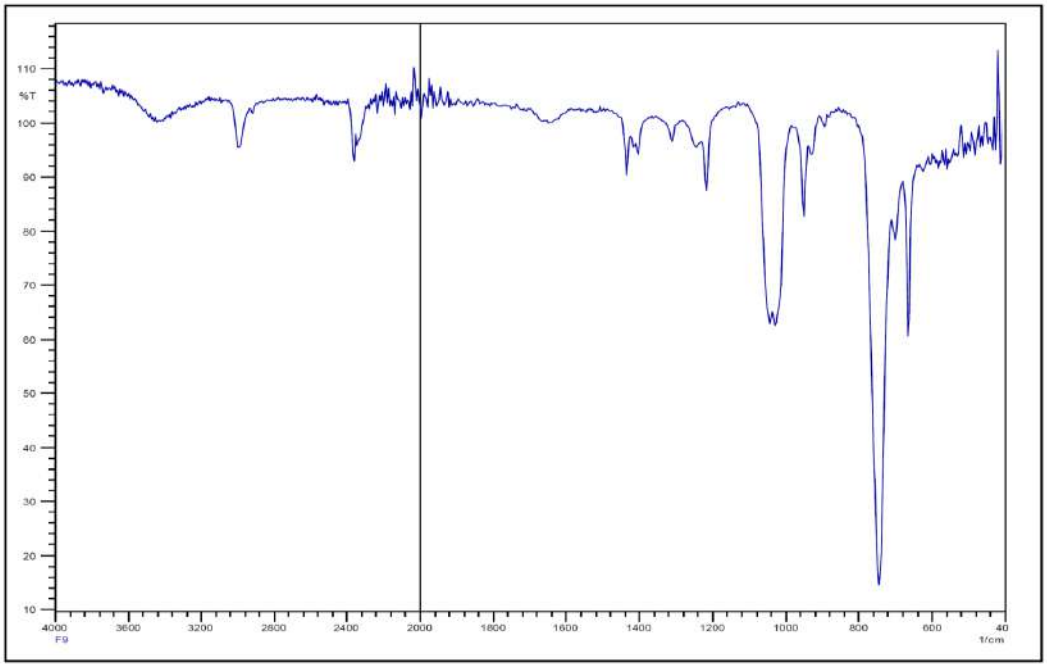


Figure 5.14 : FT-IR spectra of pure compound F_9

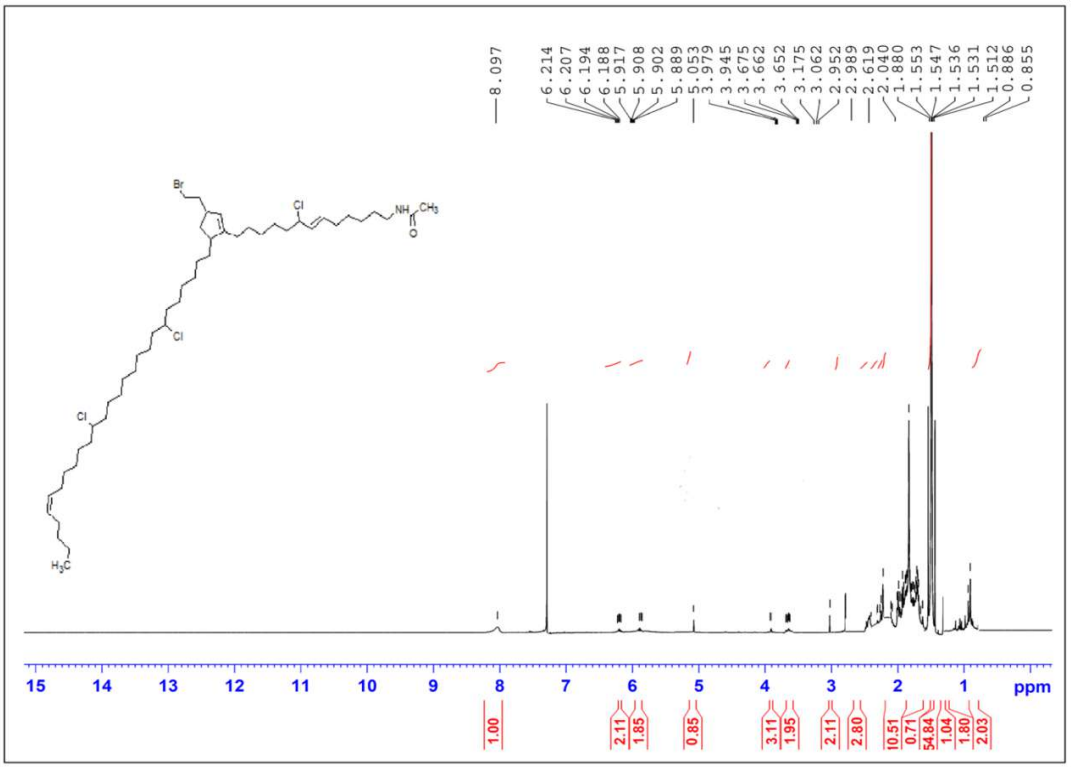


Figure 5.15: ^{1}H-NMR spectra of pure compound F$_9$

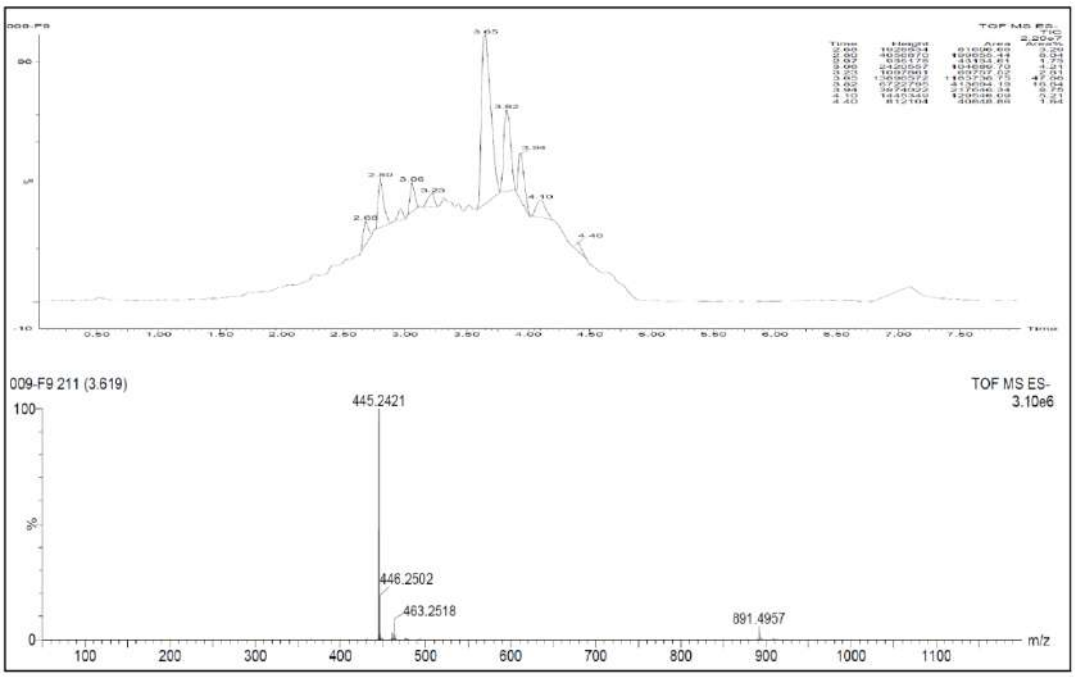


Figure 5.16: Mass spectra of pure compound F$_9$

5.2.2.5. F_{10} Sample:

Spectral characterization of compound F_{10}:6-chloro-8-(2-(3-chloro-7-(2-methylcyclopropyl) hept-6-ynyl)-4, 5-dihydro-4-(hydroxymethyl) oxazol-4-yl)octan-2-one

Physical state: Yellow

Melting point: 230-231

Solubility:DMSO, DMF and Chloroform

Stability: Hygroscopic, stable at room temperature

IR Spectra: Intense broad band near 3300 cm^{-1}due to -OH stretching vibration,1700cm^{-1} peaks indicating presence of C=O and 2890 cm^{-1}peak is due to N=C stretching vibration.

^{1}H-NMR spectra (δ ppm): 5.2(s, 1H), 3.9(s, 2H), 3.8(s, 2H), 3.4 (p, 1H), 2.4 (t, 2H) and 2.1 (s,3H).

Mass: From the mass spectra molecule base peak m/z 445.25 corresponds to molecular mass of compound F-10 (Mass= 444). The mass spectra represents the chlorine patron (m+2 peak) in spectra.

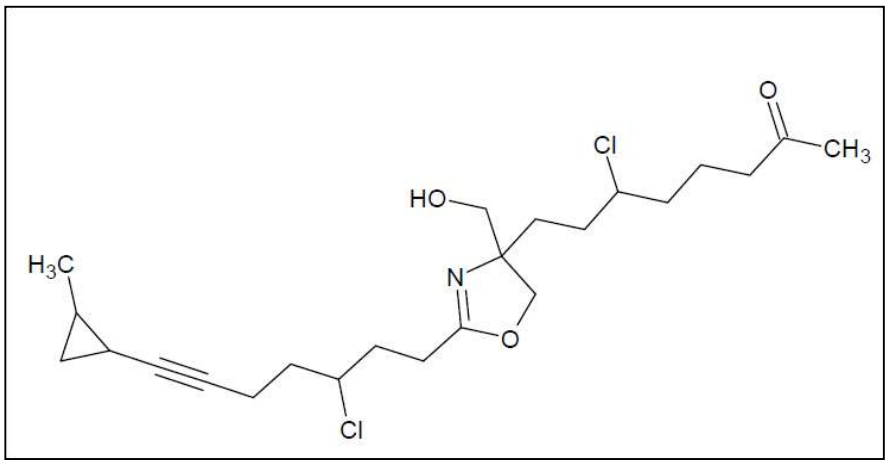


Figure 17: Structure of pure compound F_{10}

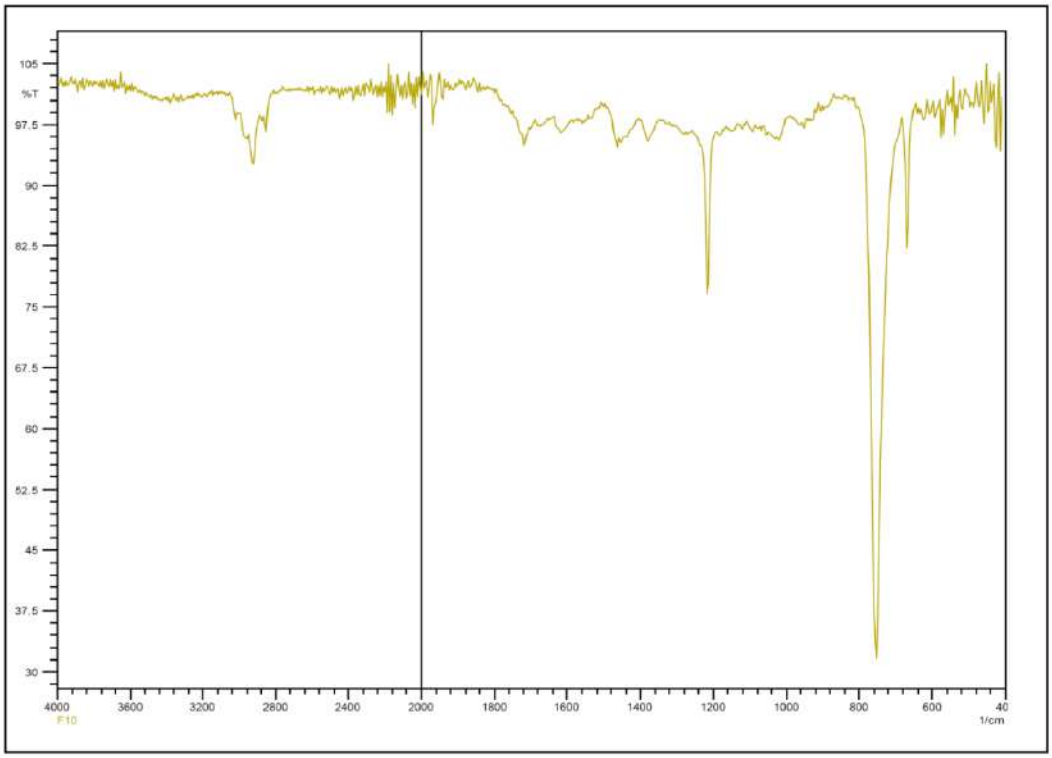


Figure 5.18 : FT-IR spectra of pure compound F_{10}

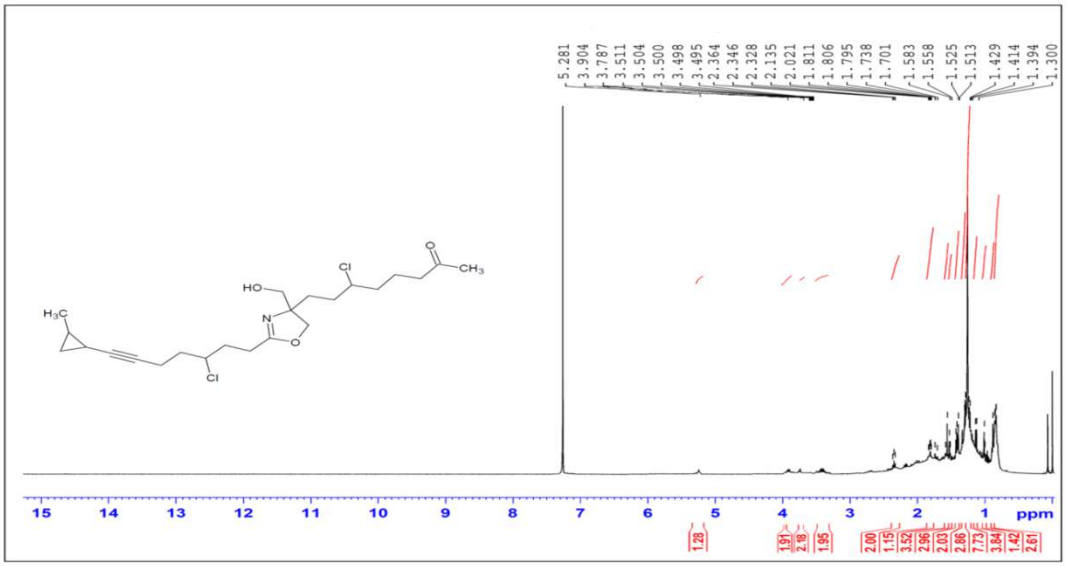


Figure 5.19: ^{1}H-NMR spectra of pure compound F_{10}

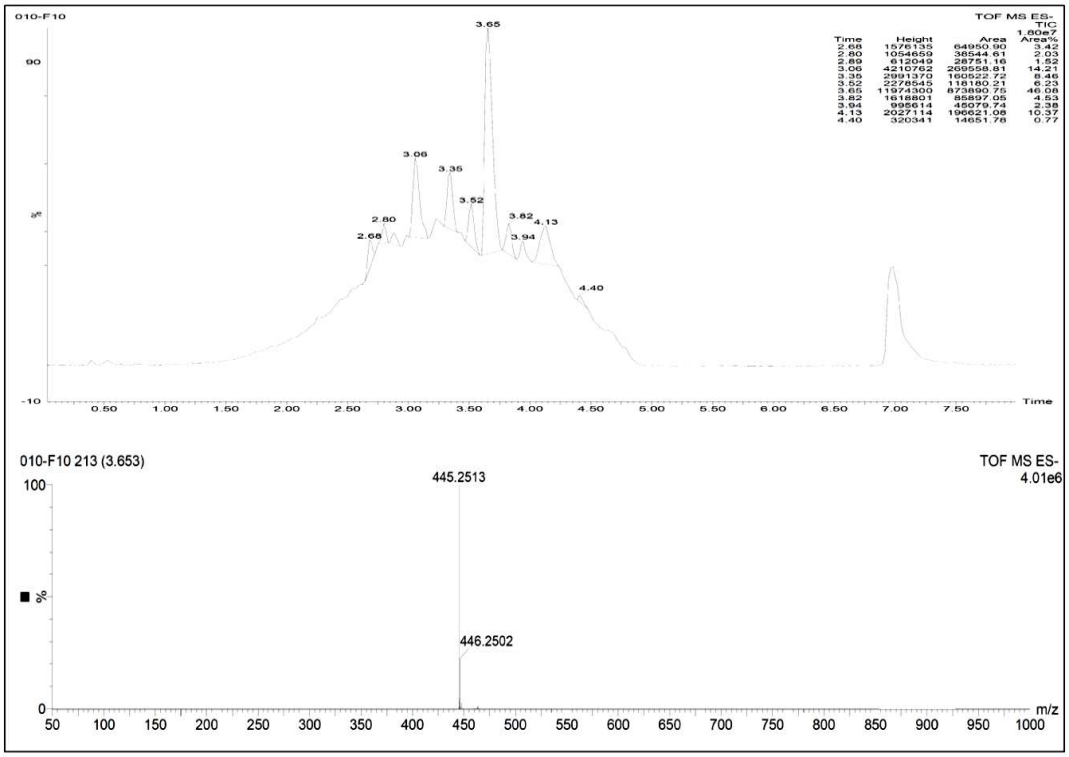


Figure 5.20: Mass spectra of pure compound F_{10}.

In this present study, five pure compounds were isolated from petroleum ether extract *H. mysoresence* .These five compounds were found to be novel compounds

5.3. Collection and screening of clinical samples for dental caries isolates.

5.3.1. Collection of infected teeth samples

In the present study, we have collected 100 teeth samples from 100 subjects suffering from dental caries. Depending upon colony morphology on selective and differential media. A total of 404 clinical isolates were recovered from the infected teeth samples (Plate 4).

5.3.2. Colony characteristics of isolates

The isolated colonies developed on blood agar, MS agar, MIS agar, MRS agar and SDA agar were observed (Plate 4, 5 and 6) and individually subcultured for further biochemical identification. Colony characteristics observed for the isolation on differential media was deduced in the Table 5.11.

PLATE 1


a) ***Annona muricata***


b) ***Anisomeles indicus***


c) ***Azadiractha indica*** **d)** ***Mangiferra indica***


e) ***Hypericum mysoresence***

PLANT SELECTED FOR THE STUDY

PLATE 2


a) *Crysophyllum roxburghii*

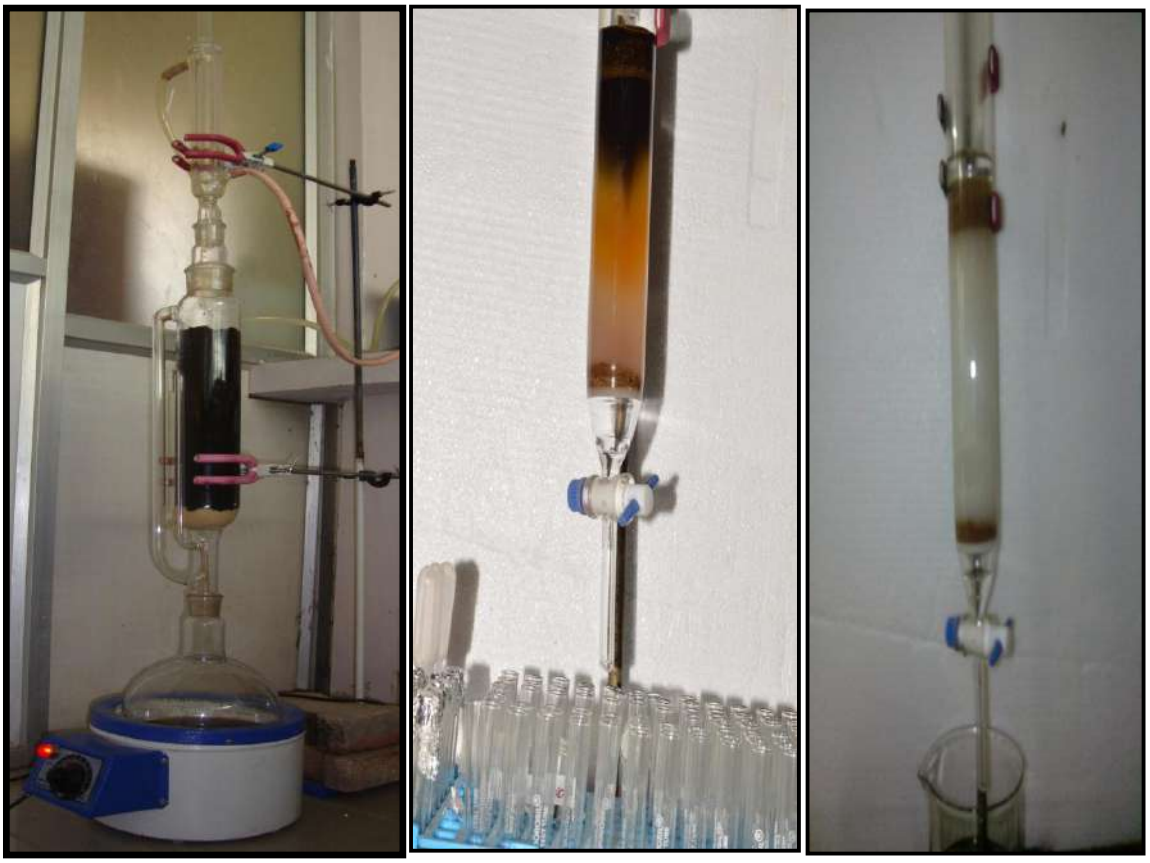

b) *Scleropyrum pentandrum*


c) *Malpighia glabra* **d) *Wrightia tinctoria***


e) Toddalia asiatica

PLANT SELECTED FOR THE STUDY

PLATE 3



a) **b)**

a) A view of soxhalet apparatus **b) A view of column chromatography**

A view of column chromatography, collecting different fractions of compounds

5.4. Biochemical characterization and determination of antibiotic sensitivity pattern of dental caries isolates against standard antibiotics.

5.4.1. Biochemical Identification of clinical isolates

5.4.1.1. Morphological and staining characteristics of isolates

Staining of the cultures isolated from caries specimens was performed to study the morphological characteristics. Gram's stained slides of all the isolates showed the presence of violet colored cocci and bacilli species (Plate 7). Cocci are typically arranged in chains and all the isolates were found to be Gram positive (Table 5.11).

5.4.1.2. KOH solubility test

To justify Gram's staining results, KOH solubility test was also conducted. There was no formation of mucoid strand when the culture was mixed with the 3%KOH and the loop was gently lifted. This indicated that all the clinical isolates were Gram positive (Table 5.11).

5.4.1.3. Optochin sensitivity of isolates

Optochin sensitivity test was conducted in order to differentiate *Streptococcus pneumoniae* and other viridans streptococci isolated from the caries samples (Plate 7). There was no inhibition of the isolates by the optochin disc as revealed by absence of inhibition zone around the disc i.e., all the isolates were found to be resistant to optochin (Table 5.12).

5.4.1.4. Catalase test

Catalase test was performed for all the 30 isolates in order to primarily differentiate them from *Staphylococcus aureus* using 3% H_2O_2. All the isolates were shown to be negative for production, as no effervescence was observed when the culture was mixed with 3% H_2O_2 (Table 5.12).

5.4.1.5Arginine hydrolysis

This test was conducted to differentiate viridans streptococci into their groups. All the isolates were not able to hydrolyze arginine as no change in the color to deep purple was observed (Plate 7). But some of the clinical isolates have hydrolysed arginine which was indicated by the change of media color into deep purple color. By

this test, it is possible to differentiation between *S. mutans* and *S. sanguis*. *S. mutans* gives negative results and *S. sanguis* shows positive results for arginine hydrolysis (Table 5.12).

5.4.1.6 Esculin hydrolysis

This test was conducted to differentiate among viridans streptococci species. All the isolates have shown to hydrolyze esculin as revealed by the color change i.e., black color (Plate 7). In our results it is also observed that some of the clinical isolates were not involved in the hydrolysis of esculin as there was no change in media color. This tests confirms the presence of *S. mitis* in the clinical samples as they are not involved in the esculin hydrolysis (Table 5.12).

5.4.1.7 Mannitol fermentation

Ability of the isolates to ferment mannitol and produce acid was checked in mannitol broth and is an identification parameter for the detection of mutans streptococci (Plate 8). A color change from red to yellow was observed in all the tubes indicating that the all isolates were capable of fermenting mannitol. Results of this test reveals that clinical isolates produced acid but no production of gas. *S. mutans* are involved in production of acid where as some of the isolates were negative for acid production (Table 5.12).

5.4.1.8Sorbitol fermentation

Fermentation of sorbitol is another test which useful in the identification of *mutans streptococci* isolated from caries samples (Plate 8). Here also, color change from red to yellow was observed in all the tubes inoculated with the isolates which are suggestive of sorbitol fermenting ability of isolates. In this test also *S. mutans* showed positive for acid production whereas other clinical isolates are not involved in acid production (Table 5.12).

5.4.1.9 VP test

This test is another important test to identify streptococci from caries samples. VP test is performed for the differentiation of *S. sanguis* from streptococcus species isolated from dental caries samples. Formation of pink/red color in the tubes after adding the reagents indicated the production of acetyl methyl carbinol. All the tubes showed pink color after addition of reagent indicating that they are VP positive (Plate

8). Some clinical isolates didn't showed production of acetyl methyl carbinol, and further these isolates were identified as *S. sanguis* (Table 5.12).

5.4.1.10Urea hydrolysis

Ability of the isolates to hydrolyze urea and produce ammonia is checked in urea broth medium. The tubes were observed for color change from yellow to pink due to hydrolysis of urease. In this study, none of the tubes have shown color change from yellow to pink. This indicated that all the isolates were negative for urease production (Table 5.12).

5.4.1.11. Growth at 5 % NaCl and sugar fermentation test.

NaCl is an inhibitory substance which may inhibit the growth of certain types of bacteria. It is a key factor to identify *L. acidophilus*. In our study, the clinical isolates recovered from MRS agar showed growth at 5% NaCl, which is the suggestive for the presence of *L. acidophilus* in the clinical sample. In our result it is also observed that, all the isolates have shown the production of acids by fermenting all the incorporated sugar in the media. This is the most important characteristics of *L. acidophilus,* which produces the acid and involved in the progression of dental caries and results in demineralization of the enamel (Table 5.13).

5.4.1.12 Germ tube test

Germ tube test was performed for the confirmation of presence of *C. albicans* in the clinical sample. In this study, it has been observed that the clinical isolates which are recovered from SDA agar showed the formation of germ tube(Plate 8), when they are inoculated with serum (Table 5.13).

The results of morphological and biochemical tests revealed the presence of *S. mutans, S. sanguis, S. mitis, S. salivarius, L. acidophilus and C. albicans* in the clinical samples. Among 100 subjects, *S. mutans* (22.27%) was found to be most predominant flora followed by *L. acidophilus* (19.80%), *S. sanguis* (19.30%), *S. mitis* (16.83%) *and S. salivarius* (14.35%). *C. albicans* ranks last with isolation percentage of about 7.42%. From the above results it is evident that the 90% of the dental caries is caused because of *S. mutans*, hence it is again proved as an initiator of dental caries and further progression may be caused by the secondary colonizers such as *L. acidophilus, S. sanguis, S. mitis, S. salivarius* (Figure 5.21).

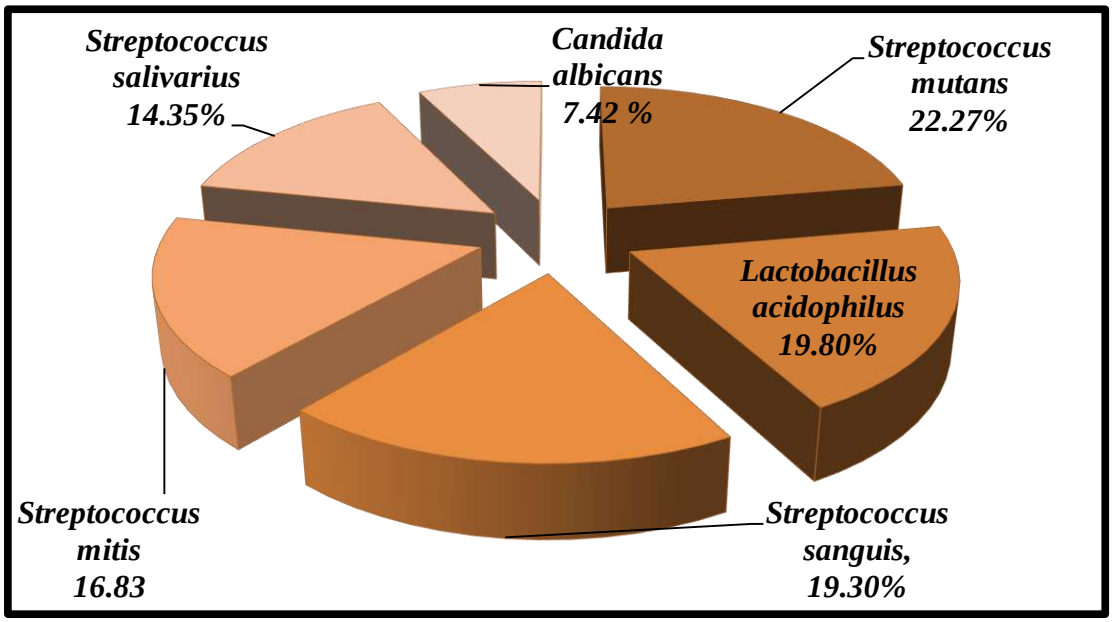


Figure 5.21: Isolation percentage of pathogens recovered from infected teeth samples.

Organisms	Colony characteristic					Gram staining	KOH Solubility (Formation of mucoid strands)
	MS media	MIS media	BA media	MRS media	SDA media		
S. mutans	0.5 to 2 mm diameter, Irregular white colonies, on the top of the colony there is a production of drop of watery like substances was observed.	-	Pin point colonies showing alpha hemolysis	-	-	Gram positive cocci arranged in chains	+
S. sanguis	Growth of colonies by adhering to the surface of the agar which is difficult to pick up without damaging the agar surface.	-	Pin point colonies showing alpha hemolysis	-	-	Gram positive cocci arranged in chains	+
S. mitis	-	Small pin point blue color colonies	Pin point colonies showing alpha hemolysis	-	-	Gram positive cocci arranged in chains	+
S. salivarius	-	Small smooth or rough gum drop colonies with 1-5mm in diameter	Pin point colonies showing alpha hemolysis	-	-	Gram positive cocci arranged in chains	+
L. acidophilus			-	Large or small white colonies	-	Gram positive bacilli	+
C. albicans	-	-	-	-	Round, oval shaped cells of about 4 to 8 mm in diameter.	-	

MS=Mutans sanguis agar, MIS= Mitis Salivarius agar Media, MRS=de Mann rogosa and sharpe agar media, SDA= Saborauds dextrose agar media

Table 5.11: Cultural characteristics of clinical isolates on different media and morphological details of isolated colonies.

Organism	Optochin	Catalase	Arginine	Esculin	Mannitol	Sorbitol	VP test	Urea hydrolysis
S. mutans	-	-	-	+	+	+	+	-
S. sanguis	-	-	+	+	-	-	-	-
S. mitis	-	-	-	-	-	-	+	-
S. salivarius	-	-	-	+	-	-	+	-

Table 5.12: Biochemical characteristics of Streptococcus species isolated from infected teeth sample.

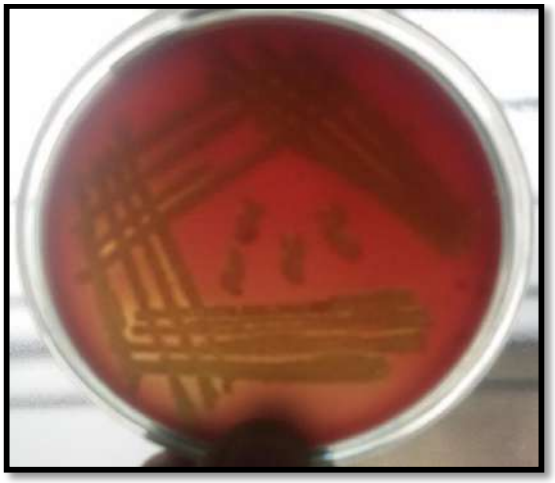
Organism	Sugar fermentation		Growth at 5% Na Cl	Germ tube test
	Production of acid	Production of gas		
L. acidophilus	+	-	+	-
C. albicans	-	-	-	+

Table 5.13: Biochemical characteristics of Lactobacillus species and Candida species isolated from infected teeth sample from.

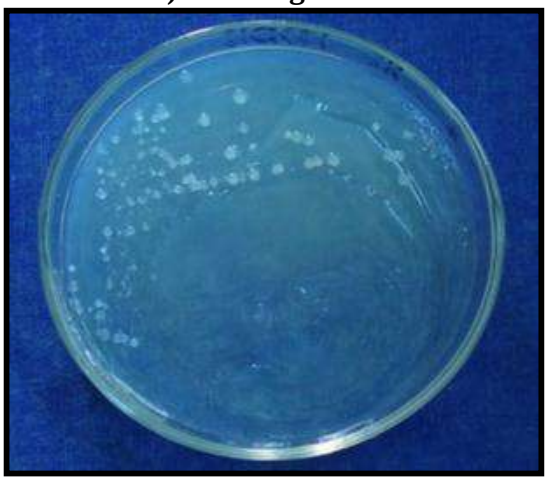
PLATE 4

Figure 1: a) Container with saline (sterile); b) Tooth sample in sterile saline; c) Teeth from dental caries subject

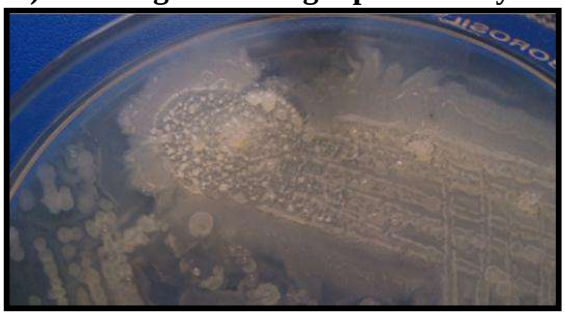
d) Blood agar



e) Blood agar showing alpha hemolysis



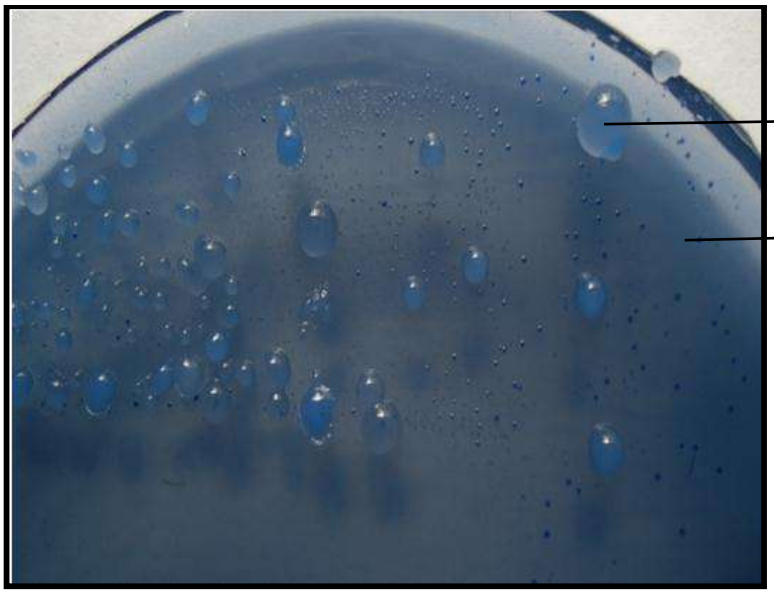
f) Mutans Sanguis agar



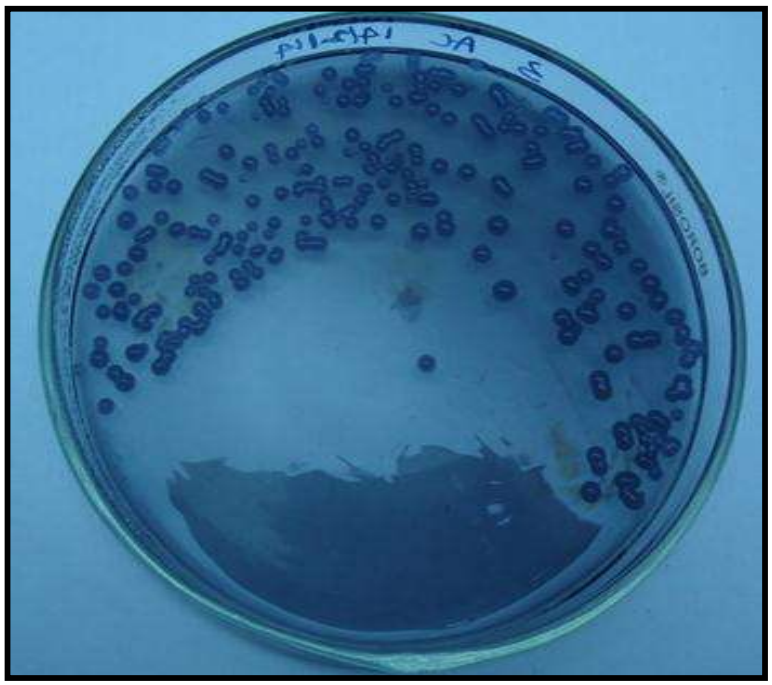
g) Colonies Showing puddles of glucan on Mutans Sanguis agar

Colonies of Streptococcus species on Blood agar and Mutans Sanguis agar

PLATE 5



a) Mitis salivarius agar

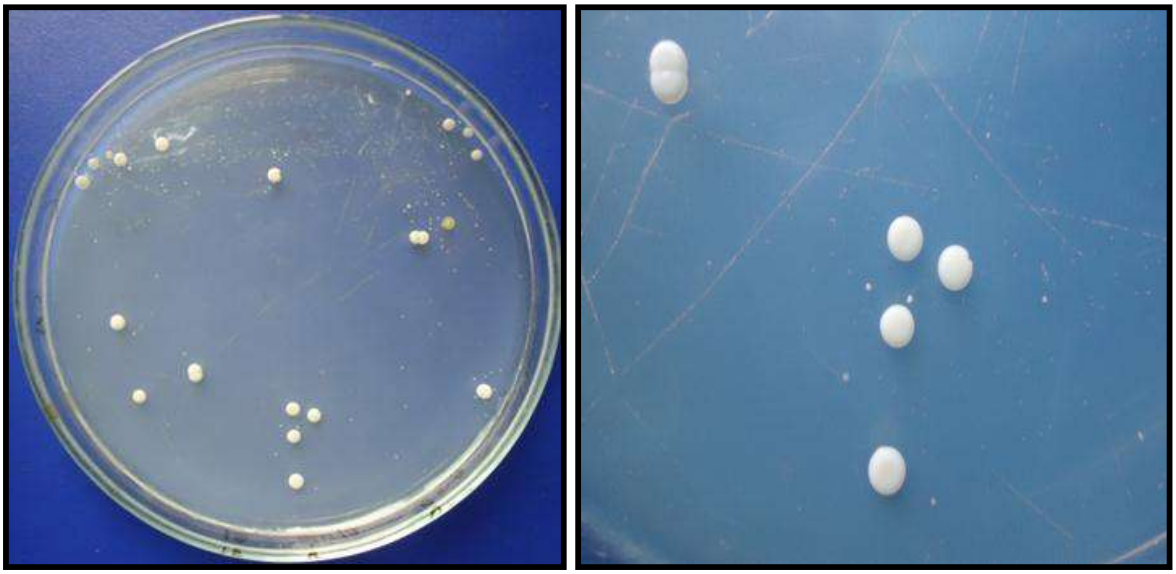


b) Colonies of Streptococcus species on Mitis salivarius agar

Colonies of Streptococcus species on Mitis salivarius agar

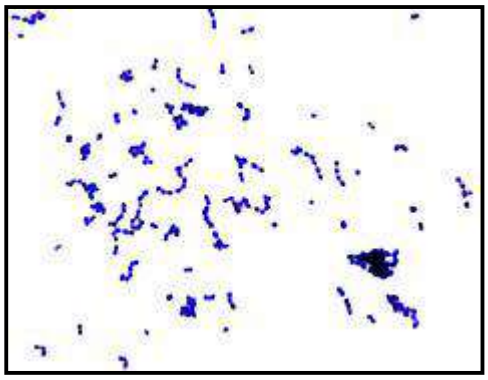
PLATE 6

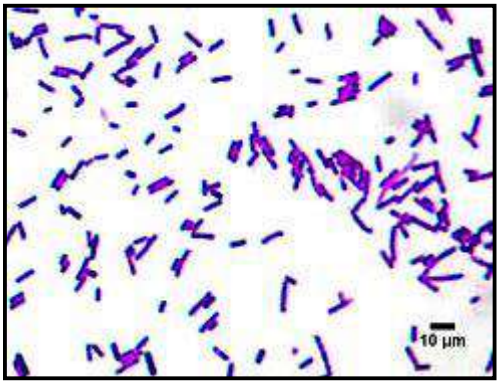
a) Colonies of Lactobacillus on de Mann Rogosa agar



b) Colonies of Candida species on Saborauds Dextrose agar

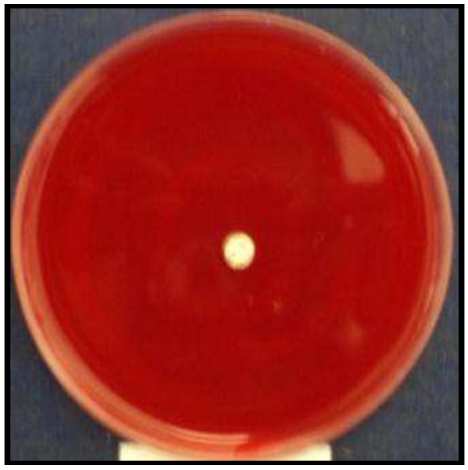
PLATE 7

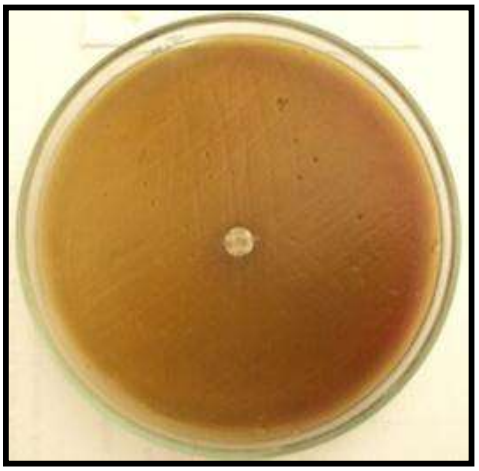




a) **b)**

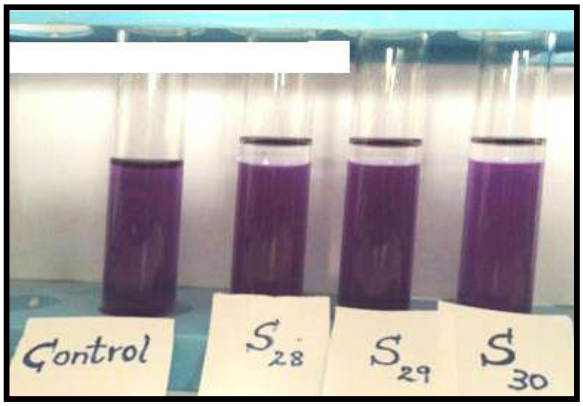
a) Microscopic field showing Gram positive cocci arranged in chains (40X). b) Microscopic field showing Gram positive bacilli (40X)

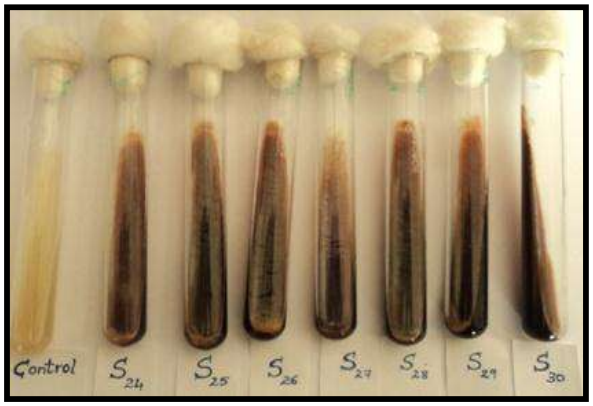




Before incubation **After incubation**

Optochin sensitivity

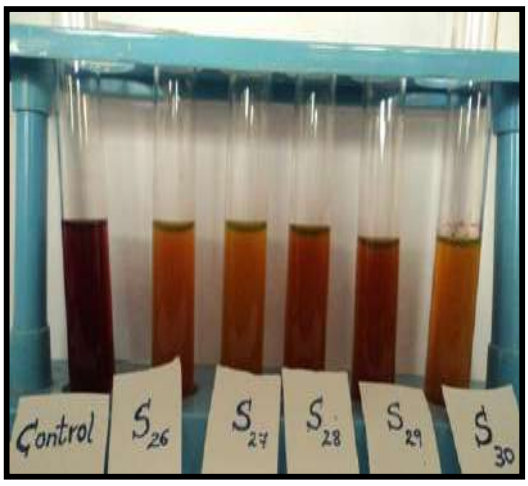




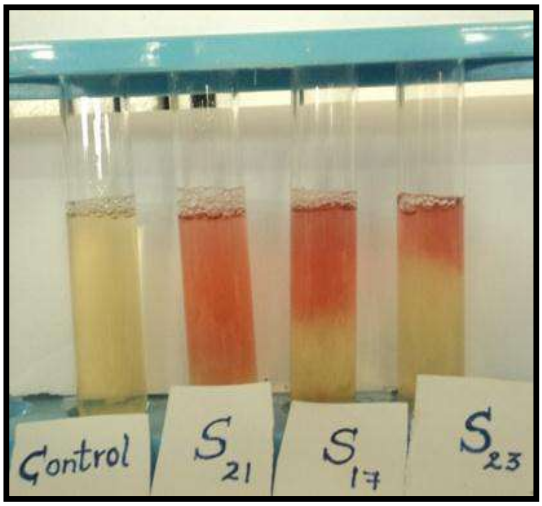
Arginine hydrolysis **Esculin hydrolysis (right)**

PLATE 8

a) Fermentation of Mannitol



b) Sorbitol fermentation



c) VP test

d) Germ tube test

5.4.2. Determination of antibacterial susceptibility pattern of clinical isolates to standard antibiotics.

Antibacterial susceptibility test of clinical isolates to standard antibiotics was determined by employing agar disc diffusion method. Results of this test revealed that, there were variations in the susceptibility patterns of the isolates to various antibiotics used. All the isolates were foundto be sensitive to all the screened antibiotics, but some of the isolates also showed intermediate susceptibility and resistance pattern.

S. mutans showed highest susceptibility with zone of inhibition ranging from 20 to 34 mm in diameter for all the antibiotics tested. Highest susceptibility was observed against Penicillin (45 mm), and least susceptibility was observed against Chloramphenicol (20 mm). *S. mutans* was found to exhibt intermediate resistance against Ampicillin (17 mm), Penicillin (17 mm), Streptomycin (15 and 19 mm). Resistance pattern was also observed among *S. mutans* strains for Erythromycin (13 and 14 mm), Norfloxacin (10 and 13 mm) and Oflaxacin (10 mm). In our study, all the isolates of *S. mutans*were found to be resistance against Polymixin (Figure 5.22 and Plate 9a).

S. sanguis showed its sensitivity for all the tested antibiotics, highest susceptibility was observed against Erythromycin with zone of inhibition 35 mm and least was observed against Gentamicin with zone of inhibition 20 mm. Intermediate resistance was also observed for the antibiotics such as Erythromycin (15 mm), Kanamycin (15 mm), Norfloxacin (15 and 18 mm), Penicillin (18 mm) and Trimethoprim (15 mm). In this study, resistance pattern was observed among *S. sanguis* against Ampicillin (13 mm) and all the strains of *S. sanguis* were found to be resistance against Polymixin (Figure 5.23 and Plate 9 b).

Penicillin was found to exert its action against *S. mitis* with highest zone of inhibition of 40 mm and lowest inhibition was observed in Erythromycin (20 mm). Intermediate resistance was observed in Kannamycin (19 mm), Norfloxacin (15 and 16 mm), Tetracyclin (19 mm), Ampicilin and Chloramphenicol with zone of inhibition of 17 mm respectively. Resistance was observed against Ampicillin (10

mm), Chloramphenicol (10 mm), Penicillin (8 and 5 mm). It is also observed that all the strains of *S. mitis* found to be resistance against Polymixin (Figure 5.24 plate 9 c).

S. salivarius showed highest sensitivity to Penicillin with zone of inhibition 40 mm and lowest inhibition against erythromycin with zone of inhibition 20 mm. In this study, *S. salivarius* did not exhibited intermediate sensitiveness to exposed antibiotics. The obtained result also reveals that, the some of the strains exhibited resistance pattern to ampicillin and polymixin (Figure 5.25 and Plate 9 d).

In our result, it clearly indicates that the most sensitive organism was *L. acidophilus* when compared to other species. This organism was found to be very sensitive to all the exposed antibiotics. Highest activity was exhibited by Penicillin (40 mm) and least activity was observed in chloramphenicol (21 mm). Intermediate resistance pattern and resistance pattern was observed only in Polymixin (Figure 5.26 and Plate 9 d).

5.5. Bioassay of extracted compounds on selected dental caries isolates

5.5.1. Antimicrobial activity of Methanol extract of medicinal plants against pathogens of dental caries

5.5.1.1. Antibacterial activity

Antibacterial activity of methanolic extract of 10 medicinal plants was evaluated by agar well diffusion method. The methanol extracts with different concentration at 10 mg/ml, 25 mg/ml and 50 mg/ml was assessed, against 20 clinical strains selected from each groups *i.e.* from *S. mutans, S. sanguis, S. mitis, S. salivarius* and *L. acidophilus*. All the plant extracts showed dose dependent activity against all the clinical isolates. Results of antibacterial activity were recorded as average zone of inhibition in millimetre (mm).

5.5.1.1.1. Antibacterial activity of methanolic extracts against clinical isolates of *Streptococcus mutans*.

Antibacterial activity of crude methanolic extract of plants was assessed against *S. mutans.* All the screened plants showed inhibitory efficacy against *S. mutans.* Among the plant evaluated, methanolic extract of *H. mysoresence* showed

most significant antibacterial activity with zone of inhibition 11.90 ± 0.718 mm, 15.30 ± 0.470 mm and 18.95 ± 0.883 mm at 10 mg/ml, 25 mg/ml and 50 mg/ml respectively. Least activity was observed in *M. glabra* with zone

of inhibition 7.2 ± 0.918 mm, 10.5 ± 1.54 mm and 15.80 ± 1.870 mm at 10 mg/ml, 25 mg/ml and 50 mg/ml respectively. Except these, all the methanolic plant extract showed moderate dose dependent antibacterial activity against *S. mutans*. The standard tetracycline (positive control) at 10 µg/ml showed inhibition zone of about 29.65 ± 0.790 mm and negative control 10% DMSO showed no zone of inhibition (Table 5.14 and Plate 10).

Similarly antibacterial activity was also done for the standard strains of *S. mutans* (MTCC- 497) and *S. mutans* (MTCC- 897). Both the strains were susceptible for the methanolic extract of plants. At 50 mg/ml methanolic extract of *H. mysoresence* showed a significant antibacterial activity against *S. mutans* 497 (19.0 ± 1.0 mm) and *S.mutans*-807 (19.60±0.57 mm) respectively. Least activity was demonstrated by *M. glabra* against *S. mutans* 497 (12.0 ±057 mm) and *S. mutans* (12.6 ±0.57 mm). Apart from these, all the plants extract showed moderate antibacterial activity against both standard strains of *S. mutans* (Figure 5.27).

5.5.1.1.2. Antibacterial activity of methanolic extracts against clinical isolates of *Streptococcus sanguis*.

The results of inhibitory activity of crude methanolic extract of plants against *S. sanguis* revealed that, all the tested plants showed a dose dependent inhibitory activity (Table 5.15).Among the plant screened,least activity was observed in *M .glabra* with zone of inhibition 5.4 ± 0.843 mm, 7.9 ± 0.875 mm and 13.90 ± 1.870 mm at 10 mg/ml, 25 mg/ml and 50 mg/ml respectively. Methanolic extract of *H. mysoresence* showed highest inhibitory activity with zone of inhibition 11.10 ± 0.394 mm, 18.05 ± 0.887 mm and 21.05 ± 0.394 mm at 10 mg/ml, 25 mg/ml and 50 mg/ml respectively. Other methanolic extracts showed moderate dose dependent antibacterial activity against *S. sanguis*. The standard tetracycline (positive control) at 10 µg/ml showed inhibition zone of about 27.50 ± 0.843 mm and10% DMSO (negative control) did not showed any zone of inhibition (Figure 5.28 and Plate 11).

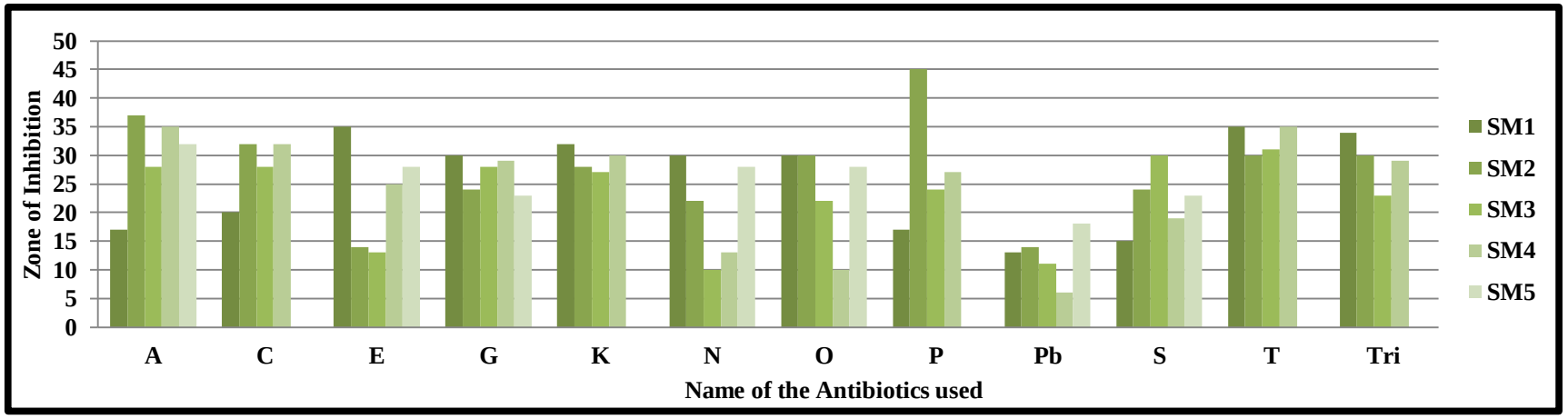


Figure 5.22: Antibacterial susceptibility pattern of *S. mutans* to standard antibiotics.

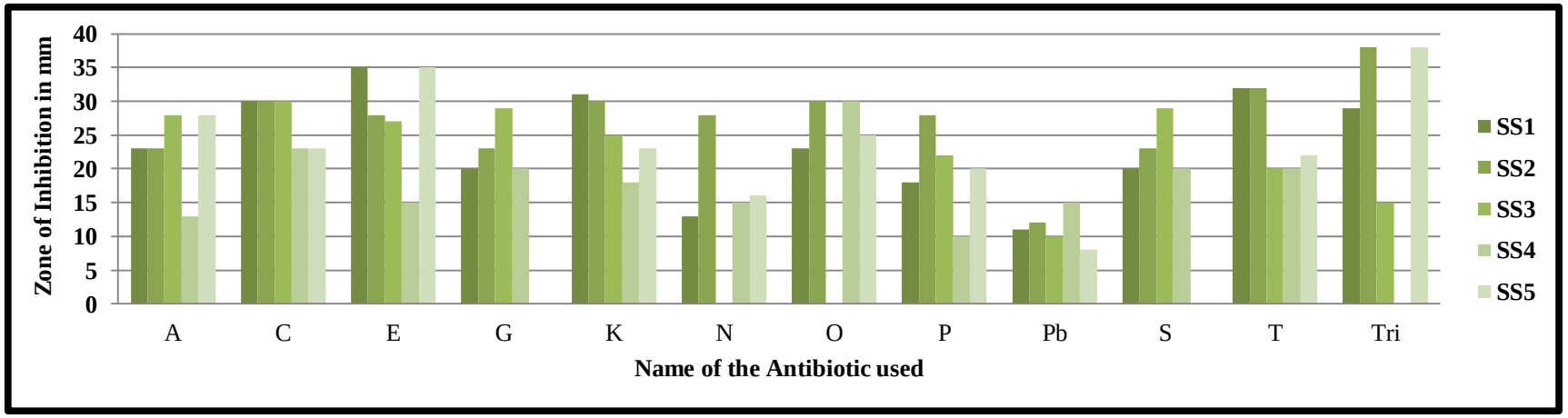


Figure 5.23: Antibacterial susceptibility pattern of *S. sanguis* to standard antibiotics.

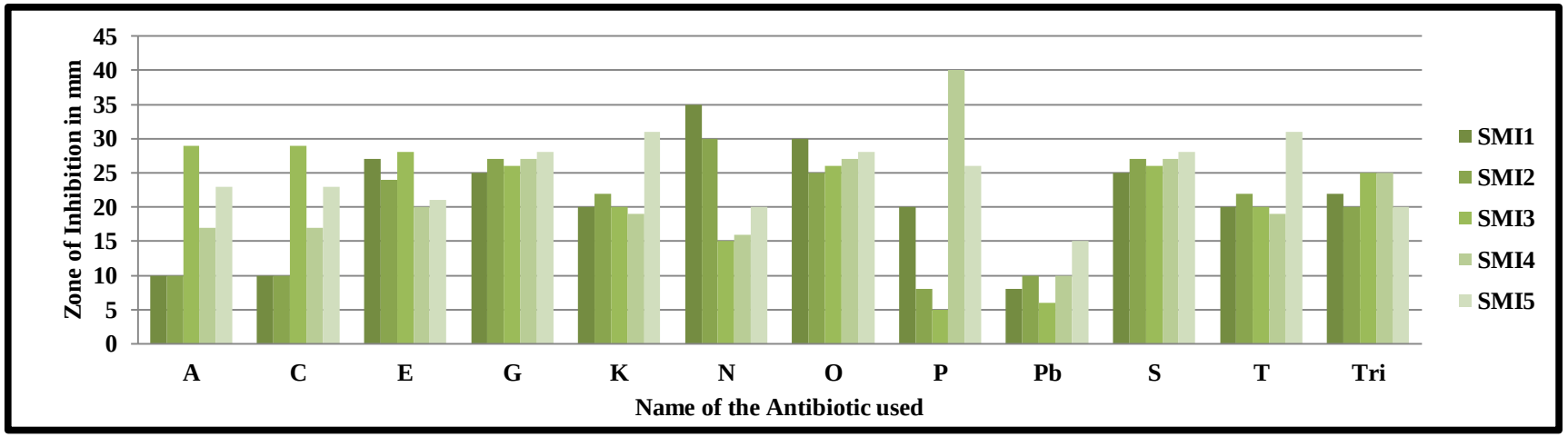


Figure 5.24: Antibacterial susceptibility pattern of *S. mitis* to standard antibiotics.

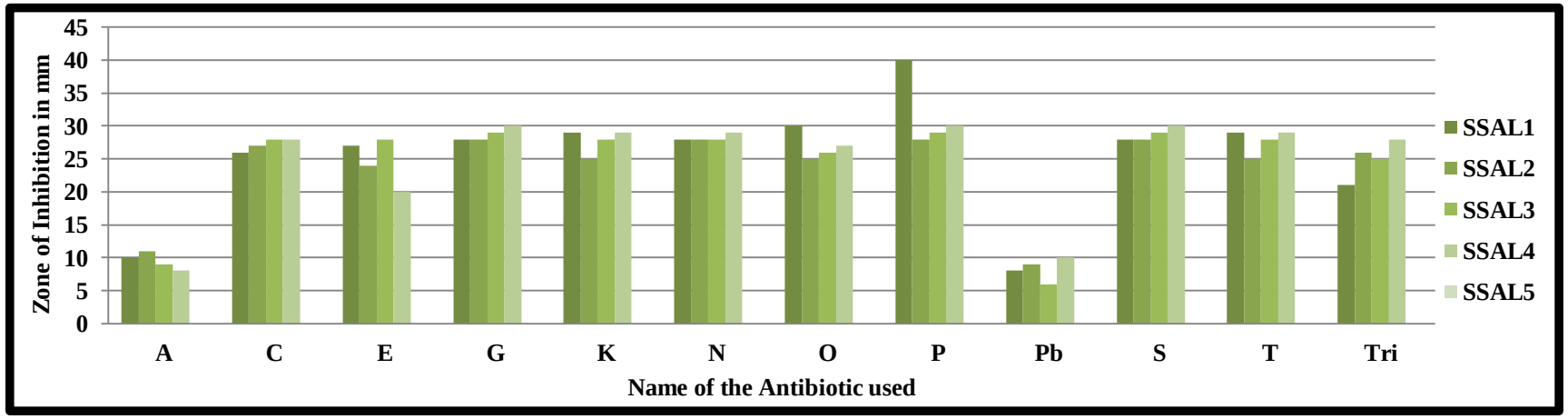


Figure 5.25: Antibacterial susceptibility pattern of *S. salvarius* to standard antibiotics.

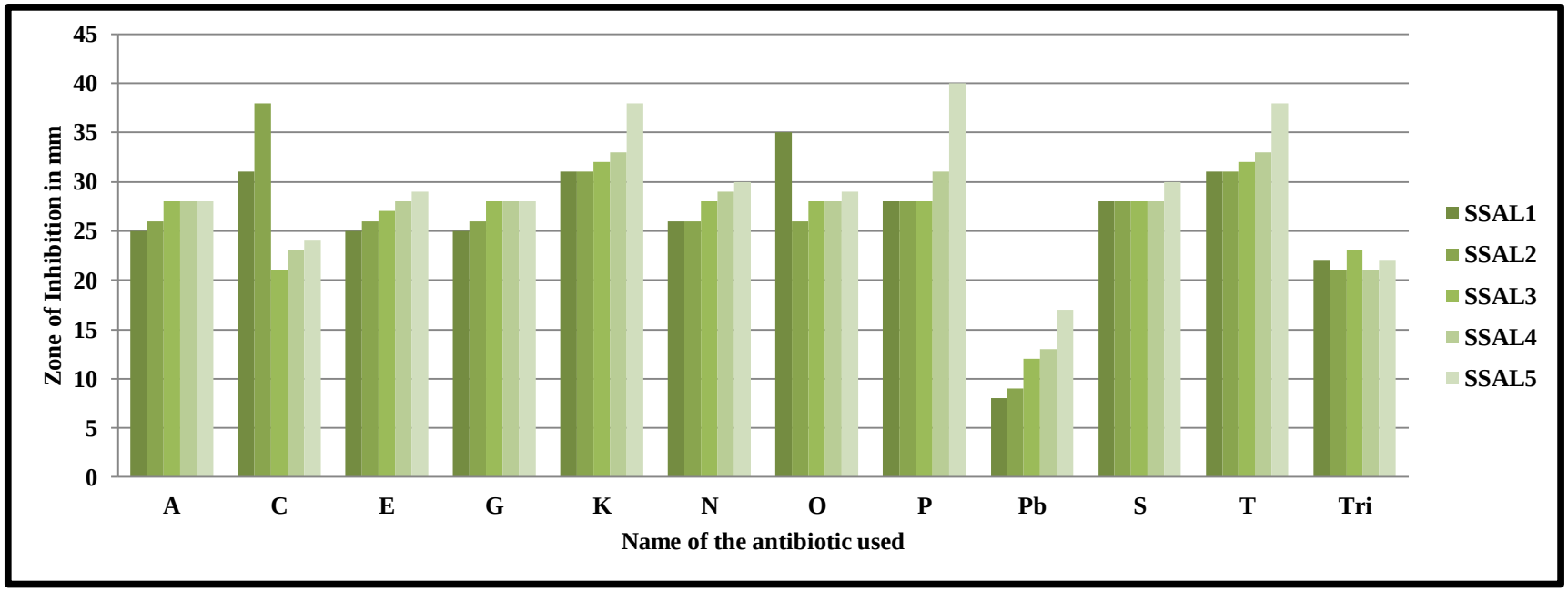
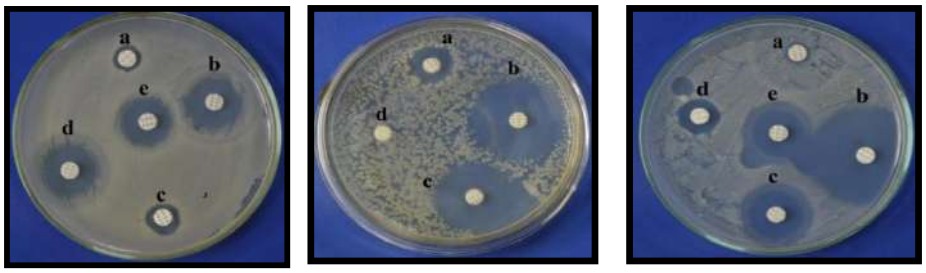
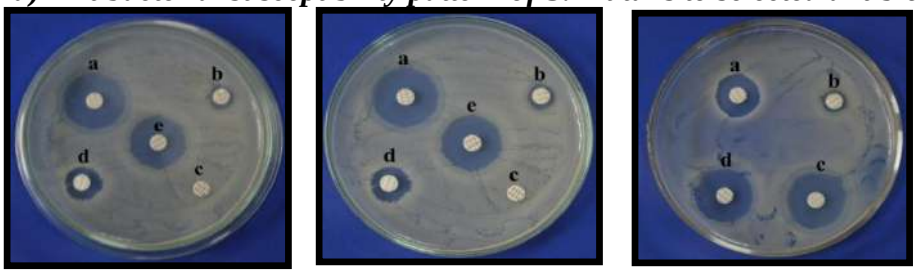


Figure 5.26: Antibacterial susceptibility pattern of *L.* acidophilus to standard antibiotics.

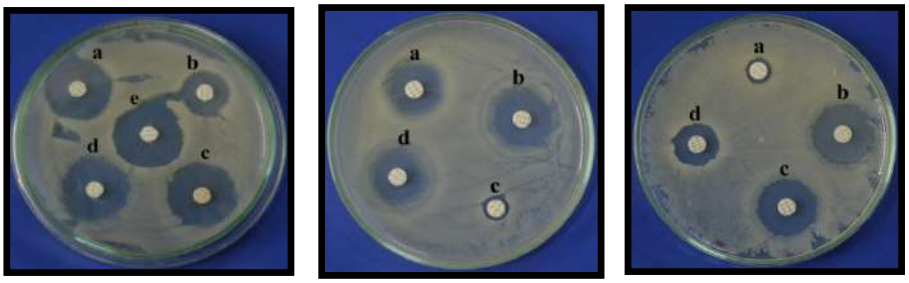
PLATE 9



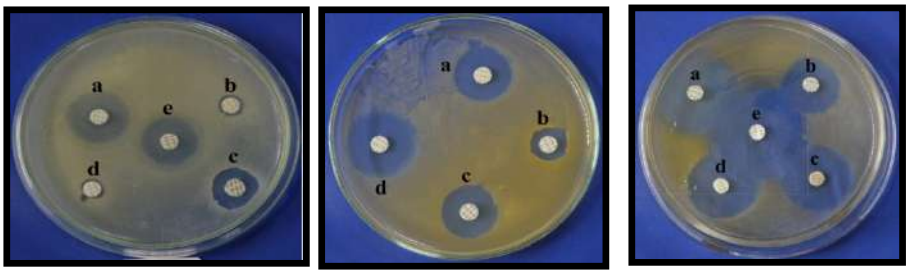
a) Antibacterial susceptibility pattern of S. mutans to selected antibiotics



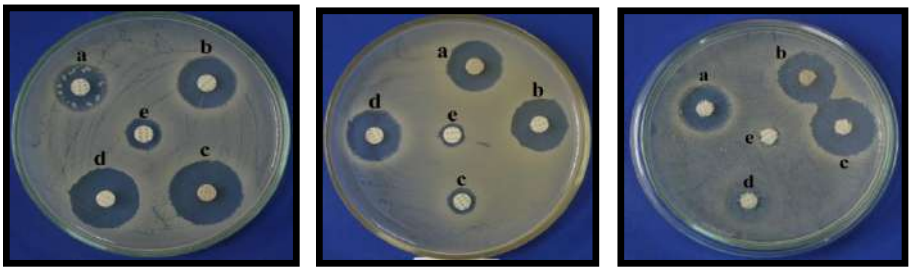
b) Antibacterial susceptibility pattern of S. sanguis to selected antibiotics



c) Antibacterial susceptibility pattern of S. mitis to selected antibiotics



d) Antibacterial susceptibility pattern of S. salivarius to selected antibiotics



e) Antibacterial susceptibility pattern of L.acidophilus to selected antibiotics

Antibacterial susceptibility pattern of dental caries pathogens to various antibiotics

5.5.1.1.2. Antibacterial activity of methanolic extracts against clinical isolates of *Streptococcus sanguis*.

The results of inhibitory activity of crude methanolic extract of plants against *S. sanguis* revealed that, all the tested plants showed a dose dependent inhibitory activity (Table 5.15).Among the plant screened, least activity was observed in *M. glabra* with zone of inhibition 5.4 ± 0.843 mm, 7.9 ± 0.875 mm and 13.90 ± 1.870 mm at 10 mg/ml, 25 mg/ml and 50 mg/ml respectively. Methanolic extract of *H. mysoresence* showed highest inhibitory activity with zone of inhibition 11.10 ± 0.394 mm, 18.05 ± 0.887 mm and 21.05 ± 0.394 mm at 10 mg/ml, 25 mg/ml and 50 mg/ml respectively. Other methanolic extracts showed moderate dose dependent antibacterial activity against *S. sanguis*. The standard tetracycline (positive control) at 10 µg/ml showed inhibition zone of about 27.50 ± 0.843 mm and10% DMSO (negative control) did not showed any zone of inhibition(Figure 5.28 and Plate 11)

5.5.1.1.3. Antibacterial activity of methanolic extracts against clinical isolates of *Streptococcus mitis*.

In vitro susceptibility of *S. mitis* to methanolic extracts of various plants was performed by following agar well diffusion method. The results revealed that, the extracts exhibited a marked antibacterial activity. Among the plant used methanolic extract of *M. indica* showed highest activity with zone of inhibition 12.65 ± 1.424 mm, followed by *H. mysoresence*11.50 ±0.945 mm at 10 mg/ml respectively. *H. mysoresence* showed highest inhibitory activity with zone of inhibition 18.05 ± 0.887 mm and 21.05 ± 0.394 mm at 25 mg/ml and 50 mg/ml respectively. Least activity was demonstrated by *M. glabra* with zone of inhibition 5.80 ± 0.910 mm, 8.80 ± 0.632 mm and 15.40 ± 1.250 mm at 10 mg/ml, 25 mg/ml and 50 mg/ml respectively. All other methanolic plant extract showed moderate dose dependent antibacterial activity against *S. mitis*. The standard tetracycline (positive control) at 10 µg/ml showed inhibition zone of about 25.06 ± 0.985 mm and 10% DMSO (negative control) did not shown any zone of inhibition (Table 5.16, Figure 5.29, Plate 12).

Tested plant	Zone of inhibition in mm			Standard	10% DMSO
	10 mg /ml	25 µg/ml	50mg/ml	10µg/ml	0
A. indicus	7.00 ± 0.790	9.80 ± 0.83	15.00 ± 0.562	29.65± 0.79	0
A. muricata	12.20± 0.894	14.90± 0 .553	16.50±0.761	29.65± 0.79	0
A. indica	12.35±1.725	13.90±1.410	17.55±0.945	29.65± 0.79	0
C. roxburghii	8.05±0.887	12.55±0.826	15.90±1.165	29.65± 0.79	0
H. mysoresence	11.90±0.718	15.30±0.470	18.95±0.883	29.65± 0.79	0
M. glabra	7.2±0.918	10.5±1.54	15.80±1.870	29.65± 0.79	0
M. indica	11.45±.999	14.35±1.040	18.65±2.277	29.65± 0.79	0
S. pentandrum	9.65±1.089	11.5±1.051	16.60±1.429	29.65± 0.79	0
T. asiatica	8.00±1.451	12.75±1.293	15.85±1.785	29.65± 0.79	0
W. tinctoria	6.85±0.875	11.50±1.147	14.50±0.513	29.65± 0.79	0

Each value is the average values of inhibition zone of 20 clinical isolates of *S. mutans*, ± standard deviation

Table 5.14: Antibacterial activity of methanolic extracts of different plants on *S. mutans*.

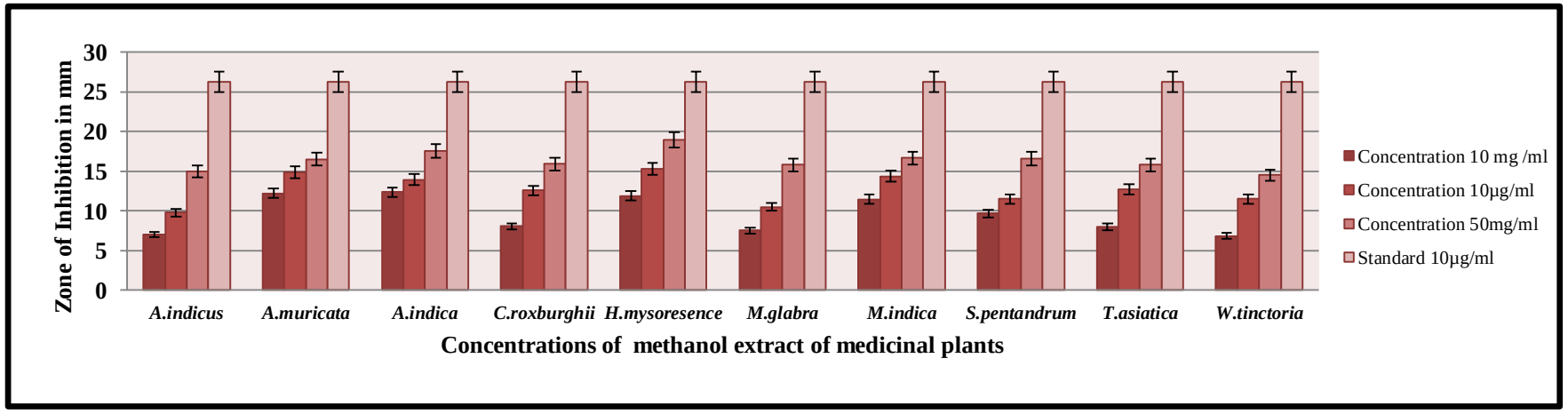


Figure 5.27: Antibacterial activity of methanolic extracts of different plants on *S. mutans*.

Tested plant	Concentration			Standard	10% DMSO
	10 mg /ml	25 µg/ml	50mg/ml	10µg/ml	0
A. indicus	7.15 ± 0.988	9.75 ±0.967	15.35 ±0.587	27.50±0.843	0
A. muricata	10.25±0.550	14.65±0.489	17.90±0.788	27.50±0.843	0
A. indica	10.80±0.768	14.45±0.826	17.65±0.813	27.50±0.843	0
C. roxburghii	7.30±0.923	10.20±0.616	14.75±1.251	27.50±0.843	0
H. mysoresence	21.05±0.394	18.05±0.887	21.05±1.021	27.50±0.843	0
M. glabra	5.4±0.843	7.9±0.875	13.90±1.100	27.50±0.843	0
M. indica	11.20±1.576	14.75±0.550	18.10±0.447	27.50±0.843	0
S. pentandrum	7±1.076	10.8±1.240	14.65±0.813	27.50±0.843	0
T. asiatica	7.60±1.046	12.00±1.298	14.32±1.360	27.50±0.843	0
W. tinctoria	7.70±0.571	11.50±0.688	14.55±0.686	27.50±0.843	0

Each value is the average values of inhibition zone of 20 clinical isolates of *S.sanguis*,± standard deviation.

Table 5.15: Antibacterial activity of methanolic extracts of different plants on *S. sanguis*.

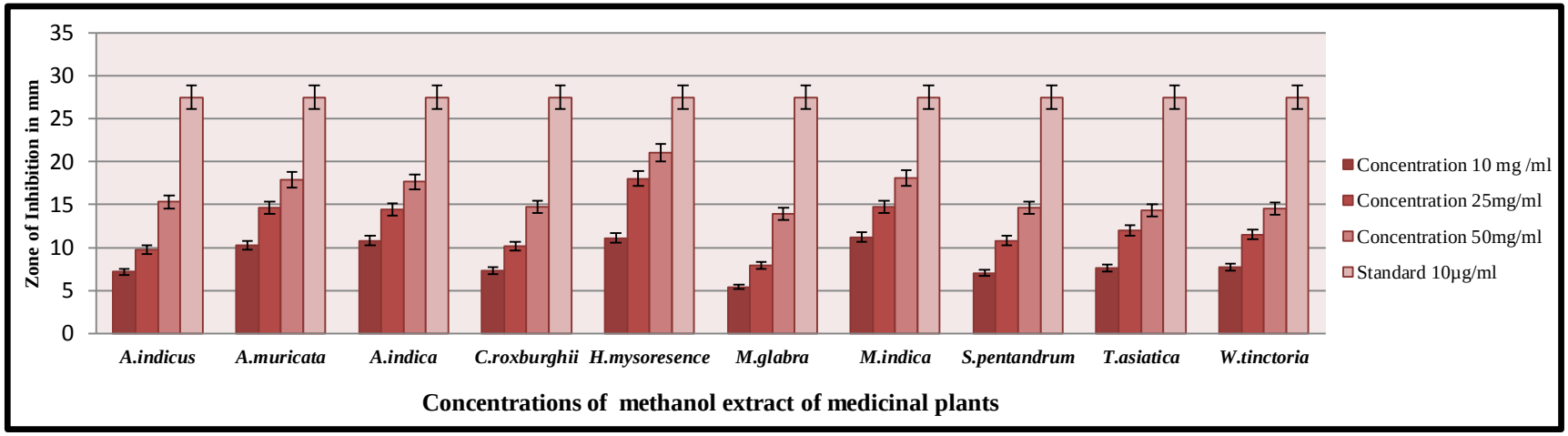


Figure 5.28: Antibacterial activity of methanolic extracts of different plants on *S. sanguis.*

Tested plant	Zone of inhibition in mm			Standard	10% DMSO
	10 mg /ml	25mg/ml	50mg/ml	10µg/ml	0
A. indicus	9.80 ±0.834	10.70 ± 0.865	14.80±0.894	25.6	0
A. muricata	10.90±0.912	14.55±0.605	17.70±0.571	25.6	0
A. indica	11.10±1.165	12.25±1.070	13.20±1.152	25.6	0
C. roxburghii	8.50±0.688	10.95±0.945	15.10±0.553	25.6	0
H. mysoresence	11.50±.945	14.95±0.510	19.85±0.933	25.6	0
M. glabra	5.80±0.910	8.80±0.632	15.40±1.250	25.6	0
M. indica	12.65±1.424	14.75±0.786	18.05±0.394	25.6	0
S. pentandrum	7.65±0.988	8.95±0.759	14.70±1.218	25.6	0
T. asiatica	10.1±2.490	15.05±1.820	18.35±0.587	25.6	0
W. tinctoria	8.30±0.733	11.40±0.681	14.25±0.444	25.6	0

Each value is the average of inhibition zone of 20 clinical isolates of *S. mitis*, ± standard deviation

Table 5.16: Antibacterial activity of methanolic extracts of different plants on *S.mitis*.

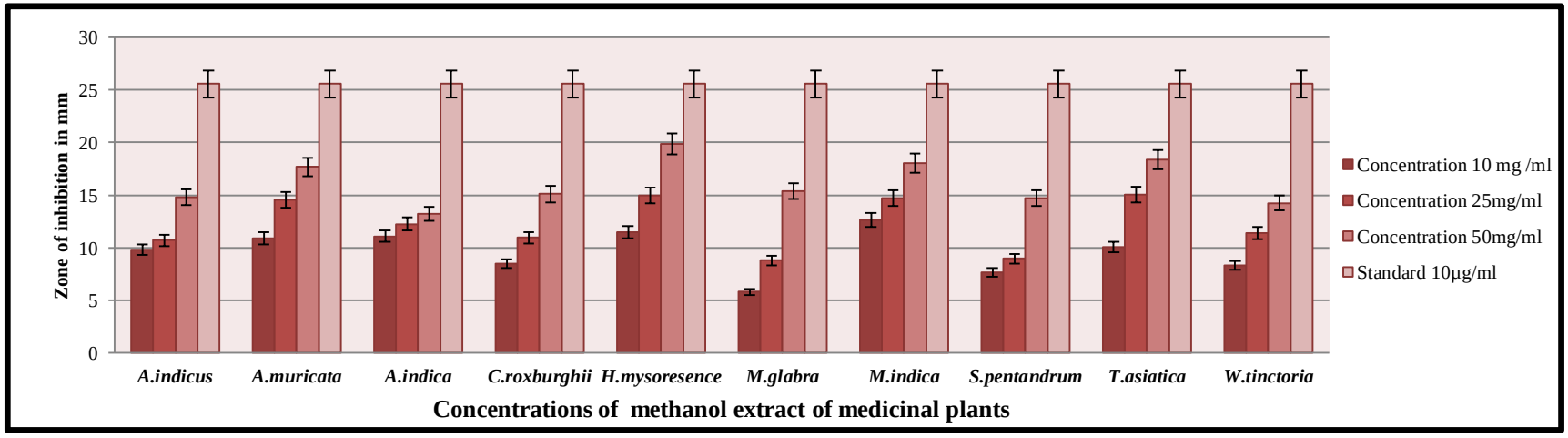
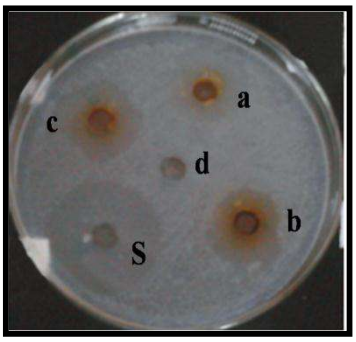
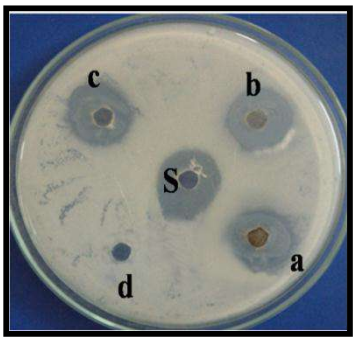


Figure 5.29: Antibacterial activity of methanolic extracts of different plants on *S. mitis*.

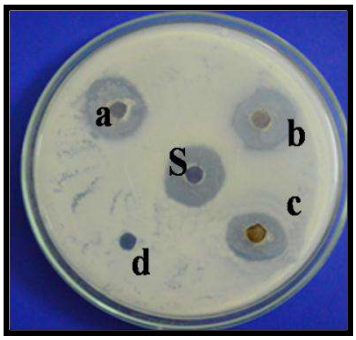
PLATE 10



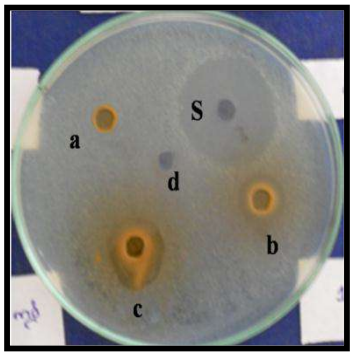
Anisomeles indicus



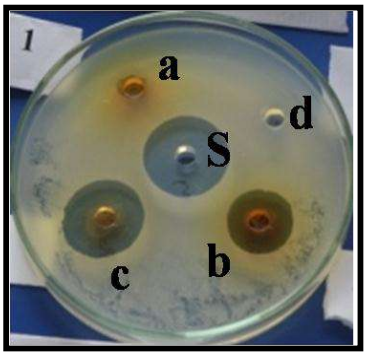
Annona muricata



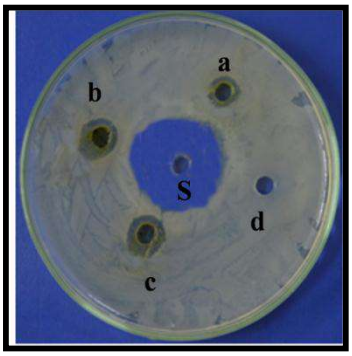
Azadiractha indica



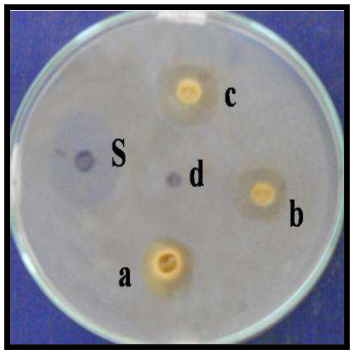
Crysophyllum roxburghii



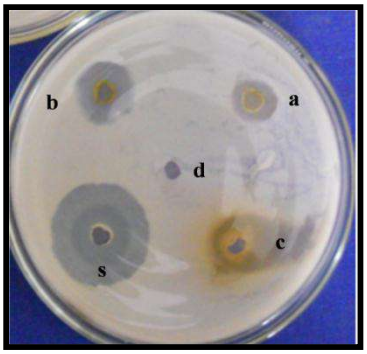
Hypericum mysoresence



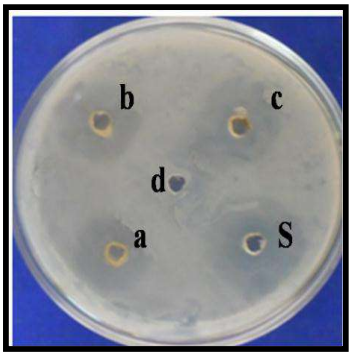
Malpighia glabra



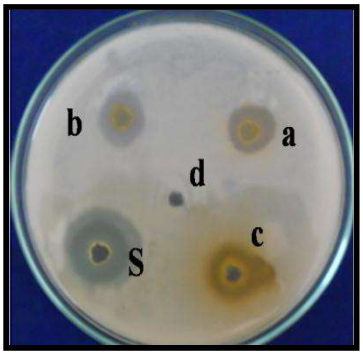
Mangiferra indica



Scleropyrum pentandrum



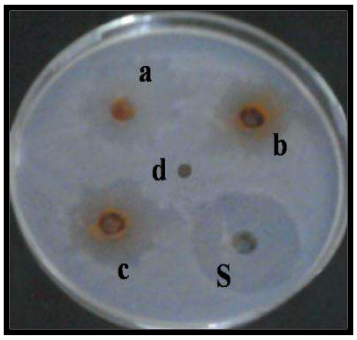
Toddalia asiatica



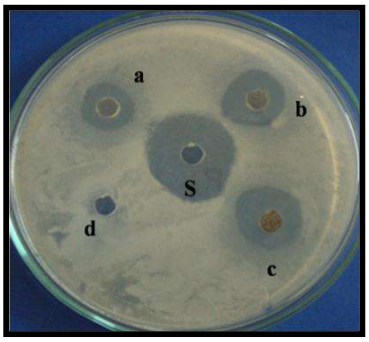
Wrightia tinctoria

Antibacterial activity of methanol extracts of medicinal plants against *S. mutans* (a-10mg/ml, b-25 mg/ml, c-50 mg/ml, d-10% DMFO, S- Tetracycline 10µg/ml)

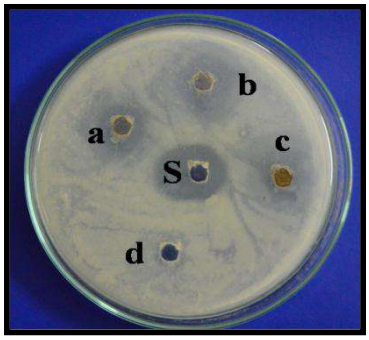
PLATE 11:



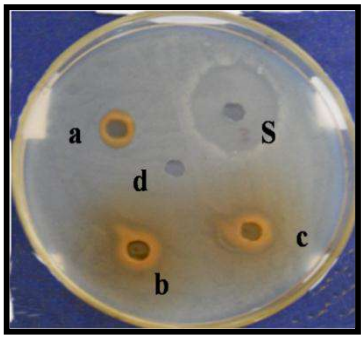
Anisomeles indicus



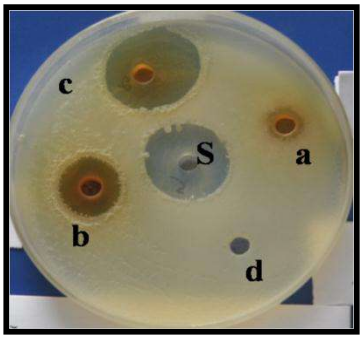
Annona muricata



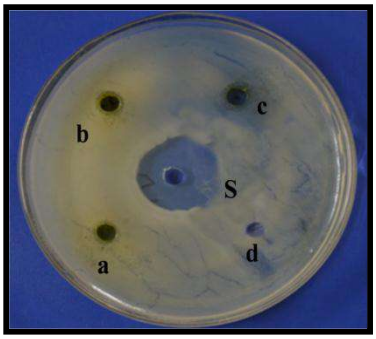
Azadiractha indica



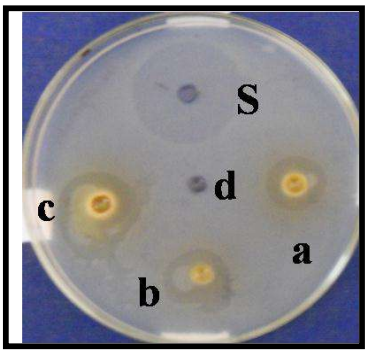
Crysophyllum roxburghii



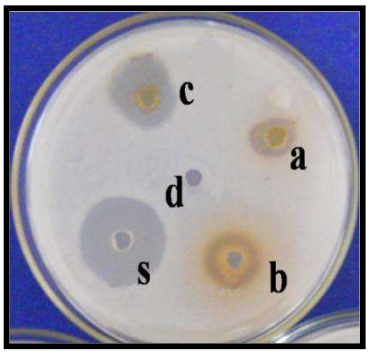
Hypericum mysoresence



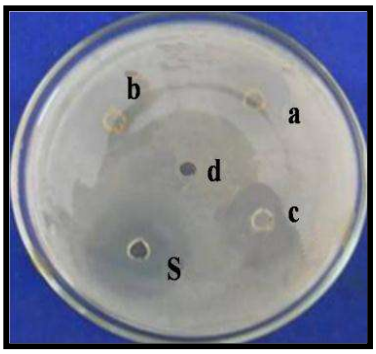
Malpighia glabra



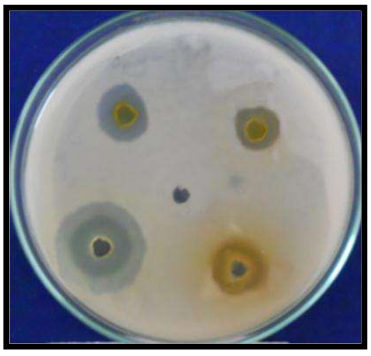
Mangiferra indica



Scleropyrum pentandrum



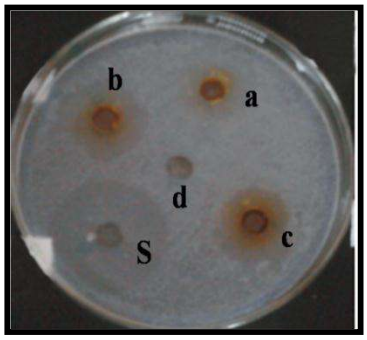
Toddalia asiatica



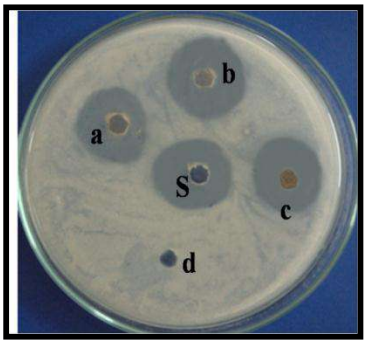
Wrightia tinctoria

Antibacterial activity of methanol extracts of medicinal plants against *S. sanguis* (a-10mg/ml, b-25 mg/ml, c-50 mg/ml, d-10% DMFO, S- Tetracycline 10µg/ml)

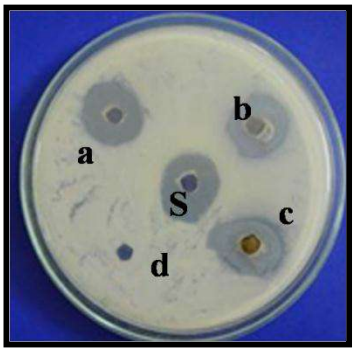
PLATE 12:



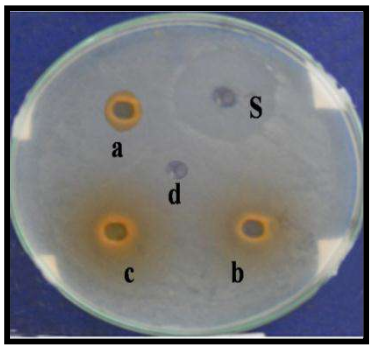
Anisomeles indicus



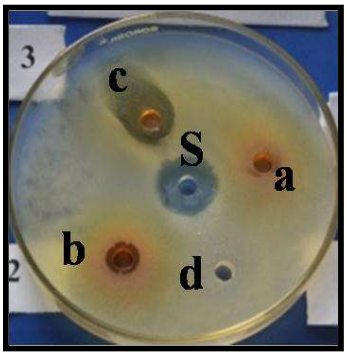
Annona muricata



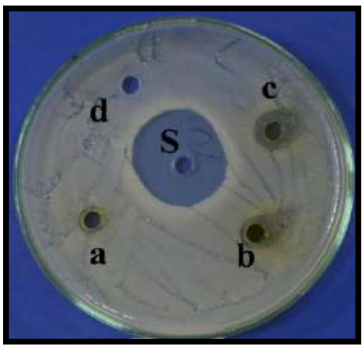
Azadiractha indica



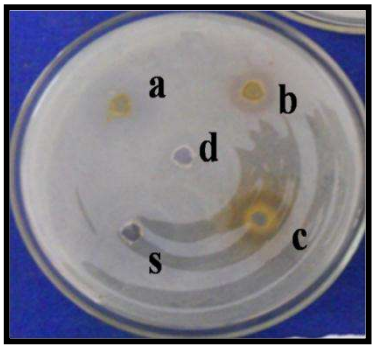
Crysophyllum roxburghii



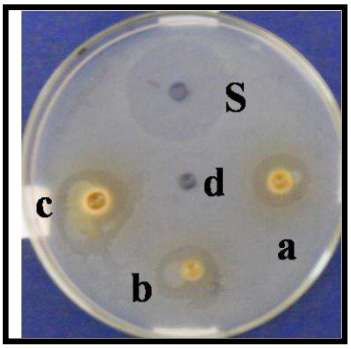
Hypericum mysoresence



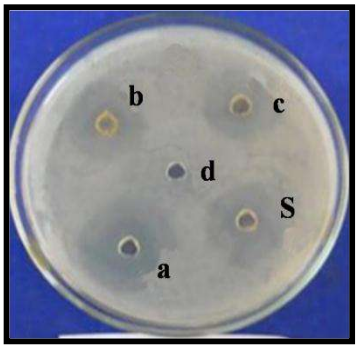
Malpighia glabra



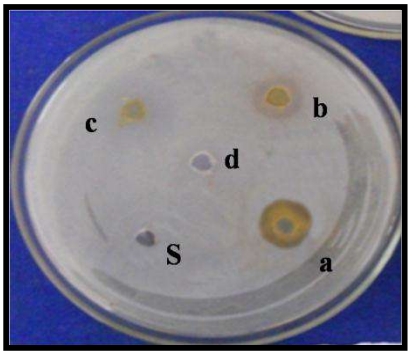
Mangiferra indica



Scleropyrum pentandrum



Toddalia asiatica



Wrightia tinctoria

Antibacterial activity of methanol extracts of medicinal plants against *S. mitis*

(a-10mg/ml, b-25 mg/ml, c-50 mg/ml, d-10% DMFO, S- Tetracycline 10µg/ml**)**

5.5.1.1.4. Antibacterial activity of methanolic extracts against clinical isolates of *Streptococcus salivarius*.

The results of antibacterial activity of crude methanolic extract of ten plants revealed that, the evaluated crude extracts possessed a potential antibacterial activity against *S. salivarius. S. salivariou s*showed highest susceptibility to methanolic extract of *M. indica* with zone of inhibition 11.40 ± 0.833 mm followed by *H.mysoresence*11.20 ± 0.834 mm at 10 mg/ml respectively. At 25 mg/ml and 50 mg/ml methanolic extract of *H.mysoresence* showed highest inhibitory activity with zone of inhibition 18.60 ± 0.503 mm and 19.25 ± 2.023 mm respectively. Least activity was observed in *M. glabra* with zone of inhibition 5.50 ± 0.70 mm, 11.1 ± 0.87 mm and 14.80 ± 0.78 mm at 10 mg/ml, 25 mg/ml and 50 mg/ml respectively. Except these, all the methanolic plant extract showed moderate dose dependent antibacterial activity against *S. salivarius* (Plate 13). Tetracycline (positive control) at 10 µg/ml showed inhibition zone of about 26.20 ± 0.513 mm and 10% DMSO (negative control) did not showed any zone of inhibition (Table 5.17 and Figure 5.30).

5.5.1.1.5. Antibacterial activity of methanolic extracts against clinical isolates of *Lactobacillus acidophilus*.

All methanolic extracts of selected plants showed a marked inhibitory activity against *L. acidophilus.* At 10 mg/ml, highest activity was observed in methanolic extract of *T. asiatica* with zone of inhibition 11.70 ± 2.557 mm followed by *W.tinctoria*11.65 ± 0.933 mm, *A.muricata*11.35 ± 0.988 mm, *A.indica*10.80 ±1.056 mm, *H.mysoresence*10.65 ± 0.933 mm, *M.indica*10.65 ± 1.424 mm and least activity was observed in *C.roxburghii*10.25 ± 0.716 mm followed by *A.indicus*8.45 ± 1.356 mm, *S.pentandrum*8.20 ± 0.834 mm and *M. glabra 6.50* ± 0.70 mm. At 25 mg/ml *H. mysoresence and M. indica*showed highest inhibitory activity with zone of inhibition 14.95 ± 0.503 mm and 14.95 ± 0.50 mm respectively. At 50 mg/ml *H. mysoresence* and *M. indica* showed highest activity with zone of inhibition 20.30 ±0.657 mm and 19.50 ± 2.013 mm respectively. At 50 mg/ml least activity was found to be in *M.glabra*15.60 ± 0.250 mm (Plate 14). The standard tetracycline (positive control) at 10 µg/ml showed inhibition zone of about 28.50 ± 1.026 mm and 10% DMSO (negative control) did not showed any zone of inhibition (Table 5.18 and Figure 5.31).

5.5.1.2. Antifungal activity of methanol extracts of medicinal plants against *Candida albicans*

Antifungal activity of methanolic extracts different plants was performed by agar well diffusion method against clinically isolated *C. albicans*. At 50 mg/ml methanolic extract of *A. muricata* showed a significant antifungal activity against *C. albicans* with zone of inhibition 12.15 ± 0.38 mm followed by *W. tinctoria* 12.7 ± 0.44 mm (Plate 15). Least activity was demonstrated by *M. glabra* with zone of inhibition 10.50 ± 0.51mm. Except these, all methanolic extracts exhibited moderate dose dependent inhibitory activity against *C. albicans* (Table 5.19 and Figure 5.32).

5.6. Bioassay of extracted compounds and purified compounds from *H. mysoresence* on selected dental caries isolates.

5.6.1. Antimicrobial activity of crude extracts of *H. mysoresence* against selected clinical isolates.

The *in vitro* antibacterial activity of different solvent extracts of *H. mysoresence* was performed against dental caries pathogens by agar well diffusion method and the results were represented in the table 5.20. At 50 mg /ml concentration, PEH showed a significant inhibitory activity against *L.acidophilus* (24.10±0.44 mm) followed by *S. mutans* (23.60±0.59 mm) *S. salivarius* (22.80±0.52 mm), *S. mitis* (21.35±1.05 mm) and *S. sanguis* (20.80 ± 1.05 mm) (Plate 16- Plate 17).

CEH at 50 mg/ml concentration showed highest activity against *L.acidophilus* (13.45±1.60 mm) and *S. mutans* (12.85±1.30 mm) followed by *S. salivarius* (12.80±1.15 mm), *S. sanguis* (12.60±1.50 mm) and *S. mitis* (12.20±0.69 mm).

MEH at 50 mg/ml concentration exhibited a very significant inhibitory effect on *L.acidophilus* (20.45±0.88 mm) and *S. salivarius* (19.55± 3.03 mm) followed by *S. mutans* (18.85±0.98 mm), *S. mitis* (18.65±1.08 mm) and *S. sanguis* (17.85±0.58 mm).

AEH at 50 mg/ml showed a moderate inhibitory activity against *L.acidophilus* (12.95±1.19 mm) and *S. sanguis* (12.25±1.20 mm) followed by *S. mitis* (11.80±1.15 mm), *S. mutans* (11.75 ± 0.85 mm) and *S. salivarius* (10.45±1.05 mm).

5.6.2. Antifungal activity of crude extract of *H. mysoresence* against *C. albicans*

Antifungal activity of different solvent extracts of *H. mysoresence* was performed by agar well diffusion method against clinically isolated *C. albicans*. At 50 mg/ml PEH showed a significant antifungal activity against *C. albicans* with zone of inhibition 13.95 ± 0.39 mm followed by MEH 10.60 ± 0.50 mm respectively. CEH and AEH did not demonstrated antifungal activity (Table 5.21).

Results of antimicrobial activity and antifungal activity suggested that crude extract obtained by petroleum ether showed promissory results and encouraged us to select PEH for purification of phytocompounds. The purification was performed by silica gel column chromatography method, yielded nineteen pure compounds. These purified compounds were screened for antimicrobial activity against clinical isolates.

5.6.3. Antimicrobial activity of purified compounds of *H. mysoresence* against clinical isolates

5.6.3.1. Antibacterial activity of purified compounds of *H. mysoresence* against clinical isolates

Antibacterial activity of pure compounds was performed by agar well diffusion method against clinical isolates of dental caries. Out of 19 pure fraction, 18 fractions at 1 mg/ml concentration demonstrated very good antibacterial activity. Among 18 compounds,F_3, F_4, F_5, F_9 and F_{10} compound showed a significant antibacterial activity against all the screened clinical isolates (Plate 18).

F_3 compound exhibited very potent antibacterial activity against *S. mutans* (15 mm) and *L. acidophilus* (11 mm) followed by *S. sanguis, S. salivarius* and *S. mitis* with zone of inhibition 10 mm respectively.

The pure compound F_4 showed antibacterial activity against *S. mitis* and *S. salivarius* with zone of inhibition of 14 mm respectively, followed by *S. mutans, S. sanguis* and *L. acidophilus* with zone of inhibition 11 mm in diameter respectively.

F_5 compound showed a marked inhibitory activity against *S. mitis* (19 mm) and *S. salivarius* (19 mm) followed by *L. acidophilus* (17 mm), *S. mutans* (17 mm) and *S. sanguis* (15 mm).

F_9 compound showed significant antibacterial activity against *S. mitis, S. salivarius* and *L. acidophilus* with 16 mm zone of inhibition respectively followed by, *S. sanguis* (12 mm) and *S. mutans* (11 mm).

Antibacterial activity of pure compound F_{10} revealed that, highest inhibition zone was observed against *L. acidophilus* (13 mm) followed by *S. mutans* (11 mm), *S. salivarius* (10 mm), *S. mitis* (10 mm) and *S. sanguis* (10 mm).

Tested plant	Zone of inhibition in mm			Standard	10% DMSO
	10 mg /ml	25mg/ml	50mg/ml	10µg/ml	0
A. indicus	8.50	11.15	14.85	26.20± 0.513	0
A. muricata	10.90	15.00	18.05	26.20± 0.513	0
A .indica	11.40	12.75	14.40	26.20± 0.513	0
C. roxburghii	10.80	12.05	14.80	26.20± 0.513	0
H. mysoresence	11.20	15.60	19.25	26.20± 0.513	0
M. glabra	5.5	11.1	14.8	26.20± 0.513	0
M. indica	9.95	14.10	17.40	26.20± 0.513	0
S. pentandrum	8.4	11.55	16.1	26.20± 0.513	0
T. asiatica	10.75	14.20	17.10	26.20± 0.513	0
W. tinctoria	10.40	11.80	15.20	26.20± 0.513	0

Each value is the average values of inhibition zone of 20 clinical isolates of *S. salivarius*, ± standard deviation

Table 5.17: Antibacterial activity of methanolic extracts of different plants on *S. salvarius*.

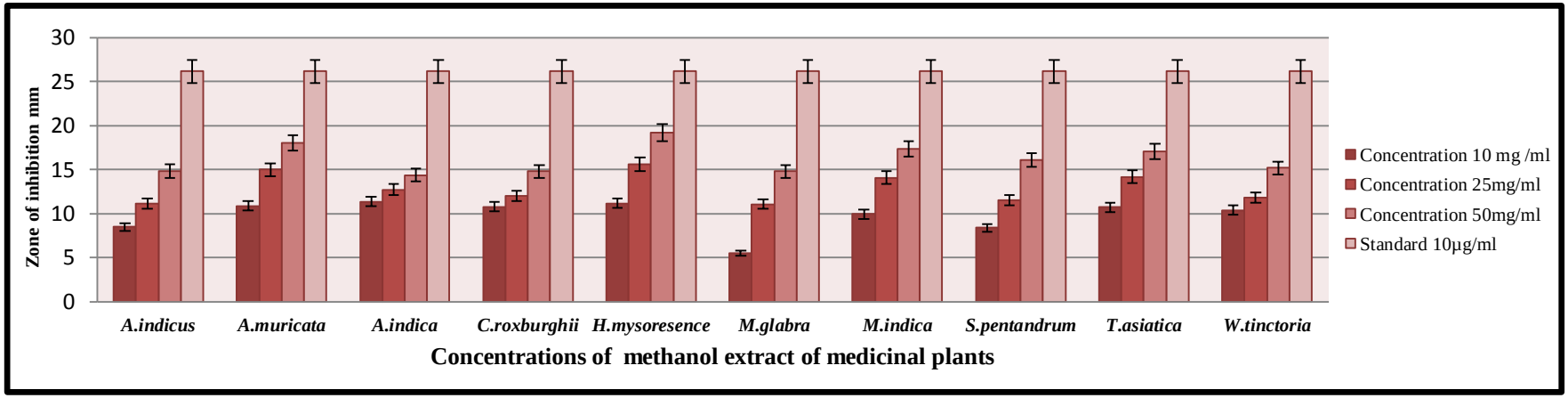


Figure 30: Antibacterial activity of methanolic extracts of different plants on *S. salvarius*.

Tested plant	Zone of inhibition in mm			Standard	10% DMSO
	10 mg /ml	25mg/ml	50mg/ml	10µg/ml	0
A. indicus	8.45±1.356	12.00±0.858	15.20±0.523	28.5±1.056	0
A. muricata	11.35±0.988	13.50±0.827	17.85±0.587	28.5±1.056	0
A .indica	10.80±1.056	13.60±1.903	16.90±1.619	28.5±1.056	0
C. roxburghii	10.25±0.716	11.05±1.099	16.25±0.851	28.5±1.056	0
H. mysoresence	10.65±0.933	14.95±0.510	20.30±0.657	28.5±1.056	0
M. glabra	6.50±0.700	11.2±0.530	15.60±0.250	28.5±1.056	0
M. indica	10.65±1.424	14.95±0.510	19.50±2.013	28.5±1.056	0
S. pentandrum	8.2±0.834	11.5±1.701	16.45±1.234	28.5±1.056	0
T. asiatica	11.70±2.557	14.15±1.424	17.40±1.603	28.5±1.056	0
W. tinctoria	11.65±0.933	12.15±0.813	16.10±0.553	28.5±1.056	0

Each value is the average values of inhibition zone of 20 clinical isolates of *L. acidophilus* ± standard deviation

Table 5.18 :Antibacterial activity of methanolic extracts of different plants on *L. acidophilus.*

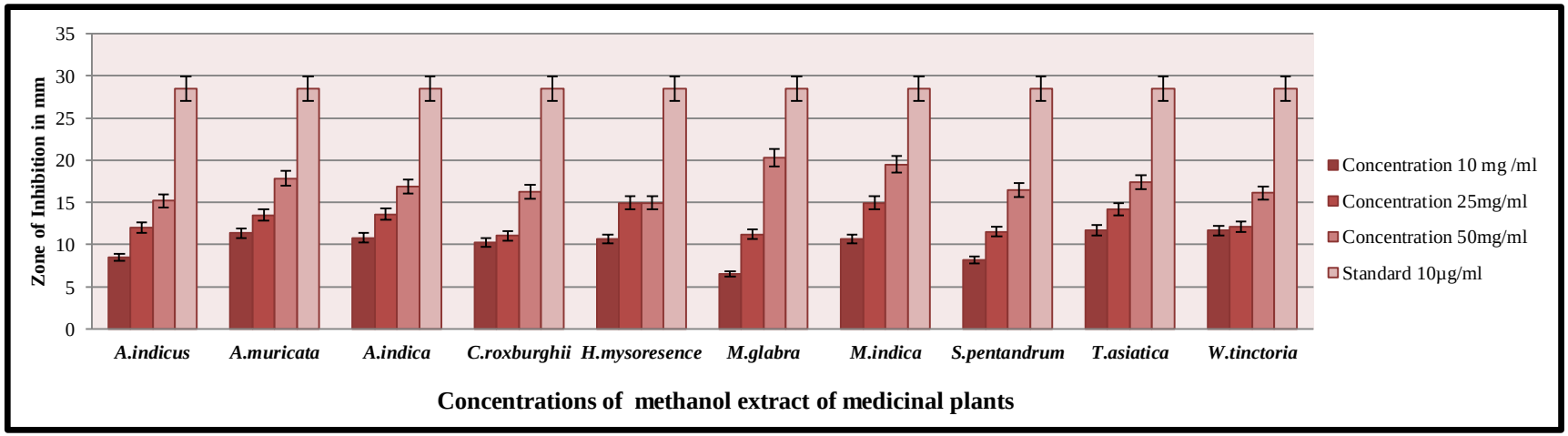


Figure 5.31: Antibacterial activity of methanolic extracts of different plants on *L. acidophilus*.

Tested plant	Concentration			Standard (Fluconozole)
	10 mg /ml	25mg/ml	50mg/ml	10µg/ml
A. indicus	8.5±0.51	10.45±0.51	12.05±0.22	21.85±0.67
A. muricata	10±0.32	12±0.0	12.15±0.38	21.85±0.67
A .indica	11.1±0.58	11.5±0.48	12.10±0.30	21.85±0.67
C. roxburghii	11.1±0.81	12.1±0.81	12.9±0.81	21.85±0.67
H. mysoresence	11.2±0.44	12.9±0.30	12.9±0.30	21.85±0.67
M. glabra	7.80±0.61	9.1±0.30	10.5±0.51	21.85±0.67
M. indica	11.1±0.36	12.2±0.6	13.0±0.00	21.85±0.67
S. pentandrum	9.9±0.64	12.0±0.32	12.8±1.03	21.85±0.67
T. asiatica	8.9±0.96	10.4±0.50	12.1±0.48	21.85±0.67
W. tinctoria	9.1±0.78	11.4±0.50	12.7±0.44	21.85±0.67

Each value is the average of inhibition zone of 20 clinical isolates of *C. albicans*± standard deviation.

Table 5.19: Antifungal activity of methanolic extracts of different plants on *C. albicans*.

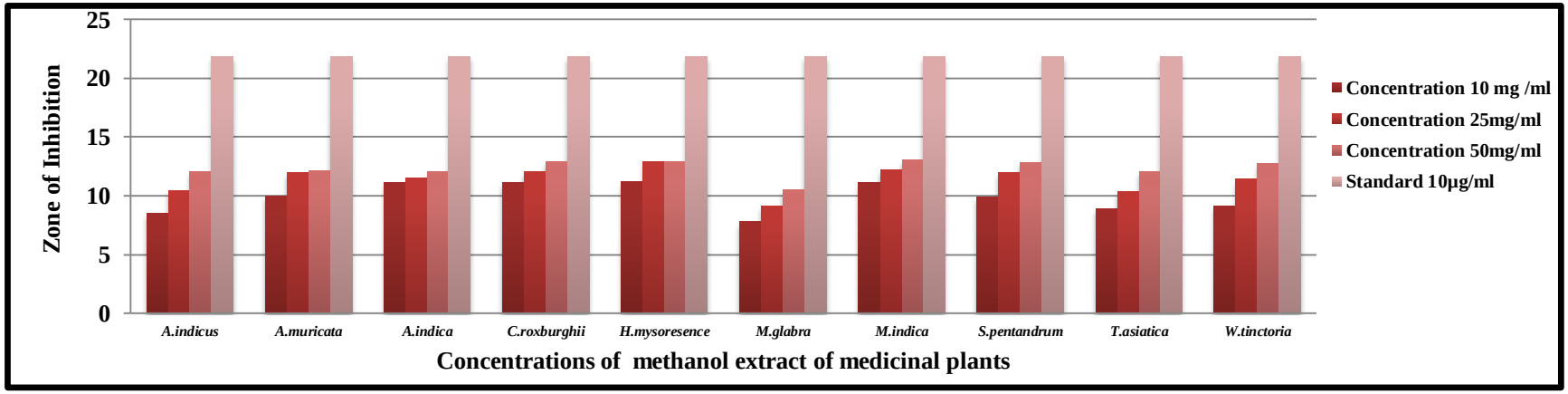
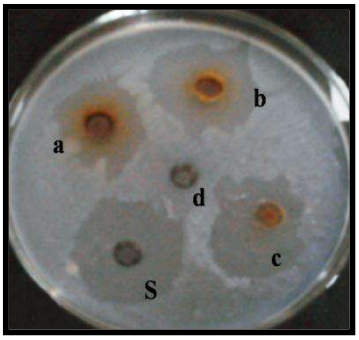
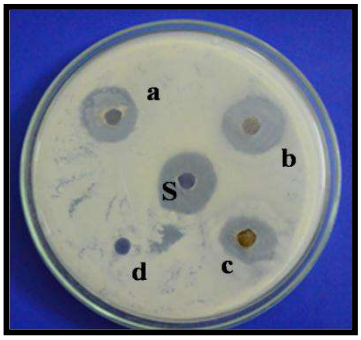


Figure 5.32: Antifungal activity of methanolic extracts of different plants on *C. albicans*.

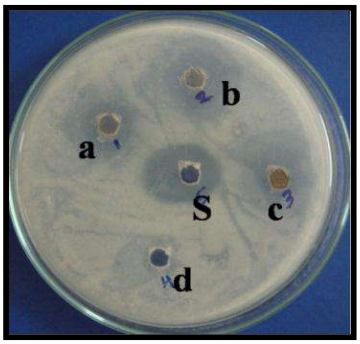
PLATE 13



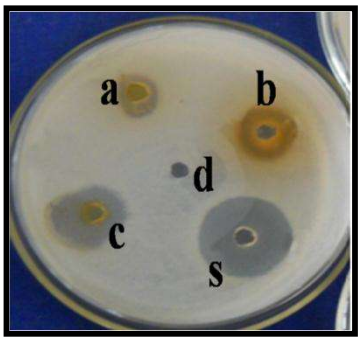
Anisomeles indicus



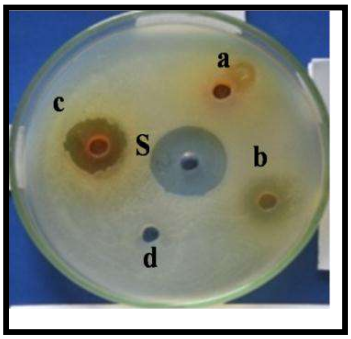
Annona muricata



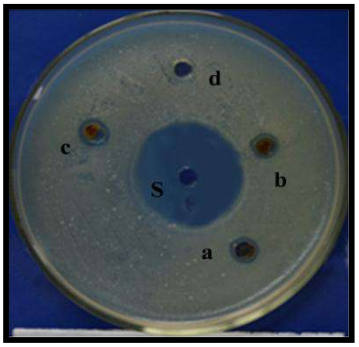
Azadiractha indica



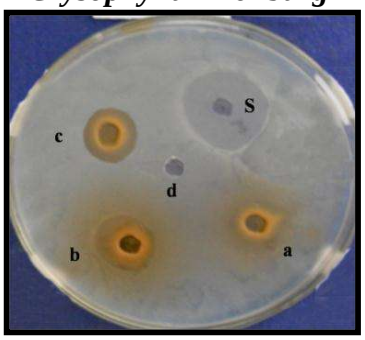
Crysophyllum roxburghii



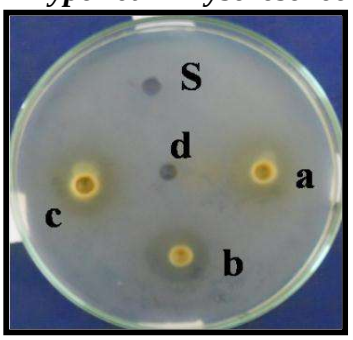
Hypericum mysoresence



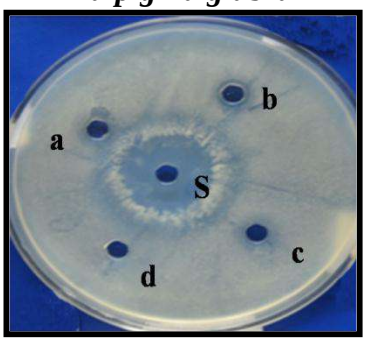
Malpighia glabra



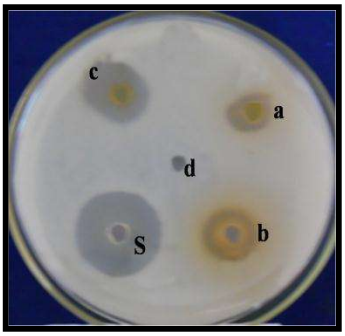
Mangiferra indica



Scleropyrum pentandrum



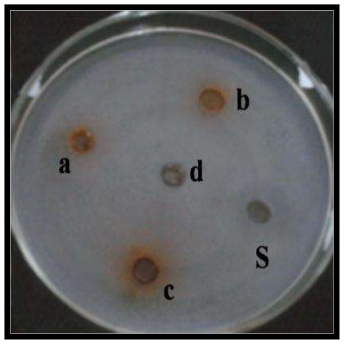
Toddalia asiatica



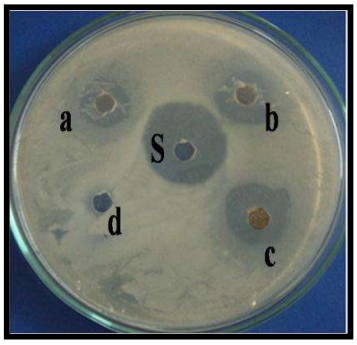
Wrightia tinctoria

Antibacterial activity of methanol extracts of medicinal plants against *S. salivarius* (a-10mg/ml, b-25 mg/ml, c-50 mg/ml, d-10% DMFO, S- Tetracycline 10µg/ml**)**

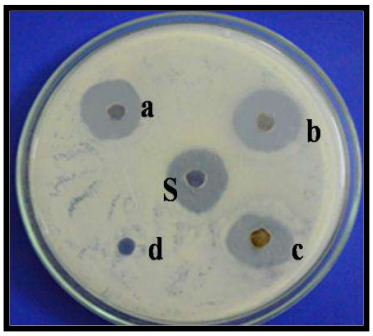
PLATE 14



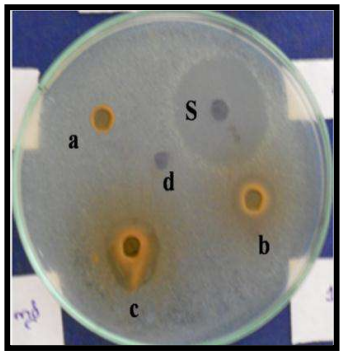
Anisomeles indicus



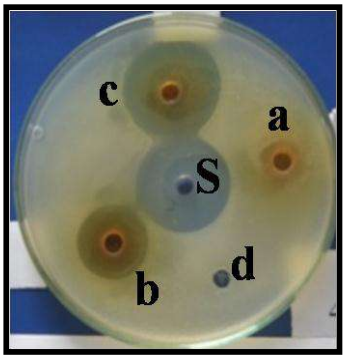
Annona muricata



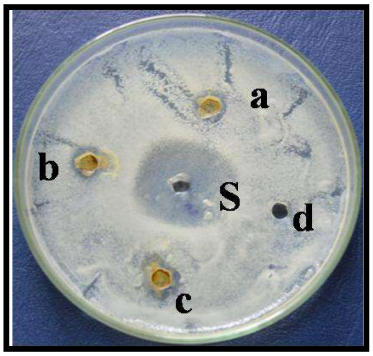
Azadiractha indica



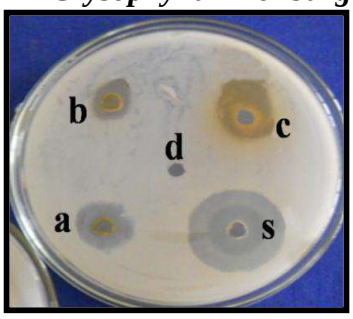
Crysophyllum roxburghii



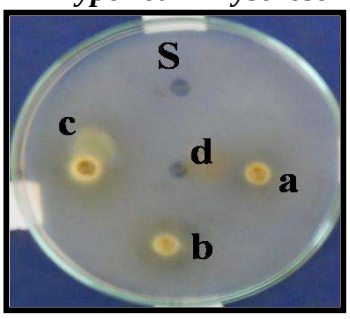
Hypericum mysoresence



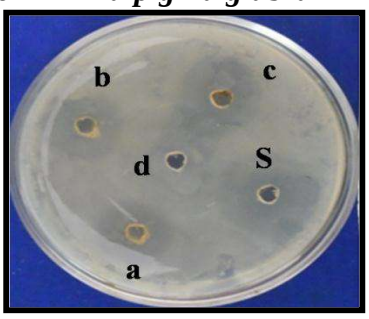
Malpighia glabra



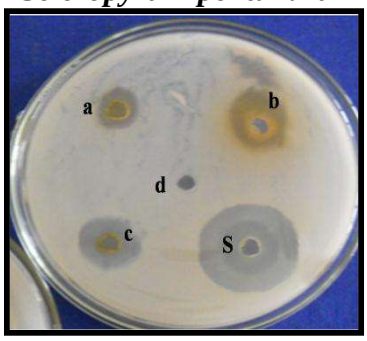
Mangiferra indica



Scleropyrum pentandrum



Toddalia asiatica



Wrightia tinctoria

Antibacterial activity of methanol extracts of medicinal plants against *L. acidophilus.* (a-10mg/ml, b-25 mg/ml, c-50 mg/ml, d-10% DMFO, S- Tetracycline 10µg/ml**)**

Test microorganism	Zone of inhibition in mm												10% DMSO	Tetracycline 10µg/ml
	Petroleum ether extract			Chloroform extract			Methanol extract			Aqueous extract				
	10%	25%	50%	10%	25%	50%	10%	25%	50%	10%	25%	50%		
S. mutans	*15.25±0.55*	*18.75±0.44*	*23.60±0.59*	7.20±1.73	10.35±0.48	12.85±1.30	10.45±0.99	14.90±0.44	18.85±0.98	8.10±0.78	9.75±1.74	11.75±0.85	0	29.65 ± 0.79
S. sanguis	15.25±0.71	19.80±1.15	20.80±1.05	8.30±0.73	10.10±0.64	12.60±1.50	10.90±1.07	14.50±0.51	17.85±0.58	7.80±2.98	10.45±0.51	12.25±1.20	0	27.50 ± 0.84
S. mitis	15.45±0.60	19.60±1.23	21.35±1.13	7.90±0.78	10.10±0.78	12.20±0.69	10.40±0.94	14.75±1.41	18.65±1.08	7.90±0.64	10.90±1.25	11.80±1.15	0	25.06 ± 0.98
S. salivarius	15.55±0.60	19.70±1.12	22.80±0.52	8.15±0.87	10.10±1.29	12.80±1.15	11.90±1.07	14.95±1.27	19.55±3.03	8.05±0.82	10.90±1.41	10.45±1.05	0	26.20 ± 0.51
L. acidophilus	15.55±0.99	20.10±0.71	24.10±0.44	7.65±1.04	11.20±1.19	13.45±1.60	11.10±1.07	15.60±0.59	20.45±0.88	8.35±0.81	9.80±0.89	12.95±1.19	0	28.5±1.056

Each value is the average values of inhibition zone of 20 clinical isolates, n= 20, ± standard deviation

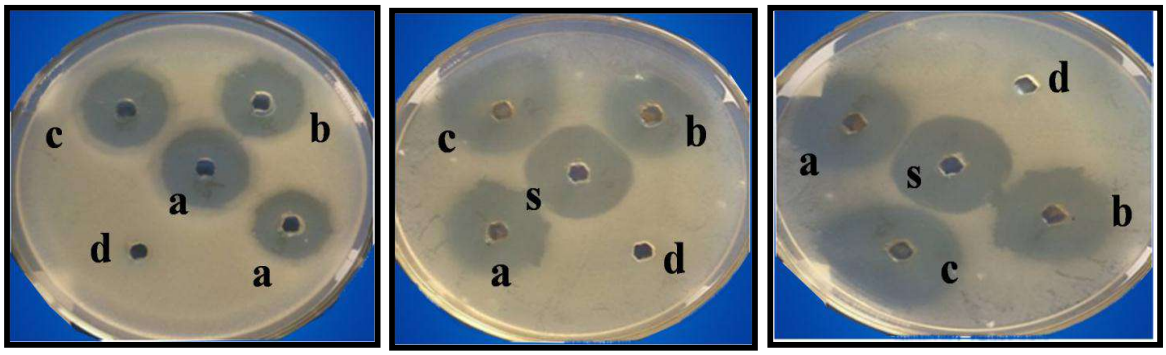
Table 5.20: Antibacterial activity of *Hypericummysoresence* against pathogens of dental caries.

Test microorganism	Zone of inhibition in mm												10% DMSO	Fluconozole10µg/ml
	Petroleum ether extract			Chloroform extract			Methanol extract			Aqueous extract				
	10%	25%	50%	10%	25%	50%	10%	25%	50%	10%	25%	50%		
C. albicans	10.80±0.41	*12.85*±0.36	*13.95*±0.39	10.10±0.30	12±0.0	12±0.1	9.80±0.52	10.90±0.30	10.60±0.50	10.85±0.48	10.75±0.44	13.55±0.60	0	21.85±0.67

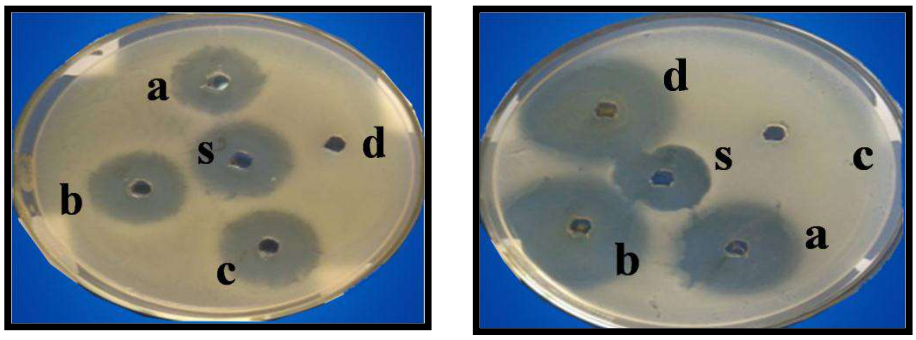
Each value is the average values of inhibition zone of 20 clinical isolates, n= 20, ± standard deviation

Table 5.21: Antifungal activity of *Hypericum mysoresence* against *C. albicans.*

PLATE 16

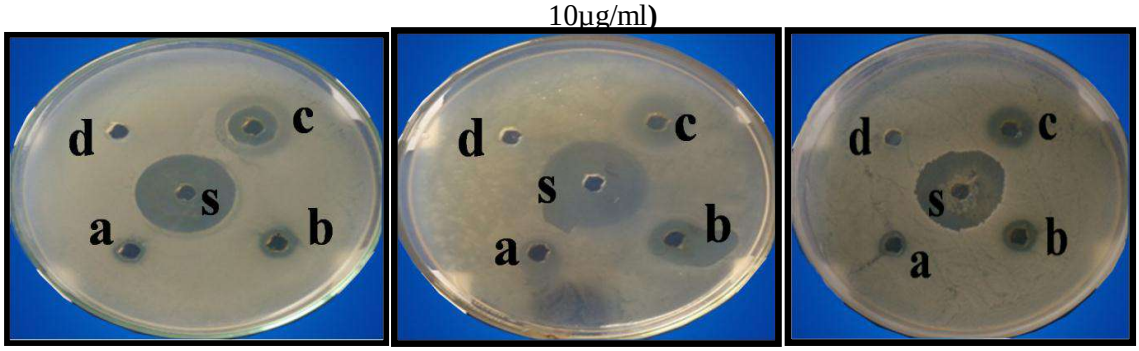


S. mutans ***S. sanguis*** ***S. mitis***

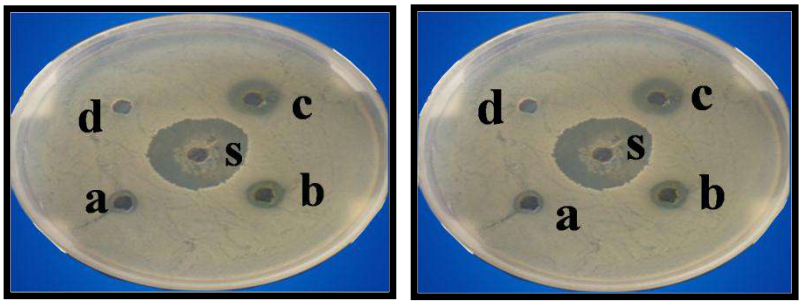


S. salivarius ***L.acidophilus***

a) Antibacterial activity of Petroleum ether extract of H. mysoresence against pathogens of dental caries (a-10mg/ml, b-25 mg/ml, c-50 mg/ml, d-10% DMFO, S- Tetracycline 10µg/ml**)**



S. mutans ***S. sanguis*** ***S. mitis***



b) Antibacterial activity of Chloroform extract of H. mysoresence against pathogens of dental caries (a-10mg/ml, b-25 mg/ml, c-50 mg/ml, d-10% DMFO, S- Tetracycline 10µg/ml**)**

Antimicrobial activity of crude ectract of H. mysoresence against dental caries pathogens

5.6.3.2.Antifungal activity of purified compounds

Antifungal activity of pure compounds was performed by agar well diffusion method against *C. albicans*. Out of 19 pure fractions, only two compounds showed significant antifungal activity. Among the pure compounds only F4 and F5 showed significant antifungal activity against *C. albicans* with zone of inhibition 9 mm and 10 mm respectively (Table 5.22).

5.6.4. Determination of MIC, MBC and MFC

Minimum inhibitory concentration is the concentration at which the extract inhibits the 90% of the growth. In this present study, MIC was done for F3, F4, F5, F9 and F10 compounds against single clinical isolates of *S. mutans, S. sanguis, S. mitis, S. salivarius and L. Acidophilus* and *C. albicans*. The MIC value for F3, F4, F9 and F10 compounds was found to be 15.62 µg/ml and the MIC value for F4 compound was found to be 7.812 µg/ml against all the tested bacteria. The MIC value for pure compounds F4, and F5 was found to be 500 µg/ml against *C. albicans* respectively and pure compound F3, F9 and F10 didn't show any inhibitory activity (Table 5.23).

Minimum bactericidal concentration is the concentration at which the extract kills the 99.9% of bacteria. In this present study, inoculated tubes of MIC were used for determination of MBC. The tubes containing different concentration used in the determination of MIC was streaked on sterile Mueller Hinton Agar plates and incubated for 24 hours at 37^0C. Inoculated plates were observed for the growth. The results reveals that, MBC value for F3, F4, F9 and F10 compounds was found to be 62.5 µg/ml against all clinical isolates. The MBC value for F5 compound was found to be 62.5 µg/ml for *S. sanguis, S. mitis, S. salivarius*and *L. acidophilus* and 15. 62 µg/ml for *S. mutans* (Table 5.23).

Minimum fungicidal concentration is the concentration at which the extract kills the 99.9% of fungus. The tubes containing different concentration of pure compounds used in determination MIC was streak inoculated on sterile Saborauds Dextrose agar plates and incubated at room temperature for 24 to 72 hours. The results revealed that, MFC value for F4, and F5 compound was found to be 1000 µg/ml whereas, all other compounds was not active (Table 5.23).

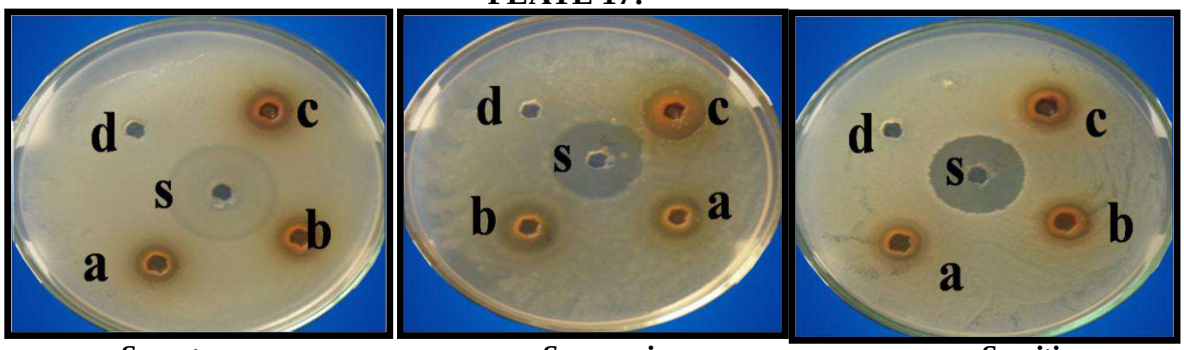
Sl. no	Name of the organism/Purified compound	Zone of inhibition in mm				
		F3	**F4**	**F5**	**F9**	**F10**
1	***S. mutans***	15	11	17	11	11
2	***S. sanguis***	10	11	15	12	10
3	***S. mitis***	10	14	19	16	10
4	***S. salivarius***	10	14	19	16	10
5	***L. acidophilus***	11	11	17	16	13
6	***C. albicans***	-	9	10	-	-

Table 5.22: Antimicrobial activity of purified compounds from petroleum ether extract of *H. mysoresenc*

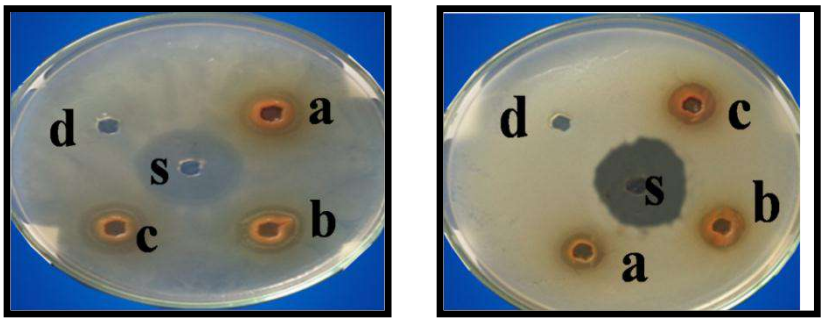
Sl No	Test organism	Purified compounds concentration in µg/ml														
		F3			**F4**			**F5**			**F9**			**F10**		
		MIC	**MBC**	**MFC**	**MIC**	**MBC**	**MFC**	**MIC**	**MBC**	**MFC**	**MIC**	**MBC**	**MFC**	**MIC**	**MBC**	**MFC**
1	***S. mutans***	15.62	62.5	-	15.62	62.5	-	7.82	15.62	-	15.62	62.5	-	15.62	62.5	-
2	***S. sanguis***	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-
3	***S. mitis***	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-
4	***S. salivarius***	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-
5	***L. acidophilus***	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-	15.62	62.5	-
6	***C. albicans***	-	-	-	1000	-	500	1000	-	500	-	-	-	-	-	-

Table 5.23: MIC and MBC values of purified compounds of *Hypericummysoresence* against pathogens of dental caries.

PLATE 17:

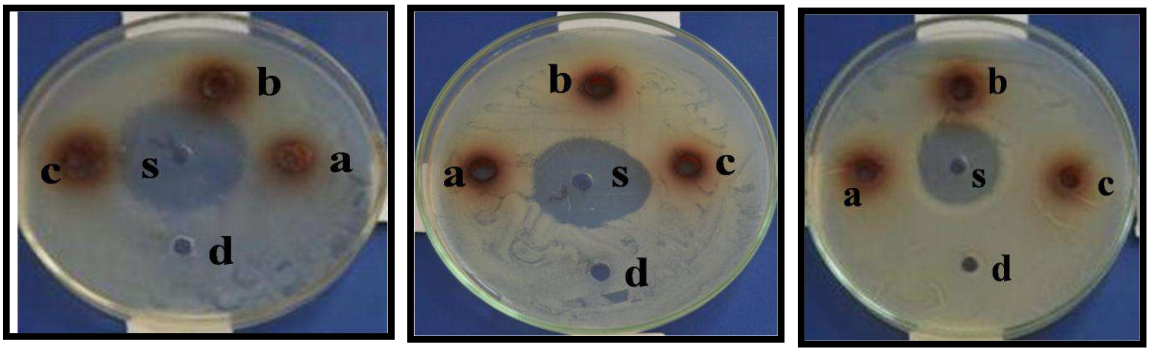


S. mutans ***S. sanguis*** ***S. mitis***

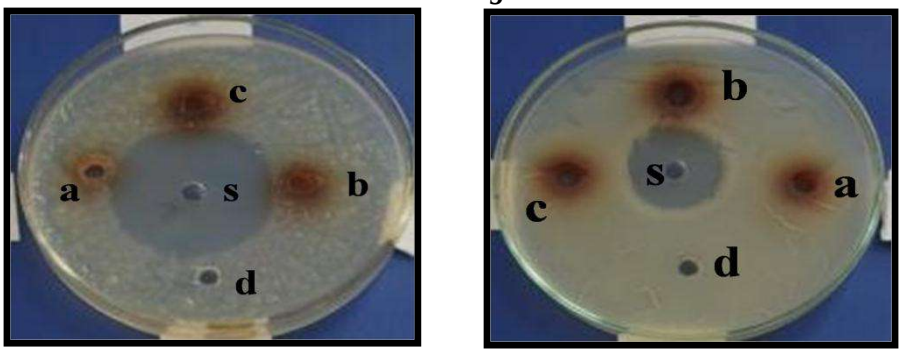


S. salivarius ***L.acidophilus***

Antibacterial activity of methanol extract of H. mysoresence against pathogens of dental caries (a-10mg/ml, b-25 mg/ml, c-50 mg/ml, d-10% DMFO, S- Tetracycline 10µg/ml**)**



S. mutans ***S. sanguis*** ***S. mitis***

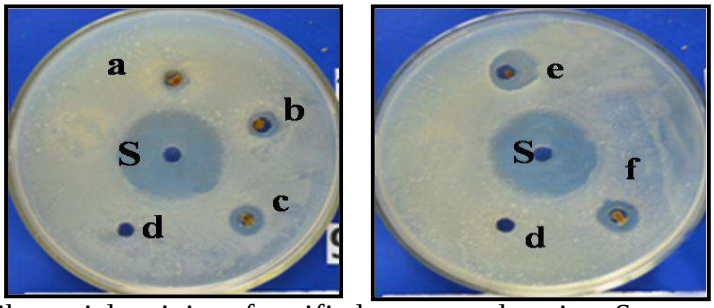


S. salivarius ***L.acidophilus***

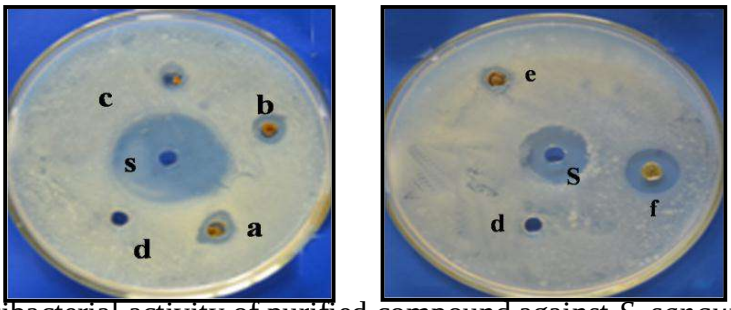
Antibacterial activity of methanol extract of H. mysoresence against pathogens of dental caries (a-10mg/ml, b-25 mg/ml, c-50 mg/ml, d-10% DMFO, S- Tetracycline 10µg/ml**)**

Antimicrobial activity of crude ectract of H. mysoresence against dental caries pathogens.

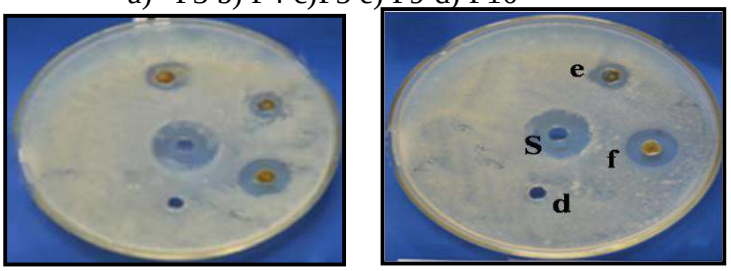
Plate 18



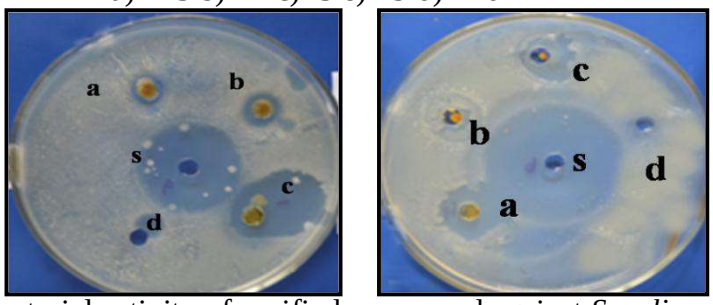
Antibacterial activity of purified compound against *S. mutans*
a) F3 b) F4 c)F5 e) F9 d) F10



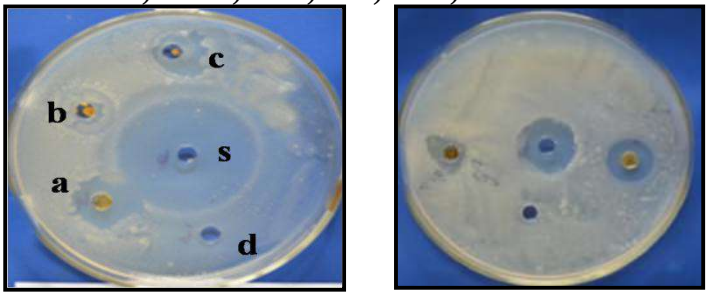
Antibacterial activity of purified compound against *S. sanguis*
a) F3 b) F4 c)F5 e) F9 d) F10



Antibacterial activity of purified compound against *S. mitis*
a) F3 b) F4 c)F5 e) F9 d) F10



Antibacterial activity of purified compound against *S. salivarius*
a) F3 b) F4 c)F5 e) F9 d) F10



Antibacterial activity of purified compound against *L. acidophilus*
a) F3 b) F4 c)F5 e) F9 d) F10

6

Discussion

Natural products have been an integral part of the ancient traditional medicine systems, e.g. Ayurvedic, Chinese and Egyptian (Sarker and Nahar, 2007). Over the years they have assumed a very central stage in modern civilization as natural source of chemotherapy as well as amongst scientist in search for alternative sources of drugs. According to the World Health Organization (WHO), a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes. Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the treatment of infectious pathologies in the respiratory system, urinary tract, gastrointestinal and biliary systems, as well as on the skin (Rios & Recio, 2005; Adekunle & Adekunle, 2009). Medicinal plants are increasingly gaining acceptance even among the literates in urban settlements, probably due to the increasing inefficacy of many modern drugs used for the control of many infections such as typhoid fever, gonorrhoea and tuberculosis as well as increase in resistance by several bacteria to various antibiotics (Levy, 1998; Van den Bogaard *et al.,* 2000; Smolinski *et al.,* 2003).

These non-nutrient herbal originated chemical entities or bioactive components are oftenly referred as phytochemicals or phytoconstituents and are responsible for protecting the plant against microbial infections or infestations by pests (Abo *et al*., 1991; Liu, 2004; Nweze *et al*., 2004; Doughari *et al*., 2009).

Current problems associated with the use of antibiotics, increased prevalence of multiple-drug resistant (MDR) strains of a number of pathogenic bacteria such as methicillin resistant *Staphylococcus aureus, Helicobacter pylori,* and MDR *Klebsiella pneumonia* has revived the interest in plants with antimicrobial properties (Voravuthikunchai & Kitpipit, 2003).

In our present study, we have made an attempt to control dental caries by naturally occurring phytochemicals produced by plants against dental caries causing pathogens.

Phytochemicals have been isolated and characterized from Leaves, stem, bark, roots and fruits. (Doughari and Obidah, 2008; Doughari *et al.*, 2009). Among the reported plants, leaves were the dominant parts in oral care uses (25.44%), followed by root (20.17%), seed/nut/fruit (18.42%), bark (14.03%), young stem/stem/rachis (12.28%), whole plant (9.65%) and gum/latex (8.77%). Among the utilization, most of the plants were used to relieve from toothache (29.82%) followed by, used as dentifrice/ toothbrush (25.43%), mouthwash/gargle (16.66%), against common dental diseases (14.03%), mouth related stomatitis/ulcer/gingivitis (12.28%) and gum bleeding/disorders (10.53%). The mode of utilization of these plants is either in the form of gargle of decoction of plant part(s), powder of dried material or toothbrush (Amit and Shalu, 2012). In this contrast, the present study is designed to explore leaf extracts of ten medicinally important plants for their inhibitory efficacy against the pathogens involved in dental caries.

6.1 Qualitative Phytochemical screening:

In this study, preliminary phytochemical assays were done for the presence or absence of phytoconsituents. Naturally, phytoconstituents are produced by the plants as their normal metabolic activities, which include primary and secondary metabolites.

The secondary metabolites are the chemicals produced by the plants in response to infection and abiotic stress. They are found in smaller quantities found only in particular genus or species. These secondary metabolites comprises of alkaloids, flavonoids, phenols, steroids, saponins, tannins, glycosides *etc*. that can have therapeutic actions in humans and which can be refined to produce drugs.

Alkaloids are the largest group of secondary chemical constituents made largely of nitrogenous compounds comprising basically of nitrogen bases synthesized from amino acid building blocks with various radicals replacing hydrogen atoms in the peptide ring. These compounds have basic properties and are alkaline in reaction, responsible for turning of red litmus paper to blue. The degree of basicity varies considerably, depending on the structure of the molecule, and presence and location of the functional groups (Sarker & Nahar, 2007). They react with acids to form

crystalline salts without the production of water (Firn, 2010). In our present study, all the selected plants showed the presence alkaloids except *W. tinctoria* .

In our present study falvonoids was found to be present in all the plants. Flavonoids are important group of polyphenols widely distributed among the plant flora. Stucturally, they are made of more than one benzene ring in its structure (a range of C15 aromatic compounds) (Kar, 2007). These compounds are derived from parent compounds known as flavans. Over four thousand flavonoids are known to exist and some of them are pigments in higher plants. Quercetin, kaempferol and quercitrin are common flavonoids present in nearly 70% of plants.

Phenolics, phenols or polyphenolics (or polyphenol extracts) are chemical components that occur ubiquitously as natural colour pigments responsible for the colour of fruits of plants. This phytochemical play an important role in plant defence against pathogens and thus are applied in the control of human pathogenic infections (Puupponen *et al*., 2008). Phenolics are classified into (i) phenolic acids, (ii) flavonoid polyphenolics (flavonones, flavones, xanthones and catechins) and (iii) non-flavonoid polyphenolics. Phenolics essentially represent a host of natural antioxidants, used as nutraceuticals.

The term saponin is derived from *Saponaria vaccaria* (*Quillaja saponaria*), a plant, which abounds in saponins and was once used as soap. Saponins therefore possess ‘soaplike’ behaviour in water, i.e. they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin. Saponins are regarded as high molecular weight compounds in which, a sugar molecule is combined with triterpene or steroid aglycone. There are two major groups of saponins and these include: steroid saponins and triterpene saponins (Kar, 2007). They possess a bitter and acrid taste, besides causing irritation to mucous membranes. Steroidal saponins are used in the commercial production of sex hormones for clinical use (Sarker and Nahar, 2007). In our study, all the methanolic leaf extract, except the extract of *A. indicus, A. indica* and *H. mysoresence* showed the formation of foam when treated with sodium bicarbonate.

In our present study Tannins were found to be present in *A. indicus, A. indica, C. roxburghii, H. mysoresence* and *M. indica* and other plants showed negative result

for the presence of tannins (Table 5.2). Tannins are widely distributed in root, bark, stem and outer layers of plant tissue which are soluble in water and alcohol. They are acidic in nature due to the presence of phenolics or carboxylic group (Kar, 2007). They form complexes with proteins, carbohydrates, gelatin and alkaloids. Tannins are divided into hydrolysable tannins and condensed tannins. Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending on the type of acid produced, the hydrolysable tannins are called gallotannins or egallitannins. Tannins are used as antiseptic and healing agents in number of diseases.

Terpenes are among the most widespread and chemically diverse groups of natural products. They are flammable unsaturated hydrocarbons, existing in liquid form commonly found in essential oils, resins or oleoresins (Firn, 2010). Terpenoids includes hydrocarbons of plant origin of general formula $(C_5H_8)_n$ and are classified as mono-, di-, tri- and sesquiterpenoids depending on the number of carbon atoms. The triterpenes (C_{30}) include steroids, sterols and cardiac glycosides with anti-inflammatory, sedative, insecticidal or cytotoxic activity (Martinez *et al.,* 2008). In our study, these terpenes are present in all plants except *A. muricata*, *M. glabra*, *W. tinctoria* (Table 5.2)..

Plant steroids (or steroid glycosides) also referred to as 'cardiac glycosides' are one of the most naturally occurring plant phytoconstituents that have found therapeutic applications as arrow poisons or cardiac drugs (Firn, 2010). The cardiac glycosides are basically steroids with an inherent ability to afford a very specific and powerful action mainly on the cardiac muscle when administered through injection into man or animal (Maurya *et al.,* 2008; Madziga *et al.,* 2010). In our study, steroids are present in *A. indicus*, *A. muricata*, *H. mysoresence*, *M. glabra*, *T. asiatica*, *W. tinctoria* and found to be absent in all the plants (Table 5.2).

Glycosides in general, are defined as the condensation products of sugars (including polysaccharides) with a host of different varieties of organic hydroxy (occasionally thiol) compounds. Glycosides are colorless, crystalline carbon, hydrogen and oxygen-containing water-soluble phytoconstituents, found in the cell sap. Chemically, glycosides contain a carbohydrate (glucose) and a non-carbohydrate part (aglycone or genin) (Kar, 2007; Firn, 2010). This group of drugs is usually

administered in order to promote appetite, aids digestion and also used to treat stomach cancer. In our study we have found the presence of cardiac glycosides only in the leaf parts of plants like, *A. muricata, M. glabra, M. indica* and *S. pentandrum* (Table 5.2).

Several methods have been employed for the detection of presence or absence of phytochemicals. In this present study, phytochemical analysis was done for methanolic leaf extracts of ten plants.

Methanol extract of *A. indicus* leaf shown the presence of alkaloids, tannins, steroids, terpenoids and flavonoids. The obtained results were in concurrence with earlier findings of Ruchi *et al*. (2015) and Shaik and Balakumar (2014). Tanins and saponins was found to be absent in *A. muricata* and results were comparable with the study conducted by Vijaymeena *et al*. (2013).

It has been observed that, the methanol extract of *A. indica* showed the presence of all the phytoconstituents but showed negative results for the presence of steroids, saponins and glycoside (Table 5.2). Similar findings were also obtained by studies conducted by Kiranmai *et al*. (2012).

C. roxburghii did not show the positive result for the presence of carbohydrates, steroids and glycosides which was comparable with the finding of Prashith *et al*. (2014), where as in case of methanol extract of *H. mysoresence*, carbohydrate and Saponins was found to be absent.

Presence of all phytoconstituents was observed in *M. glabra* in a less amount and showed negative result for the presence of tannins and terpenoids (Table 5.2). Only steroid is found to be absent in methanol extract of *M. indica* except this, all the phytoconstituents was found to be present and similar findings are also observed by Adetuyi *et al.* (2013).

Negative results were observed for the presence of Tanins and Steroids in methanol extract of *S. pentandrum* (Table 5.2) and evidence for this result was observed by the findings of Ajith babu *et al*. (2013).

It has been observed that, *T. asiatica* showed presence of all phytoconstituents except tannins and glycosides, similar findings was observed in the study conducted by Rachael *et al.* (2013).

In our study, when compared to other plants *W. tinctoria* showed the presence of less number of phytoconstituents and showed negative for the presence of carbohydrate, alkaloids, tanins, terpenoids and glycosides which was comparable with the findings of Sandhya rani *et al*. (2012).

In our present study, based on the results of antimicrobial activity against pathogens of dental caries, *H.mysoresence* was selected for further phytochemical investigation and antioxidant studies. The results revealed that, the presence of steroids, triterpenoids and absence of flavonoids in petroleum ether extract (PEH). Alkaloids and flavonoids were present in methanol extract (MEH) and aqueous extract (AEH) respectively (Table 5.7). Several investigations suggest that, the solubility of the active phytocompounds depends upon the diverse solubility capacities of different solvents with different phytoconstituents (Errum *et al.,* 2015; Vijaya lakshmi *et al.,* 2015). The differences in the observed phytochemicals within the leaf extracts of *H. mysoresence* may be due to varying degrees of solubility of the active constituents in the different solvents used (Ashok *et al.*, 2014).

6.2 Determination of total phenol and flavonoid contents and antioxidant activities.

Reactive oxygen species (ROS), such as superoxide radicals, hydroxyl (OH) radicals and peroxyl radicals, are natural by-products of the normal oxygen metabolism in living organisms, playing important roles in cell signaling (Aruoma *et al.,* 1997; Cavas *et al.,* 2005). However, excessive amounts of ROS may be a primary cause for oxidative stress resulting in biomolecular oxidation and may cause significant damage to cell structure, function and contributing to various disorders. Oxidative stress is important in the development of chronic degenerative diseases including coronary heart disease, cancer and aging (Ames *et al.,* 1983; Wiseman *et al.,* 1996). Thus, antioxidants are important inhibitors of lipid peroxidation. Plant polyphenols with antioxidant capacity could scavenge reactive chemical species as well as minimize oxidative damage resulting from excessive light exposure. Some

plant polyphenols are important components of both human and animal diets and they are found to be safe for consumption (Gulcin, 2005).

Plant materials rich in phenolics are increasingly being used in the food industry because they retard oxidative degradation of lipids and improve the quality and nutritional value of food (Kahkonen *et al.,* 1999). Phenolic compounds of plants fall into several categories; chief among these are the flavonoids which have potent antioxidant activities (Nunes *et al.,* 2012). They naturally occur in plants and are thought to have positive effects on human health. Studies on flavonoidic derivatives have shown a wide range of antibacterial, antiviral, anti inflammatory, anticancer, and anti-allergic activities (Di *et al.,* 1999 ; Montoro and Braca, 2005). Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen and various free radicals implicated in several diseases (Bravo, 1998).

Recently, phenolics have been considered powerful antioxidants *in vitro* and proved to be more potent antioxidants than Vitamin C, E and carotenoids (Rice *et al.,*1995 ; Rice *et al.,*1996). Antioxidant capacity is widely used as a parameter for medicinal bioactive components. Various methods are currently used to assess the antioxidant activity of plant compounds. DPPH radical scavenging, ferric reducing, metal chelating, nitric oxide scavenging, superoxide radical scavenging activity etc., are some of the common spectrophotometric procedures for determining the antioxidant capacities of components (Gulcin, 2010).

In this study, the antioxidant activity of methanol extract of ten plants was measured using three different assays, namely DPPH, Ferric reducing and metal chelating assays. Performing a single assay to evaluate the antioxidant properties would not give the authentic result as antioxidant activity of plant extract is influenced by many factors like, the test system and composition of extract. Therefore it is important to carry out more than one type of antioxidant capacity measurement to cover the various mechanisms of antioxidant action (Gan *et al.,* 2010).

In DPPH method, our present study indicates the variation in the antioxidant activities among methanol extracts of different medicinal plants. Almost all the extracts showed high antioxidant activity against the DPPH radical. The antioxidant activity presented by the methanolic leaf extracts of different plants follows the order:

H. mysoresence > *S. pentandrum* > *A. muricata* > *A. indica* > *W. tinctoria* > *T. asiatica* > *M. indica* > *C. roxburghii* > *A. indicus* > *M.glabra* (Table 5.4). In this study, amongst the 10 plant extracts, the methanolic leaf extract of *H. mysoresence* exhibited the highest antioxidant activity and least was observed in *M. glabra*.

The sequentially extracted crude compounds of *H. mysoresence* (PEH, CEH, MEH and AEH) showed a potent antioxidant activity in DPPH method. The IC_{50} values of all the crude extracts were calculated, the values obtained from *H. mysoresence* follows the order, AEH> PEH> MEH> CEH (Table 5.9). DPPH is nitrogen centered free radical having an odd electron which gives a strong absorption at 517 nm, its color changes from purple to yellow when DPPH odd electron paired off in the presence of radical scavenger to form the reduced DPPH-H (Cai *et al.*, 2003). In our present study, we have observed the change of color from purple to yellow which indicates the scavenging potential of the extract. Owing to this characteristic, our plant extracts has the capable of reducing this radical, which may be due to the presence of phytochemicals in the extracts particularly phenolic and flavonoid compounds.

Fe (III) reduction is often used as an indicator of electron-donating activity, which may serve as a significant reflection of the antioxidant activity (Oktay *et al.*, 2003). Compounds with reducing power indicate that, they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Chanda and Dave, 2009). In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound. Presence of reducers causes the conversion of the Fe_3^+/ferricyanide complex used in this method to the ferrous form. By measuring the formation of Pearl's Prussian blue at 700nm, it is possible to determine the unreduced Fe_3^+ ion concentration. In our present study, methanolic crude leaf extract showed the Fe_3^+ reduction which was measured at 700 nm. It was found that the reducing power increased with concentration of each sample and found to be concentration dependent. The ranking order for reducing power at 1000 µg/ml of methanol extracts was *H. mysoresence* > *S. pentandrum* > *C. roxburghii* > *M. glabra* > *A. indica* > *A. muricata* > *W. tinctoria* > *M. indica* > *A. indicus* > *T. asiatica* (Table 5.4). The crude extracts of *H. mysoresence*, also exhibited a potent ferric

reducing activity. At 1000 µg/ml the OD at 710 nm showed the maximum increase in the colour and follows the values in increasing order PEH> MEH> CEH> AEH. Previous reports suggested that the reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain (Gordon, 1990). Increasing absorbance at 700 nm indicates an increase in reducing ability. The antioxidants present in the methanol extracts of these plants caused their reduction of Fe_3^+/ ferricyanide complex to the ferrous form, and thus proved the reducing power.

In metal chelating assay, the reducing power of crude solvent extract was found to be increased with the dose. The reducing capacity of compound may serve as significant indicator of its potential antioxidant activity (Meir, 1995). The antioxidant activities have been reported to be the concomitant development of reducing power (Yang *et al.,* 2002). In metal chelating activity, Ferrozine can quantitatively form complexes with Fe_2^+. However, in the presence of chelating agents, the complex formation is disrupted with the result that, the red color of the complex is decreased. Measurement of color reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. The transition metal ion Fe_2^+ possesses the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively nonreactive radicals (Halliwell, 1994). In our present study, formation of red color decreases with increasing in the concentration of the extract, which indicates a dose dependent metal chelating activity. IC_{50} value of metal chelating activity was calculated. Depending upon the values obtained, the ranking order of the crude methanol extracts of medicinal plants was *S. pentandrum* > *H. mysoresence* > *M. indica* > *A. muricata* > *A. indica* > *W. tinctoria* > *C. roxburghii* > *T. asiatica* > *A. indicus* > *M. glabra* (Table 5.4). The highest activity was found to be in *S. pentandrum* (429.43 µg/ml) and least activity was exhibited by *M. glabra* (715 µg/ml). In our study, crude extract of *H. mysoresence* also exhibited a very promissory metal chelating activity. The IC_{50} values of all the crude extracts follows the order, MEH> PEH> CEH> AEH.

Junaid *et al.* (2013) found that leaf extract of *A. indicus* was rich in phenol and flavonoid content (106.68 µg GAE/mg and 30.80 µg GAE/mg respectively) when compared with seed extract (76.65 µg GAE/mg and 22.50 µg GAE/mg). In their

study, DPPH activity of leaf extract was found to be higher than that of seed extract in a dose dependent manner, but showed lesser activity when compared to standard. In our study, we found that, total phenol content of *A. indicus* is 51.30±0.68 mg/GAE and total flavonoid content was found to be 30.65±0.72. In our result, IC_{50} was found to be 624.65 µg/ ml which was found to be least when compared to other plant tested. The variation in the results may be due to the concentration of the sample taken for the study or may be due to the location of the plant collected.

Baskar *et al.*, (2006) investigated the antioxidant activities of leaves extract (ethanol) of *A. muricata, A. reticulata* and *A. squamosa*. Between these species they observed that highest antioxidant activity was found in the leaf extract of *A. muricata*. At 500 µg/ml the extract showed maximum scavenging activity (90.05%) of ABTS radical cation followed by scavenging of hydroxyl radicals 85.88% and nitric oxide (72.60%). In our study, leaf extract of *A. muricata* reduced DPPH to DPPHH upto 75.97% at 600 mg concentration followed by 68.60% for metal chelating activity and in reducing assay showed absorption OD of 0.571 at this concentration. These finding suggests that *A. muricata* leaves possess a potent *in-vitro* antioxidant activity suggesting its role as an effective therapeutic radical scavenging, thereby augmenting its therapeutic value.

A comprehensive study was conducted by Kiranmai *et al.* (2012) on antioxidant properties and total flavonoid contents of successive solvent extracts of different parts of *A. indica*. With respect to their study, methanolic extract of root bark was found to exhibit highest antioxidant activity followed by methanolic extract of leaf as well as seed. Garima et al. (2014) found that the leaf extract of *A. indica* showed IC_{50} of 110.36 µg/ml for DPPH activity. In our study, we have found IC_{50} value of 356.23 µg/ml for DPPH activity. The variation in the antioxidant activity may be due to the distribution of phytochemicals with different concentration.

Leaf extract of *C. roxburghii* exhibited a marked dose dependent scavenging activity against DPPH free radical with an IC_{50} value of 3.54 µg/ml and ferric reducing activity, which increased with increase in the concentration of the extract. It is also found that total phenolic content (179.05 mg Gallic acid equivalents/g of extract) was responsible for the antioxidant activity (Prashith *et al.,* 2014). In our

study also, we have found total phenol content of 49.62±0.81 mg/GAE and TPC of about 51.25±0.26 mg/GAE. The presence of these phenol and flavonoid content is responsible for a marked antioxidant activity. However the variation among the TPC and TFC values may be attributed to the variation in the phytochemicals as they depends on the locality of plant collected.

Raghu *et al.* (2014) showed that phenolic and flavonoid content of methanolic leaf extract of *H. mysoresence* was found to be 28.76 ± 1.44 mg/ml and 37.07 ± 2.90 mg/ ml respectively. In our study, total phenol content of *H. mysoresence* was 71.32 ± 0.16 mg/GAE and 61.23 ± 0.32 of total phenol content was recorded.

In their study, positive correlation was observed for the parameters like total phenolic, total flavonoid contents with that of *in vitro* antioxidant activity using different assays. Flower extract of *H. mysoresence* was found to have highest IC_{50} values, followed by leaf, stem and root extracts of *H. mysoresence* (Raghu *et al.,* 2014). In our study, even though there is a variation among the total phenol, flavonoid content and antioxidant activities was observed, but linear positive correlation was observed between these parameters. This result was in concurrence with their work and variation may be attributed with their concentration of the extract taken for the study.

Joona *et al.* (2013) in a study determined the antioxidant activity of methanolic extract of *M. indica* leaves. Results of this study reveal that, antioxidant activity of methanol extract increases with increase in concentration. Highest activity was observed in DPPH activity by scavenging 49 % of DPPH free radical at 100 µg/ml of extract concentration. In our study also, we found that, as the concentration of the extract increases antioxidant activity also get increased. This shows that, leaf extract of *M. indica* showed dose dependent antioxidant activity. Other antioxidant methods like, Metal chelting and ferric reducing assays have also showed moderate antioxidant activity. In their study, the antioxidant activity exhibited by the extract may be due to the presence of high level of flavonoids and terpenoids content in the extract. In our study also, the marked antioxidant activity may be attributed due to the presence of phytochemical in the extract particularly phenolic and flavonoid contents.

Rachael *et al.* (2013) investigated that, the antioxidant activity of methanolic leaf extract of *T. asiatica*. It was observed that the methanolic extract of *T. asiatica* leaf showed a higher activity of 77.35% at 50µg/ml and IC_{50} value of 36µg/ml. The result suggested from their study reveals, that the high antioxidant activity could be possibly due to its high phenolic content. In our study, we have found 77. 95% and 79.50% of inhibition of DPPH free radical, at the concentration of 800 µg/ ml and 1000 µg/ ml respectively. Our results are also in concordance with their study, where the antioxidant activity may be attributed to their total phenolic and flavonoid content present in the plant.

Jyothiram *et al.* (2014) found that, the methanol extract (leaf) of *W. tinctoria* showed the highest antioxidant activity. At 500 µg/ml the extract showed 61.07% of DPPH free radical inhibition and also exhibited an effective reducing power (at 500 µg/ml showed maximum absorbance of 0.571). In our study, the leaf extract of *W. tinctoria* showed 77.95% of DPPH inhibition at 600 µg/ml concentrations and also found to be 68.60 % of inhibition for metal chelating assay and reducing power with absorbance of 0.571 at the same concentration. The variation observed may be due to the concentration of the extract used.

The activities of the different plant extracts used in this study may be due to the presence of high amount of pytochemicals. These phytoconstituents exhibits the greatest antioxidant activity through the scavenging of free radicals, which participate in various pathophysiological conditions of diseases including ageing (Jyothiram *et al.*, 2014). In general, the results obtained from our study, congregate with the results of above said investigations with some variation. These variations may be due to the concentration of the extract taken, as phytochemicals and their antioxidant activity directly depends on their concentration of the extract taken.

6.3 Relationship between the total phenolic content and antioxidant activities

Phenolic and flavonoid compounds are ubiquitous bioactive compounds and a diverse group of secondary metabolites universally present in higher plants (Robards *et al.*, 1999). Phenolic compounds possess ideal structure chemistry for free radical scavenging activities because they have,

I. Phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical;

II. Extended conjugated aromatic system to delocalize an unpaired electron. (Dziedzic and Hudson, 1983; Rice *et al.,* 1996)

For flavonoids, the major factors that determine the radical-scavenging capabilities (Shahidi and Wanasundara, 1992) are:

I. Ortho-dihydroxy structure on the B ring, which has the best electron-donating properties and confers higher stability to the radical form and participates in electron delocalization.

II. Presence of 2,3-double bond with a 4-oxo function in the C ring, which is responsible for electron delocalization from the B ring.

III. 3- and 5-hydroxyl groups with the 4-oxo function in A and C rings, which are essential for maximum radical scavenging potential.

IV. 3-hydroxyl group is important for antioxidant activity. The 3-glycosylation reduces their activity when compared with corresponding aglycones. (Bors and Michel, 2002).

Several studies have shown that phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (Van Acker *et al.,* 1996). The correlation between total phenol contents and antioxidant activity have been widely studied (Klimczak *et al.,* 2007; Kiselova *et al.,* 2006; Jayaprakasha *et al.,* 2008; Kedage *et al.,* 2007). As reported, antioxidant activity of phytocompounds significantly increases with the presence of high concentration of total phenol and flavonoid content. In the present study, the correlation between total phenolic and flavonoids contents and antioxidant activity of methanolic extracts from 10 medicinal plants were analyzed. The antioxidant values correlated well with both total phenolics and total flavonoids contents. A positive relationship was found between total phenolics and antioxidant capacity in all the medicinal plants selected in this study.

In general, extracts or fractions with a radical scavenging activity showed the presence of phenolic content along with the promising correlations between them. The methanolic extracts exhibited different antioxidant capacities in relation to the method applied; thus the same material ranked differently, depending on the assay. A direct linear correlation between radical scavenging activity and phenolic content of the samples was demonstrated by linear regression analysis. In our study, linear correlation coefficient was observed in *T. asiatica* (0.999, P<0.01) followed by *W. tinctoria* (0.990, P<0.01) and *S. pentandrum* (0.986, P<0.01). The correlation between the TPC (Total Phenolic Content) and Metal chelating assay showed varied results, the best linear correlation was observed in *H. mysoresence* (0.999,P<0.01) followed by *S. pentandrum* (0.996, P<0.01) and *M. indica* (0.994). The best relation between TPC and ferric reducing assay was explained by the methanol extract of *H. mysoresence* (0.979, P<0.01) followed by *A. muricata* (0.974, P<0.01), *W. tinctoria* (0.970, P<0.01), *A. indica* and *C. roxburghii* with identical correlation value of 0.953, P<0.01. It has been observed that weak correlation was observed in between TPC and all antioxidant activities of *A. indica*. Correlation value of 0.586 was observed between TPC and DPPH activity, followed by Ferric reducing and metal chelating activity with correlation value of 0.576 and 0.535 respectively (Table 5.6).

With respect to results obtained from antioxidant activity of crude extract of *H. mysoresence*, correlation was also established between TPC and antioxidant activities (Table 5.10). A positive linear correlation was recognized in TPC and DPPH activity, PEH showed positive correlation (0.997, P<0.01) and least activity with MEH (0.840, P<0.01).

Correlation of TFC (Total Flavonoid Content) with different antioxidant methods was also performed in this study. Linear correlation was observed in between TFC, DPPH radical scavenging and metal chelating activity, but non-significant correlation was observed between TFC and ferric reducing assay in the extract of *A. indicus*. This is because of a certain flavonoid structure in which hydroxyl position may act as proton donar and show radical scavenging activity (Nickavar *et al.,* 2007). Furthermore, the extracts are very complex mixtures of many different compounds with distinct activities (Wojdylo *et al.,* 2007).

In our present study, due to the absence of flavonoid content in PEH, no correlation between TPC and other antioxidant activity was observed. TPC in CEH showed highest correlation with DPPH (0.990, <0.01), Ferric reducing activity (0.943 <0.01) and metal chelating activity (0.967, <0.01). Less correlation was observed in case of AEH between TPC and DPPH (0.711), ferric reducing (0.546) and metal chelating activity (0.497) (Table 5.10).

Several studies have reported the relationship between phenolic content and antioxidant activity. Some authors have found a correlation between the phenolic content and antioxidant activity, while others found no such relationship. The variation in the correlation value may be due to different responses of phenolic and flavonoids compounds in different assay systems. Since the molecular antioxidant responses of phenolic compounds vary remarkably, depending on their chemical structure. Antioxidant activity does not necessarily correlate with the TPC.

In our study, variation among the different antioxidant assay was found with the same extract and also with different plants extracts also. The variation of different antioxidant activities within the same sample/ or different samples, may be due to the different mechanism involved in it. Different functional groups present in phenolic and flavonoid compounds shows different antioxidant mechanism for different antioxidant assay. The antioxidant activity can be affected by substitution of hydroxyl groups on phenolics aromatic ring, because of their hydrogen donor ability (Brand *et al.*, 1995). In fact, the antioxidant activity increase with increasing degree of hydroxylation, however substitution of the hydroxyl groups with methyl (CH3), methoxyl (OCH3) groups etc., reduces its activity. So, this can explain the important antioxidant potential observed in some tested aromatic and medicinal plants compared to others (Rice *et al.*, 1995).

In our study, it has been observed that, the extract exhibiting low phenol and flavonoid content have shown promising antioxidant activity. This may be possibly due to the presence of some other phytochemicals such as, ascorbic acid, tocopherol and pigments etc., in the extract and these phytochemicals may also contribute to the antioxidant capacity other than phenol and flavonoid content (Sarabjot and Poonam,

2014) and also may be due to synergistic effect of other chemicals with phenol and flavonoid contents present in the extract (Velioglu *et al.,* 1998).

6.4. Collection and screening of clinical samples for dental caries isolates.

Dental caries is an infectious oral disease which depends on multiple parameters. It is characterized by a gradual dissolution and destruction of the mineralized tissues of the teeth. This disease begins as white spot lesions, further finds their way to enamel, dentin, root canal and finally results in the pulp's inflammation, coexisting with pain symptomatology (Aoba, 2004; Fejerskov and Kidd, 2003; Pereira, 1995). The progress of dental caries is through a complex interaction over time between acid producing bacteria and fermentable carbohydrate, and many host factors including teeth and saliva (Selwitz, *et al.,* 2007).

Since oral bacteria are considered as one of the etiologic factors involved in caries development, various microbial studies have been conducted to better understand this oral problem. Several studies have shown that, the major species that are associated with dental caries are Mutans streptococci and Lactobacillus species. The organisms of these two species are known as acid tolerant and acid producing species, which are frequently encountered in association with dental caries (Leosche and Syed, 1973).

Mutans sanguis agar is the differential media usually recommended for the isolation of *S. mutans* and *S. sanguis*. In this media more sucrose is added, as sucrose is the only sugar that *S. mutans* has the ability to utilize and able to produces extracellular polysacharride (water soluble glucan appears as drop of liquid) on the top of the colony or a puddle of polysaccharide around the colony. In our present study, *S. mutans* exhibited white, grey or yellow colour colonies which was measured 0.5 - 2 mm in diameter and also observed the production of water soluble polysaccharide on the top of the colony (Table 5.11).

In another side, on mutans sanguis agar *S. sanguis* forms smooth or rough, hard and rubbery colonies, which adhere strongly to the agar making them difficult to remove with a loop. They appear as grey, white or colourless colonies measures about 1-3 mm in diameter. In this study, some colonies showed characteristic hard and rubbery colonies which strongly adhered to the agar surface. These characteristics

features influence us to consider those colonies as *S. sanguis.* In our current study, inoculated samples on MS agar showed above said colony characters. Based on these characters, we have preliminarily identified the clinical isolates as *S. mutans* and *S. sanguis* (Table 5.11).

Mitis Salivarius agar base is used for the isolation of *S.mitis* and *S. salivarius*. This medium (with 1% potassium tellurite) is a highly selective medium, which enables to isolate streptococci from highly contaminated specimens as it inhibits a wide variety of bacteria. Occasionally *S. mutans* strains may be inhibited on Mitis Salivarius agar Base due to the high concentration of trypan blue in the medium. On this media, *S. mitis* appears as blue color tiny colony and *S. salivarius* appears as light blue colored gum drop like colonies. In this study, we have isolated *S. mitis* and *S. salivarius* on their colony characters and further confirmed by biochemical characters. In our current study, we have isolated *S. mitis* and *S. salivarius* on the basis of above said morphological characters (Table 5.11).

It is also reported that, the oral streptococci which are involved in causing dental caries are known to cause alpha hemolysis on blood agar. The property of "greening" on blood agar has been recognized for many years and bacteria which show this property are referred as viridians streptococci. (Khushbu Y and Satyam P, 2015). In our study, all the oral streptococcal exhibited partial lysis of red blood cells present in blood agar resulting in the alpha hemolysis.

In our present study, oral streptococci (*S. mutans, S. sanguis, S. mitis* and *S. salivarius*) were recovered from infected teeth samples by inoculating them into blood agar, mutans sanguis agar and mitis salivarius agar. Lactobacillus was recovered from MRS agar and Candida species were recovered from SDA agar. Depending upon colony morphology (color, shape and diameter), the above said pathogens were preliminarily isolated (Table 5.11).

6.5 Biochemical Identification of *Streptococcus* species involved in dental caries

Identification of streptococci was done by performing a set of experiments namely Gram's staining, KOH solubility test, optochin sensitivity test, arginine, esculin hydrolysis, fermentation of mannitol and sorbitol, VP test and urease test.

In our current study, all the isolates were found to be Gram positive (cocci and bacillus) and the staining result is supported by KOH solubility test. KOH solubility test is based on the lipid content of cell wall of bacteria. Gram negative bacteria contain high lipid when compared to Gram positive bacteria (Table 5.11). Hence, a culture of Gram positive bacteria will not produce mucoid thread when mixed with 3% KOH whereas Gram negative bacteria do exhibit the formation of mucoid thread when mixed with KOH (Murray *et al.*, 2003; Sundar raj, 2003).

Optochin sensitivity test is used to differentiate *S. pneumoniae* from other groups of streptococci involved in causing dental caries. An inhibition zone of at least 14mm (when 6mm disc used) is considered positive for pneumococcus identification (Murray *et al.,* 2003; Sundar raj, 2003). In our present study, our clinical isolates showed resistant to optochin disc (Table 5.12). The clinical strains showing sensitive to optochin are representatives of *S. pneumoniae* strains and these are excluded from this present study (Murray *et al.,* 2003).

Certain bacteria contain the enzymes to hydrolyze arginine. This hydrolysis results in an alkaline change in the media results in change of media colour. This test can be used for differentiation of oral streptococci. The observation was made on the basis of formation of deep purple color in the broth which was taken as positive result. The development of yellow color or no color change was considered as negative result. This test is also used to differentiate between *S. sanguis* with other organisms like *S.mutans, S.mitis* and *S. salivarius* (Murray *et al.,* 2003). In this present study, we have noticed change in media color from purple color to deep purple color, which confirms the presence of *S. sanguis*. Whereas, other clinical isolates didn't hydrolysed arginine and showed negative results (Table 5.12).

Esculin hydrolysis test is an important differential test that is used in the identification of *S. mitis*. The esculin is hydrolyzed to esculetin and dextrose. The esculetin reacts with ferric chloride present in the media and forms black color. In our present study, some of the clinical isolates are not involved in hydrolysis of esculin and they were categorized under *S. mitis* (Murray *et al.,* 2003). Except these isolates all the clinical isolates showed positive results for esculin hydrolysis forming a black color in the media (Table 5.12).

The ability of oral isolate to ferment mannitol and sorbitol with the production of acid is one of the very useful criteria for the identification of oral isolates as *S. mutans* (Table 5.12). A change in the color from red to yellow indicated the fermentation of mannitol and sorbitol by the isolate. In our study, maximum numbers of clinical isolates was not able to ferment both the sugars and are not involved in acid production. But some clinical isolates produced acid by fermenting both mannitol and sorbitol. The results of this test reveal that our clinical isolates showing acid production are *S. mutans* (Murray *et al.,* 2003).

VP test is used to determine the production of acetylmethyl carbinol. This test reactions are used in differentiation of bacteria particularly *viridans streptococci* into species/groups. Production of acetyl methyl carbinol was identified by adding VP reagents. A change in the color from yellow to pink/red is taken as positive result. In this study, some of the clinical isolates have produced acetyl methyl carbinol by the fermentation of glucose (Table 5.12). These isolates were identified as *S. mutans* and *S. salivarius*. Other clinical isolates are identified as *S. sanguis* and *S. mitis* as they are negative for acetyl methyl carbinol production (Murray *et al.,* 2003).

Urease test detects urease production by the bacteria. When urease is produced, it splits urea into carbon dioxide and ammonia. The formation of ammonia increases the pH of the medium which is indicated by the color change of phenol red from red to yellow. In our present study, all clinical isolates are not concerned in the production of CO_2 and NH_3 as there was no color change in the tubes (Murray *et al.,* 2003).

For the identification of *L. acidophilus*, based on the colony morphology on MRS agar, clinical isolates were selected and tested for Grams staining reaction, Growth at 5% NaCl, and sugar fermentation tests. In our study, it has been observed that clinical isolates from MRS agar media are Gram positive bacilli and showed growth at 5% Nacl and fermented maltose, lactose, sucrose and glucose by production of acid which is indicated by color change in the media. It is also observed that our clinical isolates showed negative results for gas production, which is indicated by absence of bubble in the Durham's tube (Table 5.13).

C. albicans is also identified on the basis of colony morphology on SDA agar and performing germ tube tests. In our present study, all the clinical isolates recovered from SDA media showed a characteristic germ tube production germ tube after treating wit human serum (Table 5.13).

In our present study, the results of morphological and biochemical tests revealed the presence of *S. mutans, S. sanguis, S. mitis, S. salivarius, L. acidophilus and C. albicans.* Among 100 subjects, *S. mutans* (22.27%) was found to be most predominant flora followed by *L. acidophilus* (19.80%), *S. sanguis* (19.30%), *S. mitis* (16.83%) *and S. salivarius* (14.35%). *C. albicans* ranks last with isolation percentage of about 7.42% (Figure 2.21). From the above biochemical results, it is evident that the 90% of the dental caries is caused because of *S. mutans,* hence it is again proved as an initiator of dental caries and further progression may be caused by the secondary colonizers such as *L. acidophilus, S. sanguis, S. mitis, S. salivarius* and *C. albicans*.

In a study, 330 patients having teeth cavities associated with or without pain, pus discharge and biofilm deposits were screened for the presence of dental caries pathogen. A total of 325 bacterial isolates were identified in which 297 isolates were Gram positive and 28 isolates were Gram negative. Of 90% Gram positive bacterial isolates, 43.77% were *S. mutans,* 31.64% were *S. aureus,* 10.77% were *S. mitis,* 8.08% were *S. albus,* 5.72% were *S. vestibularis*. Among all bacterial isolates, *S. mutans* was found to be predominant organism to cause dental caries followed by *S. aureus*. The incidence rate of Gram positive bacteria was found higher in caries than Gram negative bacteria because the most common bacteria that are found in the supragingival plaque are Gram positive cocci (*S. mitis, S. oralis, S. sanguis, S. mutans, S. gordonii, S. aureus* and *S. epidermidis*). Similar results were found in our present study also, where *S. mutans* was found to be a predominant microflora. These prominent bacteria are responsible for the plaque formation due to their interactions with each other and the tooth surface.

Similar findings were also obtained in a study conducted by Olajokun *et al.* (2008) in which 100% of bacterial isolates was Gram positive associated with caries. Among all the bacterial isolates, *S. mutans* were predominant bacteria (43.77%) associated to caries.

Maripandi *et al.* (2011) reported that, from 70 clinical samples, 87 bacterial isolates were recovered on the basis of alpha hemolysis on blood agar, Grams staining and other biochemical characters. Among 87 bacterial isolates 72% are facultative anaerobes and 28% are obligate anaerobes. In their study *S. mutans* was manitol positive, *S. salivarius* was lactose positive and *S. sanguis* was sorbitol negative. *S. mutans* was the predominant isolates (22.98%) followed by *Candida spp.,* (14.94%) and less than 10% in *S. sanguis, S. mitis* and unidentified streptococcus spp. In our study, clinical isolates of *S. mutans* showed positive for mannitol fermentation and *S. sanguis* was sorbitol negative.

Even though streptococcus species acts as a primary etiological agents in causing dental caries several reports have suggested that the progression of dental caries was achieved by the secondary invaders in the oral cavity like *Lactobacillus species, Actinomyces, Bacteroids, Spirochaetes and C. albicans*. (Dige *et al.,* 2007). In our study also, secondary colonizers such as *L. acidophilus* and *C.albicans* have been recovered and identified. These organisms within the dental plaque act as secondary colonizers and contribute in the progression of dental caries into the pulp and root canal. These secondary colonizers are known to produce acid by utilization of dietary sugars, which results in the cavitations of the dentin by *in situ* acid accumulation.

Girija *et al.,* (2012) reported that the carious dentine samples yielded a high percentage of Lactobacillus sp (47.3%) and *C. albicans* (55.5%). In a study Kaur *et al.* (2012) isolated dental caries pathogens from 90 caries patients. Based on the colony morphology and biochemical characteristics, they have isolated *S. mutans, S. aureus* and *L. acidophilus*. They found that *Streptococcus* sp. was found to be most prevalent bacteria in dental caries patients followed by *Staphylococcus* sp. and *Lactobacillus sp.*

Classically, the microorganisms involved in the genesis and development of caries are bacteria such as *S. mutans* and other cocci and rods (Parolo and Maltz, 2006). But there is evidence of the involvement of *C. albicans* in the etiology of dental caries (Loesche *et al.,* 1995; Nikawa *et al.,* 2003b; Siqueira *et al.,* 2002; Sziegolei *et al.,* 1999). In our present study also, we have isolated *C. albicans* from

the patients suffering from dental caries with isolation percentage 7.42%. Even though the prevalence of *S. mutans* and *L. acidophilus* was found to be high in our study, the data related to isolation *C. albicans* is sufficient to explain the involvement of this candidate in the progression of dental caries.

In a cros-sectional study, 60 children suffering from early childhood caries were screened for the presence of *C. albicans* and other molds. They isolated *C. albicans* on the basis of colony morphology on sabourauds dextrose agar and further confirmed by performing germ tube test. They found that *C. albicans* were isolated in 55%, mold fungi were found in 29% and no fungal growth in 16% of the clinical samples (Maryam G, *et al.,* 2011). Similar work has been reported on the isolation of *C. albicans* from the dental caries patients. In their study, presence of *C. albicans* was done by colony morphology on SD agar and germ tube tests (Shyla J et al., 2011).

Adraine *et al.,* (2011) showed that the positive correlation between the presence of caries and yeasts (*C.albicans*) in the mouth ($p = 0.0087$) and between yeasts and the number of carious teeth ($p < 0.05$).

50 patients suffering from dental caries were screened for the presence of pathogens involved in dental caries. Result showed that *S. mutants* (36%) and *L. acidophilus* (31%) were predominant followed by *S. sanguis* (16%) and *C. albicans* 12% (Jeevitha *et al.,* 2013).

6.6. Antibacterial susceptibility test of clinical isolates against standard antibiotics

The invention of antibiotics reformed the management of infectious diseases. Antimicrobial agents can either reduce the growth of microbes (bacteriostatic agents) or kill micro-organisms (bactericidal agents) by a variety of mechanisms. Nonetheless, depending on the concentration of the antibiotic the same drug could act as a bacteriostatic or a bactericidal agent. The method of choice for the clinical microbiologists for the *in vitro* antimicrobial susceptibility testing is still the disc diffusion method. Acceptance of the *in vitro* disk susceptibility method has been aided by its simplicity and rapidity. The Kirby-Bauer technique for disk susceptibility testing has been endorsed by the CLSI, which is permitted by the US FDA and is also

recommended by the WHO. In this study, Kirby-Bauer disc susceptibility technique was adapted for performing antibacterial susceptibility test of clinical isolates against selected antibiotics. Results of this test revealed that, there were variations in the susceptibility patterns of the clinical isolates to the diverse antibiotics. All the isolates were found to be susceptible to all the screened antibiotics, but some of the isolates showed intermediate susceptibility and resistance pattern. The susceptibitity of the clinical isolates may be due to the efficacy of the antibiotic and its mode of action.

β- lactam antibiotics are the most commonly prescribed drugs and these groups are broad class of antibiotics, consisting of all antibiotic agents that contain a β- lactam nucleus in their molecular structures. This group includes penicillin and its derivatives like ampicillin, amoxicillin, cephalosporins, monobactams and carbapenems. Most of the β- lactam antibiotics work by inhibiting cell wall biosynthesis in the bacterial organisms and are the most widely used group of antibiotics. In our present study, we have selected pencillin and ampicillin as a representatives of β- lactam antibiotics. In our study, all the clinical isolates of *S. mutans* and *S. sanguis* showed susceptible to ampicilin with zone of inhibition 37 mm and 28 mm respectively. One clinical isolates from *S. mutans and S. sanguis* showed intermediate resistance and resistance pattern to the exposed antibiotic respectively. Susceptibility and resistance pattern was observed in two clinical isolates of *S. mitis* and one clinical isolate exhibited intermediate resistance. In our study, it is also observed that all the clinical isolated of *S. salivarius* was found to be resistance to ampicillin.

Pencillin was very effective against *S. salivarius* and *L. acidophilus* as all the clinical isolates were found to be susceptible. Intermediate resistance was found among single isolates of *S. mutans* and *S. sanguis*. Resistance pattern was observed in a single isolate of *S. sanguis* and among two isolate of *S. mitis.* The action of β-lactam drug is particularly on cell wall of bacteria, these antibiotics inactivate the action of transpeptidase enzyme, which is very essential for cell wall synthesis. In our present study, susceptibility of clinical isolates to ampicillin and penicillin may be attributed due to blocking of cell wall synthesis by inhibiting final transpeptidation step of peptidoglycan production in the bacterial cell wall.

Several studies have also reported on mechanism in the bacterial resistance to β- lactum antibiotic. The resistance pattern obtained in this present study may be attributed due to the production of β- lactamases. These enzymes utilize zinc ions to disrupt the β-lactam ring of the antibiotic by serine ester mechanism. The enzyme first associates noncovalently with antibiotic to yield a complex. The β-lactam ring is then attacked by the free hydroxyl on the side chain of a serine residue at the active site of the enzyme, yielding a covalent acyl ester. Hydrolysis of the ester finally liberates active enzyme and inactivates drug (Seiji *et al.,* 2005)

Penicillin-binding proteins (PBPs) also react with β- lactams to give serine esters, but, unlike the similar esters formed by β- lactamases, these do not hydrolyze readily (Ghuysen, 1991). This distinction blurs in a few cases: on one hand, some b-lactamase inhibitors form stable serine esters with β- lactamases (Bush and Sykes; 1983); on the other, a few PBPs deacylate rapidly, and their weak β- lactam-hydrolyzing activity may protect the bacterial cell if drug entry is sufficiently restricted by impermeability (David, 1995). Secondarily, the change in the affinity of penicillin-binding proteins for β- lactams or overproduction of specific penicillin-binding proteins. When penicillin binds to a specific PBP at a certain fixed ratio, cell growth stops or cells die; these PBPs are called low-affinity PBPs. Alteration of the affinity of a PBP could have an important role in the mechanism of resistance to β-lactams (Seiji *et al.,* 2005).

In our present study, it is observed that all the clinical isolates of *S. sanguis* and *L. acidophilus* showed a large sensitiveness to chloramphenicol. Single strains from *S. mutans, S. salivarius* and two strains from *S. mitis* exhibited resistance pattern to chloramphenicol. Chloramphenicol is an aromatic antibiotic containing benzene derivatives. The sensitivity of the clinical isolates to this antibiotic may be due to the immediate cessation of protein synthesis (Oleg, 1963). As chloramphenicol is a bacteriostatic antibiotic which specifically ceases protein biosynthesis, a number of important biosynthetic processes, such as DNA synthesis, RNA synthesis and the formation of certain cell-wall polymers proceed in the presence of chloramphenicol for considerable periods of time (Sadao *et al.,* 1977). Chloramphenicol crunch to the 50S ribosomal subunit and obstructs ribosomal functions, such as peptidyltransferase

(PTase) activity, binding and movement of ribosomal substrates through the PTase center and translation termination (Madhavan and Bagyalakshmi, 2014).

In our study, the sensitivity of clinical isolates may be attributed to the inhibition of protein synthesis. In this present study resistance pattern is also observed among the clinical isolates, this nature of resistance may be attributed to the chloramphenicol acetyltransferase (CAT) which results in the acetylation of the antibiotic (Jaoquim *et al.,* 1999).

Erythronmycin is a macrolides group of antibiotic well known for blocking protein synthesis in bacteria. The structure of erythromycin is made up of 14-membered macrocyclic rings attached to desosamine and cladinose sugar moieties (Dhermesh *et al.,* 2011). This antibiotic is known to inhibit protein synthesis by blocking physically by protein translation initiation or by peptidyl tRNAs translocation, which in turn inhibits the elongation of peptide chain by deactivating peptidyltransferase reaction (Tardrew *et al,* 1969). Due to this mechanism of action, macrolides group of antibiotic including erythromycin are known as 50S ribosome inhibitors (Micheael *et al.,* 2010).

In our study, all the clinical isolates were found to be susceptible for erythromycin. The obtained result may be attributed to the inhibition of protein synthesis among the clinical isolates. Resistance and intermediate resistance pattern is also observed, two clinical isolates from *S. mutans* and one clinical isolate from *S.sanguis* have shown resistance and intermediate resistance to erythromycin respectively. This resistance among the clinical isolates may be due to the ribosomal modification by methylation and also may be due to the acquisition of *erm* gene (erythromycin ribosome methylase). It is known that, among the bacteria A2058 nucleotide is a key nucleotide for the binding of erythromycin. Due to *erm* gene acquisition, it encodes a ribosomal methylase, which dimethylates 23S rRNA at a single site, adenine at position 2058 (Roland and Patrice; 2002).This resistance mechanism will reduces the affinity of erythromycin for its target site which may result in preventing direct access to the target or by modification of binding site conformation.

Aminoglycosides are the broad spectrum antibiotics which acts on wide range of pathogens involved in life threatening infections. The history of this group of antibiotics begins with the discovery of streptomycin around 1944. Thereafter, by the introduction of series of compounds like kannamycin, gentamicin and tobramycin have revoutionalised the use of this class of antibiotics for the treatment of many bacterial infections particularly infection caused by gram negative pathogens (Shazi *et al.*, 2008).

In our present study we have used Aminoglycosides group of antibiotics like gentamicin, kannamycin and streptomycin to know the susceptibility pattern on clinical isolates of dental caries. All the clinical isolates showed susceptible patterns for gentamicin with zone of inhibition ranging between 26 mm and 30 mm. In case of kannamycin, one clinical isolate from *S. sanguis* and *S. salivarius* showed intermediate resistance with zone of inhibition 18 mm and 19 mm respectively. When compared to gentamicin and kannamicin, streptomycin exhibited lesser effect on clinical isolates with zone of inhibition ranging from 15mm to 30 mm. Two clinical isolates from *S. mutans* showed intermediate resistance to streptomycin one clinical isolates from *S. sanguis* and *S. salivarius* were found to be non susceptible. The susceptibility of these clinical isolates to this group of antibiotics may be due to the mode of action. Aminoglycosides are known to block protein synthesis and also known to cause disintegration of bacterial cell membrane. These group acts by impairing protein synthesis in bacteria by binding 30S subunit of ribosomes through energy dependent process or phase (EDP- II). The binding of this antibiotic perturbs the elongation of peptide chain by flawing the proofreading process resulting in premature termination of peptide chain which affects the translational accuracy (Marie *et al.*, 1999). These atypical proteins are inserted in cell membrane leading disintegration of cell membrane resulting in alteration in the pliability and further help in the aminoglycoside transport (Marie *et al.*, 1999).

The resistance of clinical isolates to aminoglycosides group of antibiotics may be attributed to alteration of ribosomal structure or by bacterial enzymes which results in the inactivation of antibiotic and also by decreased uptake of and accumulation of the drug in bacteria (Lakshmi *et al.*, 2000). In our present study resistance pattern was

not exhibited by our clinical isolates. However intermediate resistance was observed in *S. sanguis* and *S. mitis* to kannamicin and *S. mutans* to streptomycin.

Nalidixic acid is the first quinolone antibiotic which was introduced to treat infections caused by gram negative bacteria around 1962. It is made possible to treat infection caused by gram positive bacteria, when the structural modification to the quinolone antibiotics has started. These structural modification of the functional group resulted in the development of newer, second (Lomefloxacin, Norfloxacin, Ofloxacin and etc.), third (Levofloxacin, Sparfloxacin, Moxifloxacin and etc.) and fourth (Trovafloxacin etc) generation antibiotics commonly called floroquinones. These antibiotics are having broad spectrum activity due to the presence of fluorine (F) and piperazine atoms at different functional sites of quinolones.

In our study, we have checked susceptibility pattern of our clinical isolates against second generation antibiotics of fluroquinolone group ie norfloxacin and oflaxacin. The result reveals that two clinical isolates of *S. mutans* showed resistance pattern against norfloxacin and one clinical isolate against ofloxacin. Among *S. sanguis* one clinical isolate showed resistance and two clinical isolates showed intermediate resistance against norfloxacin. It is also observed that three clinical isolates from *S. mitis* have exhibited intermediate resistance against norfloxacin. Except these, all the clinical isolate was found to susceptible against both the antibiotics. The results found in this study may be attributed due to the mode of action of the antibiotic and resistance mechanism developed by the bacteria in response to this antibiotic. Susceptibility of these clinical isolates may be due to the inhibition of DNA gyrase (topoisoerase II and DNA topoisomerase IV). This DNA gyrase is known to introduce negative superhelical turns into duplex DNA with the utilization of ATP. It is known that the fluroquinolone antibiotics act on DNA by covalent attachment of DNA gyrase to DNA. This complex makes unattainable to the action of DNA polymerase which results in the blocking of DNA synthesis and replication leading to cell death.

It is also believed that quinolones acting on DNA topoisomerase II are involved in inhibiting gram negative bacteria whereas topoisomerase IV are involved

in inhibiting gram positive bacteria by interrupting with transfer of separated chromosomal DNA to their daughter cells during cell division.

In our study, resistance pattern has also been observed and resistance against fluroquinolones may be acquired from spontaneous mutation of genes responsible for encoding of DNA gyrase enzyme. Resistance may be acquired due to change in single nucleotide of gyrase gene. It is also known that, fluroquinone antibiotics, especially norfloxacin is selected to treat infection which are resistance to other classes of antibacterial agents including aminoglycosides, penicillins, cephalosporins, tetracyclines, macrolides and sulfonamides, including combinations of sulfamethoxazole and trimethoprim.

Polymixin are the polypeptide group of antibiotics exerts its effect on lipopolysaccharide (LPS) of the outer membrane of Gram negative bacteria (Alexandre *et al*., 2007). It is a polypeptide consists of cyclic heptapeptide, a linear tripeptide and fatty acid tail linked to N terminal of the tripeptide (Katz and Demain, 1977). Polymixins acts as detergent like action which is of bactericidal type. Polymixn binds to LPS of outer membrane of the bacteria through self promoted uptake pathway or Membrane Lysis Death Pathway, which displaces calcium and magnesium bridges that stabilizes LPS. Due to high affinity of these peptides towards LPS, they aggressively dislocate the Ca^{2+} and Mg^{2+} ions and disarray the conformation of the outer membrane. Further interaction of fatty acid chain with LPS leads to the inclusion of polymixins into outer membrane resulting in the changes in the portability of the outer membrane. These changes in the outer membrane leads to development of temporary cracks which allow important molecules to pass through and also involved in promoting the uptake of peptide itself results in cell death.

As the action of this polymixin antibiotic is against gram negative bacterial cell wall, they are not active against Gram positive bacteria. In our present study, all the clinical isolates were found to be resistance to polymixin and also exerted low action. The result obtained by this study may be due to our clinical isolates are gram positive bacteria and lacks LPS.

Tetracyclines are the group of antibiotics which blocks the synthesis of protein by restraining the attachment of aminoacyl-tRNA to the A site of ribosomal acceptor.

A structure of tetracyclines consists of linearly fused tetracyclic nucleus to which various functional groups are attached. The antibacterial activity of this antibiotic is very important due to upholding of linearly merged tetracycline rings, naturally occurring (a) stereochemical configurations at the 4a, 12a (A-B ring junction) and 4 (dimethylamino group) positions, and conservation of the keto-enol system (positions 11, 12, and 12a) in proximity to the phenolic D ring.

In our present study, we have checked the susceptibility of clinical isolate to tetracycline. The result of this study focuses that, all the clinical isolates exhibited susceptibility to this antibiotic and resistant pattern was not observed. The highest susceptibility was exerted by *L. acidophilus* with zone of inhibition of 38 mm and least susceptibility was observed in *S. mitis* with zone of inhibition 19 mm. The susceptibility of clinical isolates to this antibiotic may be due to it mechanism of action. Tetracycline acts by attaching to the 30S subunit of the ribosome at the A-site. This blocks the attachment of new t-RNA to A-site of the ribosome, which is very essential step during protein biosynthesis. Hence due to the binding of tetracycline to A-site, protein biosynthesis cannot occur resulting in the death of the bacterial cell.

Hamzah and Senthil (2015) isolated *S. mutans* from dental caries patients and checked their susceptibility against β- lactum antibiotics including ampicillin. In their study, no resistance pattern was recorded among the clinical isolates. The results obtained from this study, are in agreement with our findings as all the *S. mutans* isolates were susceptible to ampicillin.

Dhamodhar *et al.,* (2014) reported that all the clinical isolates of *S. mutans* were found to be resistant to ampicillin. In a study, it has been reported that all the Streptococcus species were resistant to ampicillin but showed sensitivity to other antibiotics (Kushbu and Satyam; 2015). In our study also, similar resistance pattern has been observed against all the clinical isolates of *S. salivarius*.

In a study, 114 clinical isolates of *S. mutans* was found to be sensitive to chloramphenicol with average zone of inhibition of 42.2 mm (Hamzah and Sentil Kumar; 2015). Archana, (2011) and Munna *et al.* (2015), reported that 80 % of the dental caries were found to be sensitive against chloramphenicol without any resistance strains. Similar results have been found in our study also, in which almost

all streptococcus species has exhibited sensitivity, but in our results some of the clinical isolates exerted resistance against this drug. Bello *et al.* (2013) reported intermediate resistance among the clinical isolates.

Dibua *et al*, (2011) reported that *S. mutans* exerted highest susceptibility against ciprofloxacin (23) followed by streptomycin (20), Rocephalin (10 mm) and Gentamicin (2 mm). Whereas other antibiotics did not exerted any effect on *S. mutans* like Ampiclox, Zinnacef, Erythromycin, Septrin and Amoxacillin.

The results of antibacterial sensitivity test conducted by Richa roy, (2012) revealed that viridians group of streptococci including S. mutans, S. intermedius, *S. sanguis*, and *S. salivarius* showed varying degree of sensitivity to 11 antibiotics tested. They found Penicillin resistant and Vancomycin resistant strains. It has also found that, these strains were sensitive towards Erythromycin, Meropenem, Tetracyclin, Azithromicin, Gatifloxacin and Cirofloxacin. Intermediate susceptibility towards ampicilin was observed for *S. mutans*, *S. salivarius* and *S. intermedius*, while *S. mitis* and *S. sanguis* were found to be resistant to the tested drugs. S. mitis and *S. intermedius* showed resistance to Ceftriaxone and *S. sanguis* showed intermediate susceptibility for Norfloxacin and Ceftriaxone.

Bello *et al.* (2013) reported that *S. mutans*, *S. albus*, *P. aerogenosa*, *K, pneumonia* and *P. vulgaris* were isolated from decayed teeth showed sensitivity against all the antibiotics. *S. mutans* showed highest susceptibility agaist Gentamicin (20 mm) and Ciprofloxacin (19 mm) and exhibited resistance to Amoxycillin and Tetracycline with inhibition zone 8 and 6 mm respectively.

Arul and Palanivelu (2014) reported on the antibacterial susceptibility of dental caries isolates against commercially available drugs. The sensitivity pattern of isolated pathogens showed that isolated strains were most sensitivity to second generation antibiotics. Among floroquinolone group of antibiotics, Norfloxacin showed highest inhibition of 42 mm followed ofloxacin (35 mm) and ciprofloxacin (30 mm).

β-lactum antibiotics such as Cefixime, Cefatrixone, Cephalexin were also tested against these pathogen and highest activity was exerted by Cephalexin with inhibition zone of 45 mm followed by Cefatrixone (33 mm) and Cefixime (25mm).

The extended spectrum drug such as Ampicillin and Penicillin exhibited activity with 35 mm zone of inhibition. Aminoglycosides, like Gentamicin and Amikacin showed its inhibitory effect against the isolated pathogens with zone of inhibition 32 mm and 31 mm respectively. Erythromycin was found to be most active against *S. pyogenes* and tetracycline exhibited greater zone of inhibition with 37 mm (Arul and Palanivelu, 2014).

6.7 Bioassay of extracted compounds on selected dental caries isolates.

Plants and plant extracts have a variety of phytochemicals with biological a property that enhance human health and helps to diminish the risk of chronic diseases. Drugs isolated from plants have a long history that has been recorded and practiced in both traditional and modern societies as herbal remedies. The potential uptake of these phytocompounds in the drug discovery has provided a new leads to pharmaceutical industries (Conforti *et al.,* 2008). There have been numerous records on the use of traditional plants and natural products for the management of oral diseases. Many plant based medicines are used in traditional medicine system have been recorded inventories as agents used to treat microbial infections. These plant based medicine are also been recently probed for their effectiveness against oral microbial pathogens and number of them are utilized in dental industries.

Plants produces a variety of secondary metabolites, most of them are phenols or their oxygen-substituted derivatives (Cowan, 1999). Numerous studies recommended that, the derived metabolites like flavonoid, alkaloid, terpenoids, steroids, glycoside, saponin, reducing Sugar and tannin are precisely recognized for their antibacterial property. These bioactive compounds are recognized to operate by diverse mechanism and exert antimicrobial action (Shimada T., 2006). There is a lack of information regarding the mechanism of inhibitory action of plant derived metabolites against pathogenic organisms but quite a few studies have been conducted in this direction.

Phenols and polyphenols which contain the phytocompounds like phenols, phenolic acids, quinines, flavonoids, flavonols, tannins and coumarins are well known to produce phenolic toxicity to microorganisms. The mechanism is probable by inactivation of enzymes, membrane disruption, complex dispossession and also by interrupting the adhesion binding sites of the organisms (Mason and Wasserman, 1987).

Flavonoids are known to be efficient antimicrobial phytoconstituent, which affects wide array of microbial pathogens. These flavonoids may act through inhibiting the function of cytoplasmic membrane as well as by DNA gyrase inhibition and also by inactivation of β-hydroxyacyl-acyl carrier protein dehydratase (Cushnie and Lamb, 2005; Zhang *et al.,* 2008). Several reported studies suggest that, flavonoids are recognized to inhibit *S. mutans* by distracting cellular membranes and forming soluble proteins complexes (Sakanaka, *et al.,* 1989). Tanins are other group of phytochemical, which are the group of polymeric phenolic compounds, which are produced by monomeric units of flavan or quinone derivatives. They restrict the growth of microorganisms by forming cell wall complexes, which results in microbial adhesions inactivation, membrane disruption and also by metal ion complexation.

Terpenoids and essential oils are highly enriched phytocompounds with isoprene structure having general chemical structure $C_{10}H_{16}$. They occur in different forms as sesquiterpenes, tetraterpenes, triterpenes, and diterpenes. They are known to show antimicrobial activity particularly active against viruses, bacteria and protozoans. The substantiation for its antimicrobial activity is assorted and is not fully understood but it is hypothesized to involve in membrane distraction by lipophilic compounds (Silva and Fernandes, 2010). Alkaloids are heterocyclic compounds found naturally. These compounds have wide range of antimicrobial activity. The activity of these compounds is accredited to their intercalating ability with cell wall or DNA (Cowan, 1999). Depending on these facts, antimicrobial activity of ten traditional plants was performed against isolated pathogens of dental caries by agar well diffusion method. The plants used in this study showed the presence of above said secondary metabolites. Results of our forgoing findings revealed that, all the tested methanol extracts from different plants and crude extracts from *H. mysoresence* showed the dose dependent antimicrobial activity. This antimicrobial activity may be

attributed due to the presence of secondary metabolites and their inhibitory mechanisms involved in it.

The present study was evaluated to know the antibacterial activity of methanol extract (leaf) of ten medicinal plants against *S. mutans.* The result obtained in this study, reveals that the all plant extracts showed significant antibacterial activity (Table 5.14). The highest activity was exhibited by methanol extract of *H. mysoresence* (18.95±0.883 mm) and least activity was observed in *M. glabra* (15.80±1.870 mm). In our study it is also observed that, all the extract showed dose dependent antimicrobial activity against isolated strains of *S. mutans* and also standard strains of *S. mutans* (MTCC-497 and MTCC 897). Highest inhibitory activity was observed at 50mg/ml and at this concentration, antimicrobial activity of ten medicinal plants follows the ranking order, *H. mysoresence* > *M. indica* > *A. indica* > *S. pentandrum* > *A. muricata* > *C. roxburghii* > *T. asiatica* > *M. glabra* > *A. indicus* > *W. tinctoria*. In this current study, similar results were also found against *S. sanguis* in a dose dependent pattern (Table 5.15). Among the plant screened, least activity was observed in *M. glabra* with zone of inhibition 13.90 ± 1.870 mm and methanolic extract of *H. mysoresence* showed highest inhibitory activity with zone of inhibition 21.05 ± 0.394 mm at 50 mg/ml respectively. The ranking order can be represented as *H. mysoresence* > *M. indica* > *A. muricata* > *A. indica* > *A. indicus* > *C. roxburghii* > *S. pentandrum* > *W. tinctoria* > *T. asiatica* > *M. glabra*. In our study, we have observed a variation among the susceptibility among the clinical isolates to different plant extracts tested. It is observed that, *S. mitis* showed least susceptibility to *A. indica* with zone of inhibition 13.20±1.152 mm, where as *S. mutans* and *S. sanguis* showed moderate activities against this extract. When compared to activity against *S. mutans* and *S. sanguis*, *S. mitis* showed highest susceptibility to the extract of *H. mysoresence* with slight variation in the zone of inhibition 19.85 ±0.933 at 50 mg/ml. The ranking order for *S. mitis* was found to be *H. mysoresence* > *T. asiatica* > *M. indica* > *A. muricata* > *M. glabra* > *C. roxburghii* > *A. indicus* > *S. pentandrum* > *W. tinctoria* > *A. indica* (Table 5.16).

Due to variation among the susceptibility of clinical isolates the ranking order for *S. salivarius* differs with other clinical isolates and follows the ranking order like *H. mysoresence* > *A. muricata* > *M. indica* > *T. asiatica* > *S. pentandrum* > *W.*

tinctoria > *C. roxburghii* > *A. indicus* > *M. glabra* >*A. indica*. Among the isolated streptococcus species, it is observed that maximum susceptibility was observed in *S. sanguis,* followed by *S. mitis, S. salivarius* and *S. mutans* (5.17).

In our present study, *L. acidophilus* showed highest susceptibility when compared to isolated Streptococcus species. All the extracts showed dose dependent activity against *L. acidophilus*. Highest activity was observed in *H. mysoresence* with zone of inhibition 20.30 ± 0.657 mm and least activity was exhibited by *A. indicus* with inhibition zone 15.20 ± 0.523 mm at 50 mg/ml of extract concentration. The ranking order for *L. acidophilus is H. mysoresence* > *M. indica* > *A. muricata* > *T. asiatica* > *A. indica* > *S. pentandrum* > *C. roxburghii* > *W. tinctoria* > *M. glabra* > *A. indicus* (5.18).

Present investigation was also undertaken to know the efficacy of plant extract against *C. albicans*. It has been observed that, antifungal activity exhibited by methanol extract of different plants is low when compared to antibacterial activity. At 50 mg/ml methanolic extract of *A. muricata* showed a considerable antifungal activity against *C. albicans* with zone of inhibition 12.15 ± 0.38 mm followed by *W. tinctoria* 12.7 ± 0.44 mm at 50 mg/ml. Least activity was demonstrated by *M. glabra* with zone of inhibition 10.50 ± 0.51mm. Except these, all methanolic extracts exhibited moderate dose dependent inhibitory activity against *C. albicans.* The ranking order is as follows *M. indica* > *C. roxburghii* > *H. mysoresence* > *S. pentandrun* > *W. tinctoria* > *A. muricata* >*T. asiatica* >*A. indica* >*A. indicus* > *M. glabra* (5.19). The inhibitory action of these plants might be recognized due to the presence of phenolic and flavonoid content in the extract and mechanism of action involved in it. Differences in the susceptibility of the isolates to the used plant extract may be probably related to possible genotypic variations that have to be further investigated.

In our present study, literature survey suggested that limited work has been documented on antibacterial activity of *C. roxburghii, H. mysoresence, M. glabra, S. pentandrum, T. asiatica, and W. tinctoria* and most of the plants tested, it seem to be first reported in this research work with best to our knowledge.

Dixit and Sharma, 2014 evaluated the antibacterial effect of *A. indica* leaf extract against various pathogenic bacteria like *E. coli, P. vulgaris, K. pneumoniae*

and *P. aeruginosa*. The antibacterial activity exerted by this plant is due to present of ovatodiolide, acteoside, isoacteoside, and terniflorin. The pure compound also showed inhibitory effect on *H. pylori* by bacterial adhesion inhibition and also it interrupts the invasiveness ability to human gastric epithelia cells (Roa *et al*., 2012). Vivek *et al.* (2013), demonstrated that the leaf extract of *A. indicus* showed a potent inhibitory activity against drug resistant pathogens involved in urinary tract infections. It has been also observed that, essential oil from leaf and roots of *A. indica* exhibited a effective antimicrobial activity on *E. coli, P. aeruginosa, B. pumilus* and *S. aureus* with a array of MIC values extended from 31.25 to 250 µg/ml (Ushir *el al.,* 2010). Kundul *et al.* (2013), also showed that essential oil present in this plant is active against plant fungal pathogens like *R. bataticola and P. aphanidermatum*. In our present study similar antibacterial and antifungal activity was observed in this plant. Antibacterial activity against pathogens of dental caries is first reported in our study and this activity may be attributed to the presence of secondary metabolites in this extract.

The literature reports that the secondary metabolites present in the leaf extract of *A. muricata* exposed a highest inhibitory activity against *P. aeruginosa, S. aureus, K. pneumonia, B. subtilis* and *E. coli* (Vijaymeena, *et al*., 2013; Abubacker and Deepalakshmi, 2013). They investigated on methyl ester of hexadecanoic acid from leaves of *A. muricata*, which showed antifungal activity at 10 and 15 mg/ml against *A. solani* (NCBT-118), *A. erithrocephalus* (NCBT-124), *A. albicans* (NCBT-120), *A. fumigatus* (NCBT-126) and *P. chrysogenum* (NCBT 162). In another study, methanol and aqueous extract of leaf of *A. muricata* showed a significant activity against gram positive (*B. subtilis, S. aureus* and *S. pyogenes*) and gram negative (*E. coli* and *K. pneumonia*) pathogens. Both extracts exhibited antibacterial properties but the methanolic extract was more proficient as it inhibited a wide range of organism at changeable concentrations (Solomon *et al.*, 2014). Similar antibacterial and antifungal activity was observed against our clinical isolates.

Mistry *et al.* (2015) reported a very promising antibacterial activity of methanolic leaf extract of *A. indica*. This extract is able to inhibit *S. mutans* with zone of inhibition 24.67 ± 2.517 mm. This extract also inhibited other bacteria, *E. faecalis* (27.00±1.0mm) and *S. aureus* (18.00±0.0 mm) but no action was found against *C.*

albicans. In a study, the methanol extracts of *A. indica* showed promisory antimicrobial activity against all the test organisms. The zone of inhibition for *S. mutans* (20 mm), *S. mitis* (16 mm), *S. salivarius* (10 mm), *Lactobacilli* (22 mm) and *P. intermedia* (20 mm) and *C. albicans* (10 mm). These results were in agreement with our study as this extract inhibited almost all the isolated pathogens of dental caries (Soniya, *et al.,* 2014). The investigation conducted by Gauniyal *et al.* (2014) explains that, ethanol leaf extract of *A. indica* inhibited all the isolated oral pathogens. This extract inhibited bacterial pathogens like *S. mutans* (18 mm), *E. faecalis* (19 mm), *L. acidophillus* (23 mm) and fungal pathogens *C. albicans* (20 mm) and *C. tropicalis* (19 mm). In our study, the methanol extract of this plant inhibited all the clinical isolates.

Kekuda, *et al.,* (2014) reported anticaries activity of methanolic leaf extract of *C. roxburghii* against 24 strains of clinically isolated *S.mutans*. All clinical isolates were susceptibility against all tested strains with zone of inhibition ranging from 1.2± 0.0 mm to 2.3 ± 0.2 mm.

H. mysorense is reported to possess wound healing property, anti-HSV-1 and antitumor properties in the ayurvedic system of traditional medicine and little is known for its antibacterial activity. Anusha *et al.* (2015) comparitvely studied on antibacterial activity and antifungal property of leaf and flower extracts. In their study, in comparison with flower extract leaf extract showed highest inhibitory activity against bacteria and fungi. Gram positive bacteria (*S. aureus* and *B. subtilis* with zone of inhibition 24 and 30 mm respectively) showed higher susceptibility to extracts when compared to Gram negative bacteria like *B. coagulans* (25 mm), *E. coli* (20 mm), *P. aeruginosa* (19 mm) and *S. typhi* (20 mm).

M. glabra, or Barbados cherry (Acerola) is a bush or small growing tree which produces a fruit rich in vitamin contents, flavonoids and carotenoids (Roberta *et al*., 2011). In latest years, many studies have been conducted on the role of ROS in the etiology of various disorders by concentrating only on fruit extract and limited information have been reported on biological activities of leaves, bark and root. Our study is the first to report inhibitory activity of leaf extract against dental caries pathogens.

In a study Vijayaragavan, (2013) investigated antimicrobial activity of leaves of *W. tinctoria* against six bacteria (*S. aureus, S. pyogenes, , E. coli, S. typhi, L. lactis* and *P. aeruginosa*) and three fungi (*A. niger, A. flavus, R. indicus* and *M. indicus*). Aqueous extract showed significant antibacterial activity against most of these bacteria. The most susceptible microorganism was *L. lactis* (32 mm) followed by *P. aeruginosa* (31 mm zone of inhibition in aqueous extract) and *M. indicus* (32 mm zone of inhibition in methanol extract). Lakshmi Devi *et al.* (2009) investigated the antibacterial activity of different extracts of *W. tinctoria* (Roxb.) R.Br. against three Gram positive bacteria, viz. *S. epidermidis, S. aureus* and *B. subtilis* and two Gram negative bacteria *viz. E. coli* and *P. aeruginosa* and only Gram positive bacteria showed susceptibility against methanol extract.

6.8 Bioassay of extracted compounds and purified compound from *H. mysoresence* on selected dental caries isolates

Based on the results of preliminary antimicrobial screening assays among the tested plants, *H. mysoresence* was selected as the best plant. Further, this plant was preferred and subjected for sequential extraction with four solvents (petroleum ether, chloroform, methanol and aqueous) and were initially evaluated for their anticariogenic activity against clinical isolates of dental caries. The results of this study revealed that, *L. acidophilus* demonstrated highest susceptibility for petroleum ether extract (24.10±0.44 mm), chloroform extract (13.45±1.60 mm), methanol extract (20.45±0.88) and lastly aqueous extract (12.95±1.19 mm). *S. mutans* also showed highest susceptibility to PEH (23.60 ±0.59 mm) followed by MEH (18.85±0.98 mm), CEH (12.85±1.30 mm) and AEH (11.75±0.85 mm). In our study, the highest inhibitory activity of was observed in PEH and MEH with zone of inhibition 22.80±0.52 mm and 19.55±3.03 mm respectively, but showed least susceptibility for CEH (12.80±1.15 mm) followed by AEH (10.45±1.05 mm). *S. sanguis* exhibited least susceptibility against PEH and MEH with zone of inhibition 20.80 ± 0.12 mm and 17.85 ± 0.58mm respectively. PEH demonstrated a significant antibacterial activity against *S. mitis* (21.35 ± 1.05 mm) followed by MEH (18.65 ± 1.08 mm), CEH (12.20 ± 0.69 mm) and less activity was observed in AEH (11.80 ± 1.15 mm) (Table 5.20).

Similar antibacterial properties have been previously studied by several investigators. In a study, a stronger antibacterial effect was observed with the methanolic leaves extract of *Bixa orellana L*. (achiote) with an inhibition zone of (19.97 ± 1.31) mm against *S. mutans* (Dyanne *et al.,* 2016). Dinesh *et al.* (2013) evaluated on inhibitory properties of 7 different indigenous plant extracts *viz.*. (*Moringa oleifera, Pimenia diocia, Syzgium aromaticum, Mangifera indica, Psidium guajava, Coriandrum sativum and Mentha arvensis*) against *S. mitis* and *S. mutans* by agar well diffusion method. The result of their study showed that 5 plant extracts tested exhibited antibacterial activity against *S. mitis* and *S. mutans*. Among the plant selected by them the *C. sativum, M. arvensis* plant extracts exhibited antimicrobial action against *S. mutans* only. About 5mg of *C. sativum* and *M. arvensis* extracts produced 16 mm and 15 mm zone respectively at 10mg/50µl. In a work, Prabhat *et al.* (2010), investigated on antimicrobial activity of seven medicinal plants (*T. chebula, M. elengi, A. aspera, A. catechu, A. arabica* and *G. glabra extracts*) against pathogens of dental caries. According to them, all the plants exhibited significant antibacterial activity against all pathogens, but the methanolic extract of *T. chebula* exerted maximum zone of inhibition against *S. aureus* (27 mm), *S. mutans* (23 mm) *S. mitis* (19 mm) *S. salivarius* (26 mm), *L. acidophilus* (24 mm) and *C. albicans* (26 mm). In their study, Petroleum ether extract of *M. elengi* and *A. aspera* inhibited *S. mutans*, *S. aureus and C. albicans*. Inhibitory efficacy of leaf extracts *G. glauca, P. scandens* and *E. kologa* were tested against the clinical isolates of *S. mutans*. Among the extracts, extract of *G. glauca* demonstrated a potent anticariogenic activity to higher extent followed by *P. scandens* and *E. kologa.* The inhibition zone was found to be 1.6 to 2.6, 1.1 to 1.9 and 0.8 to 1.5 cm in case of *G. glauca, P. scandens* and *E. kologa* respectively (Junaid, *et al.,* 2013). These results were in agreement with our findings as all the methanolic leaf extract used in this study showed antimicrobial activity against all the clinical isolates of dental caries. It has been reported that, the extract of *P. guajava, M. cochinchinensis, G. glabra, S. aromaticum and P. retrofractum* showed promissory antibacterial activity against *S. mutans*. The largest inhibition zone 16.7±0.5 mm in diameter was observed in *S. aromaticum* extract (Jitrada Wannachot and Sakulrat Rattanakiat, 2015). Methanolic leaf extract of *B. orellana* inhibited *S. mutans* with zone of inhibition 19.97 ± 1.31 mm and *S. sanguis* 19.97 ± 1.26 mm.

Nantiya *et al.* (2013) investigated on antimicrobial activity of *A. myriophylla* wood against cariogenic *S. mutans*. In their study, three flavonoids lupinifolin (6), 8-methoxy-7,30,40-trihydroxyflavone (7), and 7,8,30,40-tetrahydroxyflavone (8), a triterpenoid lupeol (3) as well as four sterols b-sitosterone (1), stigmasta- 5,22-dien-3-one (2), b-sitosterol (4), and stigmasterol (5) were isolated from *A. myriophylla* wood. All compounds showed a potent antibacterial activity against *S. mutans* with MIC and MBC ranging from 1–256 and 2–256 µg/ml, respectively. Among the purified compounds, lupinifolin a significant MIC and MBC values of 1 and 2 lg/ml respectively. Isolated *S. mutans* was found to be susceptibility with MIC and MBC values 0.25 – 2 and 0.5 – 8 lg/ml of Lupinifolin.

Several investigators have undertaken to work on antimicrobial activity of essential oil obtained from plant products against oral bacteria causing dental caries and periodontitis. Essential oil obtained from *T. riparia* leaves, showed significant antibacterial activity against *S. mutans, S. mitis, S. sanguis S. sobrinus, S. salivarus* and *L. casei* (Nathalya *et al*., 2015). It has been reported that, the most of the essential oils (EO) presented moderate to strong antimicrobial activity against the oral pathogens. The essential oil from *C. sativum* inhibited all oral isolates with MIC values ranging from 0.007 to 0.250 mg/mL, and MBC/MFC values from 0.015 to 0.500 mg/mL. On the other hand the essential oil of *C. articulatus* inhibited 63.96% of *S. sanguis* biofilm formation (Salete *et al.*, 2014).

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6.9 Antibacterial activity of purified compounds of *H. mysoresence* against clinical isolates

In this study, based on the spectral results the compounds were identified as **F_3** was identified as 1-((E)-7-(3-(5-chloronon-7-yn-2-yl)-5-hydroxyphenyl)-2-propylhept-4 enylamino)butan-2-one, **F_4:**(2E,9E)-7-bromo-N-((E)-3-chlorohept-4-enylidene) -13-cyclopropyl-4-hydroxytridec- 6-enamide, **F_5:** 6-chloro-1- (5-(4-chlorobutyl) -4-hydroxy-2-((E)-4-hydroxybut-2-enyl)piperidin-3-yl)-3-methyldec-8-yn-4-one, **F_9:** N-((6E)-13- (3-(2-bromoethyl)-5- ((Z)-7, 16-dichloroheptacos-22-enyl)cyclopent-1-enyl)-8-chlorotridec-6-enyl)acetamide, and **F_{10}:** 6-chloro-8-(2-(3-chloro-7-(2-methylcyclopropyl)hept-6-ynyl)-4,5-dihydro-4-(hydroxymethyl)oxazol-4-yl)octan-2-one. In this study as per our knowledge these compounds were the first reported compound from our laboratory.

In this present study the pure compounds was evaluated against clinical isolates and the results were summarized in the. The petroleum ether extract yielded 19 active fractions in which 5 compounds were found to be very active against tested clinical isolates, remaining 13 compounds showed moderate activity and one compound didn't show any inhibition zone around the well. F_3 compound exhibited very potent antibacterial activity against *S. mutans* (15 mm) and *L. acidophilus* (11 mm) followed by *S. sanguis, S. salivarius* and *S. mitis* with zone of inhibition 10 mm respectively. The pure compound F_4 exerted antibacterial activity against *S. mitis* and *S. salivarius* with zone of inhibition of 14 mm respectively, followed by *S. mutans*, *S. sanguis* and *L. acidophilus* with zone of inhibition 11 mm in diameter respectively. F_5 compound showed a marked inhibitory activity against *S. mitis* (19 mm) and *S. salivarius* (19 mm) followed by *L. acidophilus* (17 mm), *S. mutans* (17 mm) and *S. sanguis* (15 mm). F9 compound showed significant antibacterial activity against *S. mitis, S. salivarius* and *L. acidophilus* with 16 mm zone of inhibition respectively followed by, *S. sanguis* (12 mm) and *S. mutans* (11 mm). Antibacterial activity of pure compound F_{10} revealed that, highest inhibition zone was observed against *L. acidophilus* (13 mm) followed by *S. mutans* (11 mm), *S. salivarius* (10 mm), *S. mitis* (10 mm) and *S. sanguis* (10 mm). The activity of these pure compounds may attributed to the presence of phytochemicals, as the crude extracts of *H. mysoresence*

contains steroids and terpenoids. The petroleum ether is an effective organic solvent to isolate the steroids and terpenoids present in plants.

Antifungal activity of pure compounds was performed by agar well diffusion method against *C. albicans*. Out of 19 pure fractions, only two compounds showed significant antifungal activity. Among the pure compounds only F_4 and F_5 showed significant antifungal activity against *C. albicans* with zone of inhibition 9 mm and 10 mm respectively (Table 5.22).

In our present study, pure compound F_5 was found to be more potent compound.

The pure compounds F_3, F_4, F_5 F_9 and F_{10} at 15.62 µg/ml showed 99% of inhibition against all the bacterial isolates tested. These compounds also showed the MBC value of 62.5 µg/ml, at which no visual growth has been observed in the inoculated plates after incubation. However the pure compound F_5 has inhibited the growth of *S. mutans* at lower concentration of 7.82 µg/ml and demonstrated the MBC value of 15.62 µg/ml. This result showed that the compound F_5 has exerted a significant inhibitory potential against *S. mutans*, when compared to other purified compounds.

Several investigations have been reported on purified compounds by several workers. The literature reports suggest that, the pure compounds isolated from plants exerts significant antimicrobial properties against oral pathogens involved in causing dental caries. According to Sato *et al.* (1996) artocarpin and artocarpesin, a flavonoid compounds isolated from *Artocarpus heterophyllus* (Moraceae) has inhibited the growth of *S.mutans*, actinomyces and lactobacilli species, at MIC values of ranging from 3.13–12.5 µg/mL. In our present study also pure compound F_5 was found to be very active against *S. mutans* with MIC value 7.82 µg/ml and this compound is also found effective againstall the isolated pathogens at the concentration of 15.62 µg/ml (Table 5.23).

Erycristagallin isolated from the roots of *Erythrina variegata* (Leguminosae) exhibited a marked inhibitory activity against tested cariogenic bacteria with MIC values 1.56–6.25 µg/mL. This compounds also checked the intake of glucose in

S.mutans, signifying that the compound interferes with bacterial uptake of metabolites (Tsuchiya *et al* ., 1994). Similar type of result were observed in our study also. The compound F_5 was found to be very effective against cariogenic bacteria at 7.82 to 15.62 µg/ml.

Park *et al* (2003) isolated a bioactive compound kuwanon G from *Morus alba*, inhibited *S.mutans* with MIC value of 88 µg/ml which was comparable to chlorhexidine and vancomycin (1 µg/mL). When the cells of *S.mutans* are treated with kuwanon G, the preferential antimicrobial activity against cariogenic bacteria is due to the blocking of cell growth. Similar mode of action was also observed, when the cell are treated with the isopanduratin A, which was isolated from *Kaempferia pandurate* (Hwang *et al*., 2004).

Studies conducted with Catechins such as such as, epicatechin, epicatechin gallate, and epigallocatechin gallate isolated from *Camelia sinensis* (Theaceae) demonstrated various mode of inhibitory action. These compounds exhibited their effectiveness by preventing of adherence of bacteria to tooth surface, inhibiting glucosyltransferase activity and also by inhibition of amylases (Hamilton and Miller, 2001; Sasaki, *et al.*, 2004). In our study, even though we have not checked the antiadherence activity, the pure compounds have demonstrated a very potent antimicrobial activity against clinical isolates.

It has been reported that, the active compound quercetin-3-*O*-*α*-l-arabinopyranoside or guaijaverin isolated from *Psidium guajava* was effective against two strains of *S.mutans*. This compound inhibited the acid production and decrease hydrophobicity of one of the bacteria and inhibits the adherence of both bacteria to glass. This result in the decrease of cell surface hydrophobicity , which results in the antiadherence activity by checking plaque formation by early colonizers such as *S. mitis*, *S. sanguinis* and *Actinomyces* (Razak *et al*., 2006)

In a work Esmaeelian *et al.* (2007), showed that the ethanolic leaf extract of *Alcea longipedicellata* (Malvaceae), inhibited oral streptococci with MIC value of 160-200 µg/mL. This activity is due to the presence of active compound in the extract ie Malvin (malvidin-3,5-diglucoside).In our study also, similar findings have been observed as our extract also contains a potent antimicrobial compounds.

Studies on macelignan, a compound isolated from *Myristica fragrans* (Myristicaceae) suppressed the growth of cariogenic bacteria including *S. mutans* (MIC = 3.9 μg/mL, MBC = 7.8 μg/mL). It is also noticed that this compound completely eliminated *S. mutans* within one minute and displayed antibiofilm activity against *S. mutans*, *S. sanguis* and *A. viscosus* (Rukayadi and Hwang, 2008). In our study also *S. mutans* was found to be susceptibile to F_5 compound at the concentration of 7.82 μg/mL.

Naringin a polymethoxylated compound isolated from citrus fruit exhibited antimicrobial activity against bacteria causing periodontitis and other oral streptococci with MIC range 9.8-125 mg/ mL. This compound also showed superior antibacterial action against *A. actinomycetemcomitans* and *P. gingivalis* (Tsui *et al* ., 2008). Similarly, in our study also all the compounds was found to be effective with MIC range 7.82-15.62 against bacterial pathogens and for MIC for fungal pathogen at MIC range 0.5 -1 mg/ mL

Bakuchiol isolated from *P. corylifolia* has inhibited the growth Gram-positive and Gram-negative oral pathogens. It is also suppressed the growth of *S. mutans* under different range of sucrose concentration, organic acids, pH values in a temperature dependent pattern and also suppressed the growth of cells adhered to a glass surface (Katsura *et al*., 2001).

In our present study, all the compounds was found to be very active against bacterial pathogens with MIC range 7.82 – 15.62 μg/mL and shown MBC value at 62.5 μg/mL, this is due to the presence of pure active compounds. In our study, it is observed that F_4 and F_5 compounds was found to be very effective against *C. albicans* but other compounds was found to be inactive against *C. albicans*.

7

Summary

Today, discovery of new drug has become an important and difficult task, which can be utilized for the treatment of particular disease or infection. There are essentially two routes of drug discovery, first route will be synthesizing, entirely new chemicals and evaluating them for a particular pharmaceutical use. Secondly, identifying the chemical or biological origin (natural product chemistry) and evaluate it for direct or indirect use as a template for the development of a new drug. Due to rapid effect of synthetic drugs make them more advantageous than the herbal drug, but the iatrogenic side effect and antimicrobial resistance of synthetic drugs remains debatable.

Hence in search of alternatives, drugs from biological origin have become main tool in the modern drug discovery. Historically, plants have provided a great source of inspiration for the development of new novel drugs. The use of plants has been documented from so many years to treat various ailments including treating infectious diseases caused by microbes in the form of antimicrobial agents. Medicinal plants are rich sources of antimicrobial agents which are used as medicine in different countries. These drugs have made large contribution to human health and well being. The important role of phytocompounds in the utilization and development of new plant drug are 1) They may become the base for the development of a medicine, a natural blue print for the development of new drugs, 2) A phytomedicine to be used for the treatment of diseases. There is large number of illustrations on plant derived drugs (secondary metabolites) for the treatment of various diseases or infections caused by microbes. There are also a number of reports are available on medicinal plants as a rich source of antimicrobial agents, which are known to inhibit the growth and has the ability to interfere with virulence factors associated with pathogens to cause disease.

Clinical microbiologists have two main reasons of interest in the topic of antimicrobial plant extracts. First, it is very likely that, these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians. Second, the public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics. In addition, many people are interested in having

more autonomy over their medical care. Plant compounds are readily available to over-the-counter from herbal suppliers and natural-food stores, and self-medication with these substances is common place.

Considering the vast potentiality of plant as sources for antimicrobial drugs to treat various microbial infections, the present study is designed to know the extent of natural products, which will inhibit the growth of pathogens oral microflora, which are responsible to cause oral diseases, especially dental caries.

Based on literature survey and developments in the subject, the present work is designed to collect the medicinal plants having traditional medicinal values and to check their efficacy, specifically targetting oral pathogens involved in causing dental caries.

- In this present study, we have made an attempt to explore the antibacterial activity of medicinal plants to control dental caries infection *in vitro*. A total of 10 plants were included in this study and their leaf extracts were used for the preliminary investigations such as phytochemical analysis, antioxidant and antimicrobial activities.

- The selected plants showed almost all the phytoconstituents like alkaloids, phenols, tannins, saponins, steroids, terpenoids and flavonoids. These phytochemicals are found to be in promissory amount in all the screened plants, and acting as important candidates responsible for antioxidant and antimicrobial activities.

- In our study, the selected plant extracts showed the presence of all the phytochemcals mentioned above.

- In our present study, total phenol and total flavonoid contents was measured by using standard protocols. The result of this study reveals that, highest phenolic and flavonoid content was noticed in *H. mysoresence* extract followed by *S. pentandrum*.

- Methanol extracts of ten medicinal plants was also evaluated for antioxidant activity. The results of DPPH assay explains that *H. mysoresence* extract has converted DPPH to DPPHH upto 94.80% and showed a IC_{50} value of 261.90 µg/ml.

- The results of metal chelating activity revealed that, the methanol extract of *H. mysoresence* showed a potent metal chelating activity followed by *S. pentandrum.*

- The result of ferric reducing assay showed that, highest absorbace was noticed in the extract of *A. muricata, H. mysoresence, S. pentandrum.*

- In our present study, correlation between total phenol and total flavonoid was analysed with antioxidant activities. The results once again reconfirm the earlier findings that antioxidant activity is mainly due to the presence of flavonoid and phenolic contents in the plant extracts.

- The statistical analysis also reveals a positive correlation in the total phenolic and flavonoid content and antioxidant activities.

- Parallely, this work is also focused on the isolation of dental caries pathogens from the patients suffering from dental pain. Samples were collected from the patients visiting to District Mc Gann hospital, Shivamogga, Karnataka. In this study, we have isolated a total of 404 pathogenic organisms from 100 subjects. Based on the morphological and biochemical characters the isolated organism were designated as *S. mutans, S. mitis, S. sanguis, S. salivarius, L. acidophilus* and *C. albicans.*

- Among the organism, *S. mutans* was found to be the most prevalent organism in our study with isolation percentage of 22.27%. The second predominant organism was found to be *S. sanguis* (19.30%), followed by *S. salivarius* (14.35%) and finally prevalence rate of *C. albicans* was 7.42%.

- In this present study, preliminary antimicrobial activity of methanol extract of 10 plants was evaluated against clinical isolates of dental caries. The result of this study reveals that, all the extract inhibited the isolated pathogens in a dose dependent manner. This study also suggests that the methanol extract of *H. mysoresence* was found to exhibit a potent antimicrobial activity, when compared to other plant extracts.

- The results of qualitative, quantitative, antioxidant and antibacterial studies revealed that, *H. mysoresence* showed a promissory result which encourages us to select this plant for further work.

- *H. mysoresence* leaf extracts were subjected for sequential extraction procedure using solvents like Petroleum ether, Chloroform, Methanol and water to yield petroleum ether extract, chloroform extract, methanol extract, and aqueous extract respectively.

- These crude extracts were subjected for phytochemical, antioxidant, and antibacterial studies. Petroleum ether extract known to contain all the phytochemicals except alkaloid. Chloroform extract known to possess glycosides and flavonoids, methanol extract known to contain saponin. However, the aqueous extract of plant known to posses flavonoid, carbohydrate and alkaloids.

- Antioxidant activity of chloroform extract of *H. mysoresence* has shown highest activity, however a negative correlation was noticed with the total phenolic and flavonoid contents.

- Based on the antibacterial activity, the petroleum ether extract was further subjected for purification using silica gel column chromatography. The petroleum ether extract yielded 19 pure compounds and they are named as F_1, F_2, F_3 ...F_{19} respectively.

- Further, antimicrobial studies were carried out using purified compounds against dental caries pathogens. Our observations indicate that, all the fractions shown the marked antibacterial activity against the clinical isolates, except the fraction F_1.

- Based on these results, five compounds were selected to determine minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC).

- The MIC value for F_5 showed was 7.86 µg/ml for *S. mutans*, and all other compounds showed MIC value of 15.62 µg/ml against all the bacterial isolates. But, all the compounds was found to be inactive against *C. albicans* except F_4 and F_5. These two compound showed MIC value of 1000 µg/ml against *C. albicans*

- In our present study, all the compounds was found to be very active against bacterial pathogens with MIC range 7.82 – 15.62 µg/mL and shown MBC value at 62.5 µg/mL, this is due to the presence of pure active compounds. In our study, it is observed that F_4 and F_5 compounds was found to be very effective against *C. albicans* but other compounds was found to be inactive against *C. albicans.*

- MBC value for F_3, F_4, F_5, F_9 and F_{10} compounds was found to be 62.5 µg/ml. MFC value for F_3 and F_4 compound was found to be 62.5 and other compounds exhibited MFC value of 125 µg/ml against *C. albicans*.

- F_3, F_4, F_5, F_9 and F_{10} compounds were subjected for spectral studies, like IR, Mass, and ^{1}H-NMR.

- The isolated compound **F_3** was identified as 1-((E)-7-(3-(5-chloronon-7-yn-2-yl)-5-hydroxyphenyl)-2-propylhept-4 enylamino)butan-2-one, **F_4:**(2E,9E)-7-bromo-N-((E)-3-chlorohept-4-enylidene)-13-cyclopropyl-4-hydroxytridec-6-enamide, **F_5:** 6-chloro-1-(5-(4-chlorobutyl)-4-hydroxy-2-((E)-4-hydroxybut-2-enyl)piperidin-3-yl)-3-methyldec-8-yn-4-one, **F_9:** N-((6E)-13-(3-(2-bromoethyl)-5-((Z)-7,16-dichloroheptacos-22-enyl)cyclopent-1-enyl)-8-chlorotridec-6-enyl)acetamide, and **F_{10}:** 6-chloro-8-(2-(3-chloro-7-(2-methylcyclopropyl)hept-6-ynyl)-4,5-dihydro-4-(hydroxymethyl)oxazol-4-yl)octan-2-one

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Conclusion and Future Prospectus

The present work is aimed in the isolation of pathogens from dental caries patients and their susceptibility to the different extracts of plant origin. This study shows considerable evidence that different plant extract has inhibited the growth of dental caries pathogens (*in vitro*). The crude extracts from different plant and purified compounds from *H. mysoresence* can be used as a potential therapeutic agent in the control and preventation of dental caries infection. These compounds can be utilized in the formulation of tooth paste and tooth powder and also can be used a tablets to treat other infection other than dental caries.

This work provides a platform for the other researchers working in the same and makes them to think on the utilization of these new purified compounds of plant origin. These fractions may inhibit the organisms by interfering with other pathophysiology aspects.

Further, work is needed, on *in-vivo* antibacterial activity, anti-adherence activity; antibiofilm activity by targeting Glucosyltransferase (GTase) enzyme can be explored.

These compounds can also be utilized in the treatment of infections caused by antibiotic resistance bacteria.

The novel compounds isolated from *H. mysoresence* from our laboratory can be exploited in drug design and formulation studies. Further these compounds should be subjected to animal and human studies to determine, whether they offer therapeutic benefits, either alone or in combination with conventional agents, which indirectly help to reduce the overall burden of oral diseases worldwide.

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References

Abdulla, M., Reshma, VJ., Punit, B, and Priyanka, CK. (2014). Role of Antioxidants in Dentistry. The Journal of Dental Panacea, 1 (2): 62-67.

Abo, KA., Ogunleye, VO, and Ashidi, JS. (1991). Antimicrobial poteintial of *Spondias mombin*, *Croton zambesicus* and *Zygotritonia crocea*. Journal of Pharmacological Research, 5(13): 494-497.

Adekunle, AS., and Adekunle, OC. (2009). Preliminary assessment of antimicrobial properties of aqueous extract of plants against infectious diseases. Biology and Medicine, 1(3): 20-24.

Adetuyi, FO., Dada, IBO, and Ibrahim, TA. (2013). Phytochemical Analysis and Antioxidant Activities of Hot and Cold Water Extracts of Mango (*Mangifera indica L.*) Leaf and Stem Bark. Journal of Basic Medical Sciences, 1(2).

Adriane, CMM., Marcia, Luzia, FM, and Terezinha, IES. (2011). Prevalence of yeast species in the oral cavity and its relationship to dental caries. Acta Scientiarum Health Sciences Maringa, 33 (1): 107-112.

Aiuto, DF., Parkar, M, and Andreou, G. (2004). Periodontitis and systemic inflammation: control of the local infection is associated with a reduction in serum inflammatory markers. Journal of Dental Research, 83: 156-160.

Ajila, CM., Aalami, M., Leelavathi, K, and Rao, UJSP. (2010). Mango peel powder: a potential source of antioxidant and dietary fiber in macaroni preparations. Innovative Food Science and Emerging Technologies. 11: 219-224.

Ajith babu TK., Ganesan, V, and Sajith, P. (2013). An anatomical and phytochemical studies of *Scleropyrum pentandrum* (Dennst.) Mabb. Asian Journal of Phytomedicine and Clinical Research. 1(1): 40-46.

Alejandra, HH., Luis, FO., Luis, F, and Antonio, DC. (2014). Susceptibility of *Porphyromonas gingivalis* and *Streptococcus mutans* to Antibacterial Effect from *Mammea Americana*. Advances in Pharmacological Sciences, 1-6.

Alexandre, PZ., Luciano, Z., Goldani, JL, and Roger, LN. (2007). Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review, Journal of Antimicrobial Chemotherapy, 60: 1206–1215

Al-Hebshi, NN., Nielsen, Q, and Skaug, N. (2005). *In vitro* Effects of Crude Khat Extracts on the Growth, Colonization, and Glucosyltransferases of *Streptococcus mutans*. Acta Odontologica Scandinavica. 63: 136–142.

Ames BN. (1983). Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. Science., 23; 221: 1256-64.

Amit, B, and Shalu, VB. (2012). Ethno-dentistry: popular medicinal plants used for dental diseases in India. Journal of Intercultural Ethnopharmacology, 1(1): 62-65.

Amoroso, P., Avila, FA, and Gagliardi, CMO. (2003). Prevalence of different *streptococci* species in the oral cavity of children and adolescents. Brazilian Journal of Oral Science, 2 (4): 164-168.

Anca, T., Laurian, V., Ilioara, O., Daniela, B, and Mircea. (2011). HPLC analysis of Salicyclic derivatives from natural products, Farmacia, 59 (1): 1-5.

Antony, B., Rekha, B., Shetty, AK., Kuruvilla, T, and Ramanathan, K. (2010). Semiquantitation and characterization of *Streptococcus mutans* from patients undergoing orthodontic treatment. Journal of Bioscience and Technology, 1 (2): 59-63.

Anupama, P., Aparna, S., Vynkatesh, K., Bhagwan, R, and Mohan, SK. (2012). Efficacy of Some Medicinal Plants against human dental pathogens. Indian Journal of Natural Products and Resources, 3(1): 123-127.

Aoba T. (2004). Solubility properties of human tooth mineral and pathogenesis of dental caries, 10(5): 249-57.

Aoba T. (2004): Solubility properties of human tooth mineral and pathogenesis of dental caries. Oral Diseases.10: 249-257.

Archana, D., Singh, V, and Bhatt, AB. (2011). Antibiotic Sensitivity pattern of streptococcus against commercially available drugs and comparision with extract of *Punica granatum*. International Journal of Pharma and Bio Sciences, 2(2): 504-508.

Archana, D., Singh, V, and Bhatt, AB. (2011). Antibiotic sensitivity pattern of Streptococcus against commercially available drugs & comparison with extract of *Punica granatum.* International Journal of Pharmaceutical Biology, 2(2): 505- 508.

Arul ASJ, and Palanivelu, P. (2014). *In-vitro* evaluation of Antimicrobial potency of commercially available drugs against Dentinal Caries microbes. Research Journal of Recent Sciences, 3: 218-223.

Arul, A., Sri, KJ, and Palanivelu, P. (2013). *In-vitro* Evaluation of Antimicrobial potency of commercially available drugs against dentinal caries microbes. Vol. 3 (ISC-2013), 218-223.

Aruoma, IO, and Cuppette, SL. (1997). Antioxidant methodology *in vivo* and *in vitro* concept. Illinois: AOAS press.

Asghari, J., Ondruschka, B, and Mazaheritehrani, M. (2011). Extraction of bioactive chemical compounds from the medicinal Asian plants by microwave irradiation. Journal of Medicinal Plants Research, 5: 495-506.

Ashok, GV., Priya, SB, and Pranita, AG. (2014). Evaluation of Antibacterial and Phytochemical Analysis of *Mangifera indica* Bark Extracts. International Journal of Current Microbiology and Applied Sciences, 3(5): 567-580

Balakrishnan, M., Robin, SS, and John, RT. (2005). Dental caries is a preventable infectious disease. Australian Dental Journal, 45(4): 235-245.

Banas, JA., Russell, RRB, and Ferretti, JJ. (1990). Sequence analysis of the gene for the glucan-binding protein of *Streptococcus mutans*. Infection and Immunity. 58 (1990) 667–673.

Baskar, R., Rajeshwari, V, and Satish kumar, T. (2007). *Invitro* antioxidant studies in leaves of *Annona species*. Indian Journal of Experimental Biology, 45: 480-485.

Bauer, AW., Kirby, WMM., Sherris, JC, and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology, 45: 493-496.

Baydar, NG., Sagdic, O., Ozkan, G, and Cetin, S. (2006). Determination of antibacterial effects and total phenolic contents of grape (*Vitis vinifera L.*) seed extracts. International Journal of Food Science and Technology, 41: 799-804.

Beighton, D., Russell, RRB, and Hayday, H. (1981). The Isolation and Characterization of *Streptococcus mutans* Serotype A from Dental Plaque of Monkeys *(Macaca fascicularis)*. Journal of General Microbiology, 124: 271-279.

Bello, OO., Egberongbe, HO., Adesetan, TO, and Adenekan, AM. (2013). Antibiotic Sensitivity Profiles of Bacteria Isolated from Decayed Teeth. Scholars Academic Journal of Pharmacy, 2(6): 424-428

Bender GR., Sutton, SCV, and Marquis, RE., (1986). Acid tolerance, proton permeabilities and membrane ATPase of oral *streptococci*. Infection and Immunity, 53: 331–38.

Berkowitz, R.J., Turner, J, and Green, P. (1981). Maternal salivary levels of Streptococcus mutans and primary oral infection of infants. Archives of Oral Biology, 26:147-149.

Bhuveneshwari P. (2014). Antioxidants in Oral health care. Journal of Pharmaceutical Science and Research, 6(4): 206-209.

Birkhed, D, and Heintze, U. (1989). Salivary secretion rate, buffer capacity and pH in human saliva: clinical chemistry and microbiology. Boca Raton, FL: CRC Press, (1): 25-73.

Bors, W, and Michel, C. (2002). Chemistry of the antioxidant effect of polyphenols. Annals of New York Academy of Science, 957: 57-69.

Bouallagui, Z., Bouaziz, M., Han, J., Boukhris, M., Rigane, G., Friha, I., Fki1, HJ., Ghorbel, H., Isoda, H, and Sayadi, S. (2012). Valorization of olive processing by-products: characterization, investigation of chemico-biological activities and identification of active compounds. Journal of Arid Land Studies, 22: 61-64.

Brain, KR, and Turner, TD. (1975). The practical evaluation of phytopharmaceuticals. Wright Scientechnica, Bristol. pp 36-45.

Brand Williams, W., Cuvelier, and M., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity, Lebensm.-Wiss. u- Technol, 28: 25-30.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. Nutrition Reviews 1998, 56:317–333.

Brogden, KM, and Guthmiller, JM. (2008). Polymicrobial disease, Washington: ASM Press.

Burne, RA., Chen, YY, and Wexler, DL. (1996). Cariogenicity of *S.mutans* strains with defects in fructan metabolism assessed in a program-fed specific pathogen-free rat model. Journal of Dental Research, 75 : 1572–77

Burton, R, and Richard, JL. (2000). Dental plaque formation. Microbes and infection, 2: 1599-1607.

Bush, K, and Sykes, B. (1983). b-Lactamase inhibitors in perspective. Journal of Antimicrobial Chemotherpy, 11: 97–107.

Caetano, DAC., Araujo, CR., Lima, VLAG., Maciel, MIS, and Almeida, DME. (2011). Evaluation of antioxidant activity of agro-industrial waste of acerola (*Malpighia emarginata D.C.*) fruit extracts. Ciencia e Tecnologia de Alimentos. 31: 769-775.

Cai, YZ., Sun, M, and Corke, H. (2003). Antioxidant activity of betalains from plants of the Amaranthaceae. Journal of Agriculture and Food Chemistry, 51: 2288– 2294.

Cai, Z., Lee, FSC., Wang, XR, and Yu, WJ. (2002). A capsule review of recent studies on the application of mass spectrometry in the analysis of Chinese medicinal herbs. Journal of Mass Spectrometry, 37: 1013–24.

Calderone, RA. (2012). Candida and Candidiasis. Washington: ASM Press.

Cannell, RJP. (1998). Natural Products Isolation. New Jersey: Human Press Inc;165–208.

Caufield, PW., Li, Y., Dasanayake, A, and Saxena, D. (2007). Diversity of lactobacilli in the oral cavities of young women with dental caries. Caries Research, 41(1): 2-8.

Cavas, L, and Yurdakoc, K. (2005). An investigation on the antioxidant status of the invasive alga *Caulerpa racemosa* var. cylindracea (Sonder) Verlaque; Huisman, Boudoresque (Caulerpales, Chlorophyta). *Jornal of Experimental Marine Biology and Ecology.* 2005, *325*: 189–200.

Ceballos, R., Seethalakshmi, M., Sankoli., Shubharani, R., Sivaram, V., Ganti, SM, and Ruben, M. (2013). Evaluation of phytochemicals and *in vitro* antioxidant studies of *Toddalia asiatica* leaf. Medicinal Plants, 5(4).

Chanda, S, and Dave, R. (2009). *In vitro* models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. African Journal of Microbiology Research, 3(13): 981- 996.

Chang, S., Tan, C., Frankel, EN, and Barrett, DM. (2000). Low-density lipoprotein antioxidant activity of phenolic compounds and polyphenol oxidase activity in selected clingstone peach cultivars. Journal of Agricultural and Food Chemistry, 48: 147–151.

Chiremba, C., Rooney, LW, and Trust, BJ. (2012). Microwave-assisted extraction of bound phenolic acids in bran and flour fractions from sorghum and maize cultivars varying in hardness. Journal of Chromatography, 1012: 119–128.

Cisar, JO., Barsumian, EL., Siraganian, RP., Clark, WB., Yeung, MK., Hsu, SD., Curl, SH., Vatter, AE, and Sandberg, AL. (1991). Immunochemical and functional studies of *Actinomyces viscosus* T14V type 1 fimbriae with monoclonal and polyclonal antibodies directed against the fimbrial subunit. Journal of General Microbiology, 137: 1971–1979.

Clarke, JK. (1924). On the bacterial factor in the aetiology of dental caries. Brazilian Journal of Experimental Pathology, 5: 141–147.

Conforti, F., Sosa, S., Marrelli, M., Menichini, F., Statti, G.A., Uzunov, D., Tubaro, A., Menichini, F, and Loggia, R.D. (2008). In vivo anti-inflammatory and in vitro antioxidant activities of mediterranean dietary plants. Journal of Ethnopharmacology. 116, 144–151.

Conforti, F., Sosa, S., Marrelli, M., Menichini, F., Statti, GA., Uzunov, D., Tubaro, A., Menichini, F, and Loggia, RD. (2008). *In vivo* anti-inflammatory and *in vitro* antioxidant activities of Mediterranean dietary plants. Journal of Ethnopharmacology, 116(1): 144-151.

Corralesa, M., Toepflb, S., Butza, P., Knorrc, D, and Tauschera, B. (2008). Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: a comparison. Innovative Food Science and Emerging, 9: 85–91.

Cotelle, N. (2001). Role of flavonoids in oxidative stress. Currrent Topics in Medical Chemistry, 1: 569-590.

Cowan, MM. (1999). Plant Products as Antimicrobial Agents. Clinical Microbiology reviews, 12(4): 564–582.

Coykendall, AL. (1974). Four types of *Streptocpccus mutans* based on their genetic, antigenic and biochemical characteristics. Journal of General Microbiology, 83: 327- 338.

Cushnie, TP., and Lamb, AJ. (2005). Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents, 26:343-356.

Da Silva, ACB., Cruz, JS., Sampaio, FC, and de Araujo, DAM. (2008). Detection of oral streptococci in dental biofilm from Caries-active and Caries-free children. Brazilian Journal of Microbiology, 39: 648-651.

Daniels, TE., Silverman, SJ., Michalski, JP., Greenspan, JS., Path, MCR, and Sylvester, RA. (1975). The oral component of Sjogren's syndrome. Oral Surgery, 39: 875-885.

Das, K., Tiwari, RKS, and Shrivastava, DK. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. Journal of Medicinal Plants Research, 4 : 104-111.

David, ML. (1995). b-Lactamases in Laboratory and Clinical Resistance. Clinical Microbiology Reviews, 557–584.

De Soet, JJ., van Loveren, C., Lammens, AJ., Pavicic, MJ., Homburg, CH, and Ten Cate, JM. (1991). Differences in cariogenicity between fresh isolates of Streptococcus sobrinus and Streptococcus mutans. Caries Research, 25: 116-122.

Deng, GF., Shen, C., Xu, XR, Kuang, RD., Guo, YJ., Zeng, L., Gao, LL., Lin, X., Xie JF., Xia, EQ., Li, S., Wu, S., Chen, F., Ling, WH, and Li, HB. (2012). Potential of fruit wastes as natural sources of bioactive compounds. International Journal of Molecular Sciences, 13: 8308- 8323.

Devendra, BN., Srinivas, N, and Kusuma, SS. (2012). A comparative pharmacological and phytochemical analysis of in vivo & in vitro propagated Crotalaria species, Asian Pacific Journal of Tropical Medicine, 37-41.

Dhamodhar, P., Sreenivasa, M., Channarayappa, Shantha kumar, SS, and Indiresha, HN. (2014). Prevalence, Characterization and Heterogeneity Studies on *Streptococcus mutans* Isolated from Bangalore Urban Population. International Journal of Pharmacology and Bio Sciences, 5 (3): 122 – 128.

Dhermesh, V., Sourabh, J, and Jitender, KM. (2011). Synthesis, Characterization and evaluated for Antibacterial Activity of Novel Erythromycin Derivatives. Pelagia Research Library, 2 (6):172-184.

Dhruw, C., Rajmani, H., Bhatt, Renu, and Verma Pradeep. (2012). Isolation of Dental Caries Bacteria from Dental Plaque and Effect of Tooth Pastes on Acidogenic Bacteria. Open Journal of Medical Microbiology, 2012(2): 65-69.

Di, CG, Mascolo, N., Izzo, AA, and Capasso, F. (1999). Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Science, 65:337–353.

Dibua, EU, and Nnamani, PO. (2011). Anticariogenic potentials of clove, tobacco and bitter kola. Asian Pacific Journal of Tropical Medicine, 814-818.

Dige, I., Nilsson, H., Kilian, M, and Nyvad, B. (2007). *In situ* identification of streptococci and other bacteria in initial dental biofilm by confocal laser scanning microscopy and fluorescence *in situ* hybridization, European Journal of Oral Science, 115: 459-467.

Dinesh, MD., Uma, MS., Anjali, VM., Neetushree, S., Meenatchisundaram, and Shanmugam. (2013). Inhibitory Properties of Aqueous Extracts of Selected Indigenous Medicinal Plants against Dental Caries Causing *Streptococcus mutans* and *Streptococcus mitis.* African Journal of Basic and Applied Sciences, 5 (1): 08-11.

Dong, YZ., Yi. W., Jian., Yi, Xu., Guo, HW., Long, L, and Xiao, HY. (2016). Ultrasound extraction of polysaccharides from mulberry leaves and their effect on enhancing antioxidant activity. Carbohydrate Polymers, 137 : 473–479.

Doughari, JH, and Manzara, S. (2008). *In vitro* antibacterial activity of crude leaf extracts of *Mangifera indica Linn.* African Journal of Microbiology Research, 2: 67-72.

Doughari, JH, and Obidah, JS. (2008). Antibacterial potentials of stem bark extracts of *Leptadenia lancifoli.* against some pathogenic bacteria. Pharmacologyonline, 3: 172-180.

Doughari, JH., Human, IS., Bennade, S, and Ndakidemi, PA. (2009). Phytochemicals as chemotherapeutic agents and antioxidants: Possible solution to the control of antibiotic resistant verocytotoxin producing bacteria. Journal of Medicinal Plants Research, 3(11): 839-848.

Drucker, DB, and Melville, JH. (1968). Fermentation end products of cariogenic and noncariogenic *Streptococci*. Arch Oral Biol, 13: 565–70.

Duarte, S., Gregoire, S., Singh, AP., Vorsa, N., Schaich, K., Bowen, WH, and Koo, H. (2006a). Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms. FEMS Microbiology Letter, 257: 50–56.

Dunny, GM. (1973). Isolation and characterization of plasmid deoxyribonucleic acid from *Streptococcus mutans*. Journal of Bacteriology, 114: 1362-1364.

Dyanne MF., Gabriela, UU., Rosella, CC., Stefany, CG., Frank, MT, and Juana, VM. (2016). Antibacterial activity of *Bixa orellana L. (achiote)* against *Streptococcus mutans* and *Streptococcus sanguinis*. Asian Pacific Journal of Tropical Biomedicine, 6 (5): 400–403.

Dziedzic, SZ, and Hudson, BJF. (1983). Polyhydroxy chalcones and flavanones as antioxidants for edible oils. Food Chemistry. 12: 205-212.

Dzink, JL, and Socransky, SS. (1985). Comparative *in vitro* activity of sanguinarine against oral microbial isolates. Antimicrobial Agents and Chemotherpy, 27: 663–665.

Eberhardt, TL., Li, X., Shupe, TF, and Hse, CY. (2007). Chinese Tallow Tree (*Sapium Sebiferum*) utilization: Characterization of extractives and cell-wall chemistry. Wood Fiber Science, 39: 319-324.

Edwardson, S. (1974). Bacteriological studies on deep areas of carious dentine. Odontology Reviews, 25: 333-337, 1974. Suppl. 32.

Ellen, RP. (1976). Establishment and distribution of *Actinomyces viscosus* and *Actinomyces naeslundii* in human oral cavity. Infection and Immunity, 14:1119-1124, 1976.

Eloff, JN. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants?. Journal of Ethnopharmacology. 6 0: 1–8.

Enweani, B., Inyang, NJ., Ibeke, MA., Ibeke, and Anyasi, NE. (1999). The prevalence of bacteria in dental caries inpatients attending dental clinics in Ekpoma and its environment. Journal of Applied Sciences, 2(1): 219-229.

Erdogan, S., Ates, B., Durmaz, G., Yilmaz, I, and Seckin, T. (2011). Pressurized liquid extraction of phenolic compounds from Anatolia propolis and their radical scavenging capacities. Food and Chemical Toxicology, 49: 1592–1597.

Erin,L., Gross., Clifford, J., Beall., Stacey, R., Kutsch., Noah, D., Firestone., Eugene. J., Leys., Ann, L, and Griffen. (2012). Beyond *Streptococcus mutans*: Dental Caries Onset Linked to Multiple Species by 16S rRNA. Community Analysis Plos One, 7(10): 1-11.

Erum, I., Kamariah, AS., Linda, BL, and Lim. (2015). Phytochemical screening, total phenolics and antioxidant activities of bark and leaf extracts of *Goniothalamus velutinus* (Airy Shaw) from Brunei Darussalam. Journal of King Saud University – Science, 27: 224–232

Esmaeelian, B., Kamrani, YY., Amoozegar, MA., Rahmani, S., Rahimi, M, and Amanlou, M. (2007). Anti-cariogenic properties of malvidin-3,5-diglucoside isolated from *Alcea longipedicellata* against oral bacteria. International Journal of Pharmacology, 3(6): 468–474.

Fan, XH., Cheng, YY., Ye, ZL., Lin, RC, and Qian, ZZ. (2006). Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. Analytica Chemica Acta, 555: 217–224.

Fejerskov, O, and Kidd, EAM. (2003). Dental caries, the disease and its clinical management. London, Blackwell Munksgaard Publications.

Fejes, S., Blazovics, A., Lugasi, A., Lemberkovics, E., Petri, G, and Kery, A. (2000). *In vitro* Antioxidant activity of *Anthriscus cerefolium* l. (hoffm.) extracts. Journal of Ethnopharmacology, 69: 259-265.

Firn, R. (2010). Nature's Chemicals. Oxford University Press, Oxford, 74-75.

Foster, BC., Arnason, JT, and Briggs, CJ. (2005). Natural health products and drug disposition. Annual review of pharmacology and Toxicology, 45: 203-226.

Fourrier, E., Cau-Pottier, E., Boutigny, H., Roussel, DM., Jourdain, M, and Chopin, C. (2000). Effects of dental plaque antiseptic decontamination on bacterial colonization and nosocomial infections in critically ill patients. Intensive Care Medicine, 26:1239-1247.

French, GL., Talsania, H., Charlton, RH, and Phillips, I. (1989). A physiological classification of viridans streptococci by use of the API-20STREP system. Journal of Medical Microbiology, 28: 275-286

Fuleki, T, and Ricardo, SJM. (1997). Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario. Journal of Agricultural and Food Chemistry, 45: 1156- 1160.

Gamal, M, and El-Sherbiny. (2014). Control of growth *Streptococcus mutans* isolated from saliva and dental caries. *International journal of current microbiology and applied science,* 3 (10): 1-10

Gamboa, F., Estupinan, M, and Galindo, A. (2004). Presence of *Streptococcus mutans* in Saliva and its relationship with dental caries: Antimicrobial susceptibility of the isolates. Universitas Scientiarum, 9: 23-27.

Gamboa, F., Mabel, E, and Adriana, G. Presence of *Streptococcus Mutans* in saliva and its relation with dental caries: Antimicrobial susceptibility of the isolates. Universitas Scientiarum, 9: 23-27.

Gan, RY., Xu, XR., Song, FL., Kuang, L, and Li, HB. (2010). Antioxidant activity and total phenolic content of medicinal plants associated with prevention and treatment of cardiovascular and cerebrovascular diseases. Journal of Medicinal Plants Research, 4:2438–2444.

George, B., Kaur, C., Khurdiya, DS, and Kapoor, HC. (2004). Antioxidants in tomato (*Lycopersium esculentum*) as a function of genotype. Food Chemistry, 84: 45–51.

Ghaleb, A., Yousef, S., Kamel, A, and Ali, B. (2012). Assessment of antifungal activity of herbal and conventional toothpastes against clinical isolates of *Candida albicans.* Asian Pacific Journal of Tropical Biomedicine, 2(5): 375–379.

Ghuysen, JM. (1991). Serine b-lactamases and penicillin-binding proteins. Annual Reviews in Microbiology, 45: 37–67.

Giovanni, DF., Valeria, R., Anna, DM, and Armando, Z. (2014) Triterpenoids from *Gymnema sylvestre* and Their Pharmacological Activities. Molecules, 19: 10956-10981.

Girija, AS., Smiline., Suba, KP., Hariprasad, P, and Raguraman, RA. (2012). A preliminary study on the screening of emerging drug resistance among the caries pathogens isolated from carious dentine. Indian Journal of Dental Research, 23 (1): 26-30.

Godowski, KC. (1989). Antimicrobial action of sanguinarine. Journal of Clinical Dentistry, 1: 96–101.

Gomez, AM., Lopez, CP, and De, lOEM. (1996). Recovery of grape seed oil by liquid and supercritical carbon dioxide extraction: A comparison with conventional solvent extraction. The Chemical Engineering Journal and the Biochemical Engneering Journal, 6: 227-231.

Gordon, MH. (1990). The mechanism of antioxidant action in vitro. In Food antioxidants. Edited by Hudson BJ. London: Elsevier Applied Science; 1990: pp. 1–pp. 18.

Gorinstein, S., Martin,BO., Park, YS., Haruenkit, R., Lojek, A., Ciz, M., Caspi, A, and Libman, TS. (2001). Comparison of some biochemical characteristics of different citrus fruits. Food Chemistry. 74: 309–316.

Gregoire, S., Singh, AP., Vorsa, N, and Koo, H. (2007). Influence of cranberry phenolics on glucan synthesis by glucosyltransferases and *Streptococcus mutans* acidogenicity. Jourrnal of Applied Microbiology, 103: 1960–1968.

Grossi, SG, and Genco, RJ. (1998). Periodontal disease and diabetes mellitus: a two-way relationship. Annals of Periodontology. 3: 51-61.

Gulcin, I., Huyut, Z., Elmastas, M, and Aboul-Enein, HY. (2010). Radical scavenging and antioxidant activity of tannic acid. Arab Journal of Chemistry, 3:43–53.

Gulçin, I., Berashvili, D, and Gepdiremen, A. (2005). Antiradical and antioxidant activity of total anthocyanins from *Perilla pankinensis* decne. Journal of Ethnopharmacol. 101: 287–293.

Gurenlian, JR. (2006) Inflammation: the relationship between oral health and systemic disease. Access. 20 (4): 1-9.

Gurenlian, JR. (2007).The Role of Dental Plaque Biofilm in Oral Health. Journal of Dental Hygiene, 81(5): 1-11.

Halliwell, B. (1994). Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet, 344: 721- 724, 1994.

Hamburger, M, and Hostettmann, K. (1991). Bioactivity in plants: The link between phytochemistry and medicine. Phytochemistry, 30: 3864-3874.

Hamilton-Miller JMT. Anti-cariogenic properties of tea (*Camellia sinensis*). Journal of Medical Microbiology, 50(4): 299–302.

Hamzah, AS, and Senthikumar, R. (2015). Identification and Antibiogram Profile of *Streptococcus mutans* and *Streptococcus sobrinus* from Dental Caries Subjects. Journal of Applied Pharmaceutical Science, 5(6): 054-057.

Harborne, J.B. (1993). Phytochemical Methods. (2nd *ed*.) Chapman and Hall, London, 288.

Harborne, JB. (1978). Phytochemical methods (3rd *ed*.) Chapman and Hall, London. 60 - 203.

Harmala, P., Vuorela, H., Tornquist, K, and Hiltunen, R. (1992). Choice of solvent in the extraction of *Angelica archangelica* roots with reference to calcium blocking activity. Plant Medicine, 58: 176-183.

Harris, GS., Michalek, SM, and Curtiss, R. (1992). Cloning of a locus involved in *Streptococcus mutans* intracellular polysaccharide accumulation and virulence testing of an intracellular polysaccharide-deficient mutant. Infection and Immunity, 60 (8): 3175–85

Hartmann, T. (2008). The lost origin of chemical ecology in the late 19th century. Proceedings of the National Academy of Sciences of the United States of America, 105(12): 4541-4546.

Hattori, M., Kusumoto, IT., Namba, T., Ishigami, T, and Hara, Y. (1990). Effect of tea polyphenols on glucan synthesis by glucosyltransferase from *Streptococcus mutans*. Chemical Pharma Bulletein, 38: 717–720.

Heinrich, M., Barnes, J., Gibbons, S, and Williamson, EM. (2004). Fundamentals of Pharmacognosy and Phytotherapy, Churchill Livingstone, Edinbrugh, 245–252.

Herastuti, S., Mari, F., Hiroshi, M, and Futoshi, N. (2016). Effect of roselle calyx extract on *in vitro* viability and biofilm formation ability of oral pathogenic bacteria. Asian Pacific Journal of Tropical Medicine, 9 (2): 119–124

Hillman, JD., Dzuback, AL, and Andrews, SW. (1987). Colonization of the human oral cavity by a Streptococcus mutans mutant producing increased bacteriocin. Journal of Dental Research, 66: 1092–94.

Hirasawa, M., Shouji, N., Neta, T., Fukushima, K, and Takada, K. (1999). Three kinds of antibacterial substances from *Lentinus edodes* (Berk) Sing (Shiitake, an edible mushroom). Internatioonal Journal of Antimicrobial Agents. 11: 151–157.

Huang, D., Ou, B, and Prior, RL. (2005). The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry, 53:1841-1856.

Islam, KM., Howlader, MA., Kundu, GC., Bulbul, IJ, and Ahsan, MR. (2010). Free radical scavenging activity of chloroform and ethyl acetate extracts of leaves of *Piper betle Linn*. Libyan Agriculture Research Center Journal International, 1: 384-387.

Jackman, RL., Yada, RY., Tung, MA, and Speers, RA. (1987). Anthocyanins as food colorants - a review. Journal of Food Biochemistry, 11: 201-247.

Jain, I., Jain, P., Bisht, D., Sharma, A., Srivastava, B, and Gupta, N. (2015). Use of traditional Indian plants in the inhibition of caries-causing bacteria--*Streptococcus mutans*. Brazillian Dental Journal, 26(2):110-5.

Jain, P., Jain, D., Bisht, A., Sharma, B., Srivastava, N, and Gupta. (2015). Use of Traditional Indian Plants in the Inhibition of Caries-Causing Bacteria - *Streptococcus mutans*. Brazilian Dental Journal, 26(2): 110-115.

Jaoquim R., Liliana, C, and Lauro, N., Dolores, C., Joseph, MS., Mohammed, H., Juan, JB., Jordi, V. (1999). Mechanism of resistance to Ampicillin, Chloramphenicol and Quinolone in multidrug resistant *Salmonella typhimerium* strains isolated from fish. Journal of Antimicrobial Chemotherapy, 43: 699-702

Jayaprakasha, GK., Girennavar, B, and Patil, BS. (2008). Radical scavenging activities of Rio Red grapefruits and Sour orange fruit extracts in different *in vitro* model systems. Bioresource Technology. 99(10): 4484-4494.

Jayaprakasha, GK., Selvi, T, and Sakariah, KK. (2003). Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. Food Research International. 36: 117-122.

Jayapriya, F, and Gricilda, S. (2015). GC-MS analysis of bio-active compounds in methanolic leaf extracts of *Justicia adhatoda (Linn.) G.* Journal of Pharmacognosy and Phytochemistry, 4(1): 113-117.

Jeevitha, T., Prabhu, N., Radha, M, and Abishek, R. (2013). Prevalence pattern of dental caries – A hospital based study and understanding the risk factors. Journal of Microbiology and Biotechnology Research, 3 (6): 33-36.

Jeffcoat, MK., Geurs, NC, and Reddy, MS. (2001). Periodontal infection and preterm birth: results of a prospective study. Journal of American Dental Association. 132: 875-880.

Jenkinson, HF, and Demuth, DR. (1997). Structure, function and immunogenicity of streptococcal antigen I/II polypeptides, Molecular Microbiology. 23: 183–190.

Jenkinson, HF, and Lamont, RJ. (1997). Streptococcal adhesion and colonization, Critical Reviews in Oral Biology and Medicine, 8: 175–200.

Jenkinson, HF. (1994). Cell surface protein receptors in oral streptococci. FEMS Microbiology Letters. 121: 133–140.

Jeon, JG., Klein, MI., Xiao, J., Gregoire, S., Rosalen, PL, and Koo, H. (2009). Influences of naturally occurring agents in combination with fluoride on gene expression and structural organization of *Streptococcus mutans* in biofilms. BMC Microbiology, 9: 228.

Jitrada, W, and Sakulrat, R. (2015). *In Vitro* Antibacterial Activity of Selected Herbal Extracts on *Streptococcus mutans*. The International Conference on Herbal and Traditional Medicine (HTM 2015), 28-30,

Johnson, WB., Buquo, L., Zeinali, M, and Ligler, FS. (2006). Prevention of nonspecific bacterial cell adhesion in immunoassays by use of cranberry juice. Anals of Chemistry, 78: 853-7.

Joona, K., Sowmia, C., Dhanya, KP, and Divya, MJ. (2013). Preliminary Phytochemical Investigation of *Mangifera indica* leaves and screening of Antioxidant and Anticancer activity. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 4(1): 1112 to 1118.

Junaid, S., Dileep, N., Rakesh, KN., Pavithra, GM., Vinayaka, KS, and Prashith Kekuda, TR. (2013). Anticariogenic Activity of *Gnidia glauca (Fresen.) Gilg, Pothos scandens L.* and *Elaegnus kologa Schlecht*. Journal of Applied Pharmaceutical Science, 3(03): 20- 23.

Jyotiram, AS., Panchal, CV., Suhas, P., Poul, BN, and Patel, JR. (2014). In vitro antioxidant and antiinflamatory activity of *Wrightia tinctoria* leaves, World Journal of Pharmacy and Pharmaceutical Sciences, 3(1): 964-972.

Kafka, TI., Sinanoglou, V, and Lazos, ES. (2007). On the extraction and antioxidant activity of phenolic compounds from winery wastes. Food Chemistry, 104: 1206-1214.

Kahkonen, MP., Hopia, AI., Vuorela, HJ., Rauha, JP., Pihlaja, K., Kujala, TS, and Heinonen, M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. Journal of Agricultural Food Chemistry, 47:3954–3962.

Kalpesh, BI., Jenabhai, B., Chauhan., Akanksha, A., Garg, and Arpit, MT. (2012). Antibacterial and phytochemical studies on *Calotropis gigantia (L.) R. Br.* Latex against selected cariogenic bacteria. Saudi Journal of Biological Sciences, 19: 87–91.

Kamboj, VP. (2000). Herbal medicine. Current Science. 78(1): 35-39.

Kar, A. (2007). Pharmaocgnosy and Pharmacobiotechnology (Revised-Expanded Second Edition). New Age International Limted Publishres, New Delhi, 332-600.

Katsura, H., Tsukiyama, RL., Suzuki, A, and Kobayashi, M. (2001). *In vitro* antimicrobial activities of bakuchiol against oral microorganisms. Antimicrobial Agents and Chemotherapy, 45(11): 3009–3013.

Katz E, Demain AL. (1977). The peptide antibiotics of Bacillus: chemistry, biogenesis, and possible functions. Bacteriology Reviews, 41: 449-74.

Kaur, HP., Neha, SP, and Wadhwa, P. (2014). Antibacterial Efficacy Of Clove (*Syzygium aromaticum*), Harad (*Terminalia chebula*) and Chewing Sticks Against Cariogenic Bacteria . International Journal of Biology, Pharmacy and Allied Sciences, 3(6): 974-984.

Kedage, VV., Tilak. JC., Dixit, GB., Devasagayam, TPA, and Mhatre, MA. (2007). Study of antioxidant properties of some varieties of grapes (Vitis vinifera L.). Critical Review in Food Science and Nutrition, 47: 175-185.

Kemp, W. (1991a). Ultraviolet and visible spectroscopy. In Organic spectroscopy, Edn Kemp, W Macmillan Press Ltd, London, 243-252.

Kemp, W. (1991b). Infrared spectroscopy. In Organic spectroscopy, Edn Kemp, W Macmillan Press Ltd, London, 19-56.

Kemp, W. (1991c). Nuclear magnetic resonance spectroscopy. In Organic spectroscopy, Edn Kemp, W Macmillan Press Ltd, London, 101-240.

Keyes, PH. (1960). The infectious and transmissible nature of experimental dental caries, findings and implications. Archieves of oral biology. 1:304-320.

Khushbu, Y, and Satyam, P. (2015). Antibiogram profiles against polymicrobial pathogens among dental caries patients at Janaki Medical College teaching hospital. Nepal International Journal of Applied Dental Sciences, 1(4): 156-162.

Kiranmai, M., and Usha Sri, B., Mohammed, I, and Mahendra Kumar, CB. (2012). Antioxidant activity and total flavonoids content of different parts of *Azadirachta indica A. Juss*. Journal of Medicinal Plants Research, 6(46): 5737-5742.

Kiselova, Y., Ivanova, D., Chervenkov, T., Gerova, D., Galunska, B, and Yankova, T. (2006). Correlation between the in vitro antioxidant activity and polyphenol content of aqueous extracts from Bulgarian herbs. Phytotherapy Research, 20(11): 961- 965.

Klier, CM., Kolenbrander, PE., Roble, AG., Marco, ML., Cross, S, and Handley, PS. (1997). Identification of a 95-kDa putative adhesin from Actinomyces serovar WVA963 strain PK1259 that is distinct from type 2 fimbrial subunits. Microbiology, 143: 835–846.

Klimczak, I., Malecka, M., Szlachta, M, and Gliszczynska SA. (2007). Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. Journal of Food Compost, 20: 313-322.

Klinke, T., Guggenheim, B., Klimm, W, and Thurnheer, T. (2011). Dental caries in rats associated with *Candida albicans*. Caries Research, 45: 100-106.

Koo, H., Duarte, S., Murata, RM., Scott, AK., Gregoire, S., Watson, GE., Singh, AP, and Vorsa, V. (2010a). Influence of cranberry proanthocyanidins on formation of biofilms by *Streptococcus mutans* on saliva-coated apatitic surface and on dental caries development *in vivo*. Caries Research, 44: 116–126.

Koo, H., Hayacibara, MF., Schobel, BD., Cury, JA., Rosalen, PL., Park, YK., VaccaSmith, AM, and Bowen, WH. Inhibition of *Streptococcus mutans* biofilm accumulation and polysaccharide production by apigenin and *tt* - farnesol. J Antimicrob Chemother, 52: 782–789.

Lagerlof, F, and Oliveby, A. (1994). Caries-protective factors in saliva. Advances in Dental Research, 8:229-238.

Lakshman, RB. (2012). Phytochemical screening, quantitative estimation, total phenolics and total flavonoids, antimicrobial evaluation of *Cyamopsis tetragonoloba*. International Journal of Research in Pharmaceutical and Biomedical Sciences, 3 (3): 1139-1142.

Lakshman, RB. (2012). Phytochemical Screening, Quantitative Estimation Total Phenolics and Total Flavonoids, Anti-Microbial Evaluation of *Cyamopsis tetragonoloba*. International Journal of Research in Pharmaceutical and Biomedical Sciences, 3(3): 1139-1142.

Lakshmi, PK., Jalal, H, and Shahriar, M. (2000). Aminoglycosides: Perspectives on Mechanisms of Action and Resistance and Strategies to Counter Resistance. Antimicrobial agents and Chemotherapy, 44(12): 3249–3256.

Lakshmikantha, K., Akalanka, Dey., Satyavathi, K, and Bhojaraju3, P. (2014). GC-MS analysis of bio-active compounds in methanolic extract of *Lactuca runcinata* DC. Pharmacognosy Research, 6 (1): 58–61.

Lalla, E., Lamster, IB, and Feit, M. (2000). Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. Journal of Clincal Invest. 105: 1117-1124.

Levy, SB. (1998). The challenge of antibiotic resistance. Scientific American, 278:32-39.

Li, H., Chen, B, and Yao, S. (2005). Application of ultrasonic technique for extracting chlorogenic acid from *Eucommia ulmodies Oliv*. (*E. ulmodies*). Ultrasonics Sonochemistry, 12: 295–300.

Linke, HA, and LeGeros, RZ. (2003). Black tea extract and dental caries formation in hamsters. International Journal of Food Science Nutrition, 54: 89–95.

Liu, RH. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of Action. Journal of Nutrition, 134(12 Suppl):3479S-3485S.

Loe, H. (1993). Periodontal disease: the sixth complication of diabetes mellitus. Diabetes Care, 16: 329-334.

Loesche, WJ, and Syed, SA. (1973). The predominant cultivable flora of carious plaque and carious dentine.Caries Research, 7:201–16.

Loesche, WJ., Schork, A., Terpenning, MS., Chen, YM, and Stoll, J. (1995). Factors which influence levels of selected organisms in saliva of older individuals. Journal of Clinical Microbiology, 33 (10):1-10, 2550-2557.

London, J, and Allen, J. (1990). Purification and characterization of a Bacteroides loescheii adhesin that interacts with prokaryotic and eucaryotic cells. Journal of Bacteriology. 172: 2527–2534.

Long, L, and Edwards, J. (1972). Detailed structure of a dextran from a cariogenic bacterium. Carbohydrate Research, 24: 216–17.

Loo, CY., Mitrakul, K, and Voss, IB. (2003). Involvement of an inducible fructose phosphotransferase operon in *Streptococcus gordonii* biofilm formation. Journal of Bacteriology, 185: 6241–54.

Madhavan, HN, and Bagyalakshmi, R. (2014). Farewell, Chloramphenicol? Is this True? A Review. Research and Reviews: Journal of Microbiology and Biotechnology. 3(1), 85-115.

Madhumitha, G, and Saral, AM. (2011). Preliminary phytochemical analysis, antibacterial, antifungal and anticandidal activities of successive extracts of *Crossandra infundibuliformis*. Asian Pacific Journal of Tropical Medicine. 192-195.

Madziga, HA., Sanni, S, and Sandabe, UK. (2010). Phytochemical and Elemental Analysis of *Acalypha wilkesiana* Leaf. Journal of American Science, 6(11): 510-514.

Mahfuza, K., Ekramul, I., Rafikul, I., Aziz, AR., Khurshid, A., Proma, K, and Mamunur, R., Shahnaj, P. (2013). Estimation of total phenol and *in vitro* antioxidant activity of *Albizia procera* leaves. BMC Research Notes, 6(12): 1-7.

Malecka, M. (2002). Antioxidant properties of the unsaponifiable matter isolated from tomato seeds, oat grains and wheat germ oil. Food Chemistry, 79: 327-332.

Marie, P., Mingeot, L, Youri, G, and Paul, MT. (1999). Aminoglycosides: Activity and Resistance. Antimicrobial agents and Chemotherapy, 43 (4): 727–737.

Maripandi., Arun, KT., Ali, A, and Salamah, A. (2011). Prevalence of dental caries bacterial pathogens and evaluation of inhibitory concentration effect on different tooth pastes against Streptococcus spp. African Journal of Microbiology Research, 5 (14): 1778-1783, 18.

Marsh, PD, and Bradshaw, DJ. (1995). Dental plaque as a biofilm. Journal of Microbiology, 15(3):169-75.

Marsh, PD, and Martin, MV. (2009). Oral Microbiolog, 5th edition. Edinburgh. Churchill Livingstone Elsevier, 2009.

Marsh, PD., Featherstone, A., McKee, AS., Hallsworth, AS., Robinson, C., Weatherell, JA., Newman, HN. and Pitter, AF. (1989) A microbiological study of early caries of approximal surfaces in schoolchildren. Journal of Dental Research, 68:1151-1154.

Martinez, MJA., Lazaro, RM., Del Olmo, LMB, and Benito, P.B. (2008). Anti-infectious activity in the anthemideae tribe. Studies in Natural Products Chemistry, 35: 445-516.

Maryam, G., Seyed, AAS., Haniyeh, E, and Samaneh, G. (2011). Prevalence of *Candida albicans* in dental plaque and caries lesion of early childhood caries (ECC) according to sampling site. Caspian Journal of Internal Medicine, 2(4): 304-308.

Mason, TL, and Wasserman, BP. (1987). Inactivation of red beet betaglucan synthase by native and oxidized phenolic compounds. Phytochemistry, 26:2197–2202.

Masuda, N., Tsutsumi, N., Sobue, S, and Hamada, S. (1979). Longitudinal survey of the distribution of various serotypes of *Streptococcus mutans* in infants. Journal of Clinical Microbiology, 10(4): 497-502.

Matsumura, M., Izumi, T, and Matsumoto, M. (2003). The role of Glucan-binding proteins in the cariogenicity of *Streptococcus mutans*. Microbiology and Immunology, 7(3): 213–15

Matthias, H, and Kurt, H., (1991). Bioactivity in plants: the link between phytochemistry and medicine. 30 (12): 3864-3874

Maurya, R., Singh, G, and Yadav, PP. (2008). Antiosteoporotic agents from Natural sources. Studies in Natural Products Chemistry, 35: 517-545.

Meir, S., Kanner, J., Akiri, B, and Hadas, SP. (1995). Determination and involovement of Aqeous reducing compounds in Oxdative Defense systems of various senescing Leaves. Journal of Agricultural Food Chemistry, 43, 1995, 1813- 1817.

Michael, AK.,, Daniel, J.D, and James, JC. (2010). How antibiotics kill bacteria: from targets to networks. Nature Reviews in Microbiology, 8: 423-435.

Miller, WD. (1973). The microorganisms of the human mouth: the local and general diseases which are caused by them. Basel; New York: S. Karger; 1973. Reprint of the 1890 ed. Published by The S. S. White Dental Mfg. Co., Philadelphia.

Moldovan, R., Oprean., Benedec., Hanganu, D., Duma, M., Onigab, I, and Vlase, L. (2014). LC-MS analysis, antioxidant and antimicrobial activities for five species of menthe cultivated in Romania. Journal of Nanomaterials and Biostructures, 9 (2): 559 – 566.

Montoro, P., Braca, A., Pizza, C, and De Tommasi, N. (2005). Structure-antioxidant activity relationships of flavonoids isolated from different plant species. Food Chemistry, 2005, 92:349–355.

Mroczek, T, and Mazurek, J. (2009). Pressurized liquid extraction and anticholinesterase activitybased thin-layer chromatography with bioautography of Amaryllidaceae alkaloids. Analytica Chimica Acta, 633: 188–196.

Muna, J., Essam, A., Makky, Mashitah, M, and Yusoff. (2015). Impact of Antimicrobial Agents against Oral Isolates. International Conference on Agricultural, Ecological and Medical Sciences, Antimicrobial agents and Chemotherapy, 10-11.

Murali, KT., Rajender, V, and Manoj, K. (2013). *In vitro* Determination of antioxidant activity of *Physalis angulata* Linn. International Journal of Pharma and Biosciences, 4(3): 541 – 549.

Murali, KT., Rajender, V, and Manoj, K. (2013). *In vitro* Determination of Antioxidant Activity of *Physalis angulata lnn,* International Journal of Pharmaceutical and Biosciences, 4(3): 541 – 549.

Murray, PR., Baron, EJ., Jorgensen, JJ., Pfaller, MA, and Yolken, RH. (2003). Manual of Clinical Microbiology, 8th ed. ASM Press: Washington, DC, 1-69.

Muthuswamy, S., Rupasinghe, HPV, and Stratton, GW. (2008). Antimicrobial effect of cinnamon bark extract on *Escherichia coli O157:H7, Listeria innocua* and fresh-cut apple slices. Journal of Food Safety, 28: 534–49.

Nada, HA., Mudallal, A., Essam, FA., Jumaily, A., Nidhal, AAM, and Wahid AAS. (2008). Isolation and identification of Mutans Streptococci bacteria from human dental plaque samples. Journal of Al-Nahrain University, 11(3): 98-105.

Naima, SL., Muhammad, RK, and Maria, S. (2012). Antioxidant Activity, Total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla*. BMC Complementary and Alternative Medicine, 221:1-12.

Nakano, K., Tsuji, M, and Nishimura, K. (2006). Contribution of cell-surface protein antigen PAc of *Streptococcus mutans* to bacteremia. Microbes Infection, 8(1): 114–21.

Nalina, T, and Rahim, ZHA. (2007). The crude aqueous extracts of *Piper betle L* and its antibacterial effect towards *Streptococcus mutans*. American Journal of Biochemistry and Biotechnology, 3: 10-5.

Nantiya J., Sonesay, T., Surasak, L., Sirilux, H., Supayang, P., Voravuthikunchai., Boon Y., Sukanya, D., Sanan, and Subhadhirasaku. (2013). Antibacterial substances from *Albizia myriophylla* wood against cariogenic *Streptococcus mutans.* Archives of Pharmaceutical Research, 36:723–730.

Nascimento, MM., Lemos, JAC, and Abranches, J. (2004). Adaptive acid tolerance response of *Streptococcus sobrinus.* Journal of Bacteriology, 186(19): 6383–90.

Nathalya, IM., Carlos, EC., Letícia, F., Wilson, RC., Rodrigo, CS., Veneziani, C., Henrique, GM., Antonio, E, and Miller, C.(2015) Antimicrobial activity of the essential oil of *Tetradenia riparia* (Hochst.) Codd. (Lamiaceae) against cariogenic bacteria. Brazilian Journal of Microbiology, 46, (2):519-525.

Naveen, G., Santosh, S., Anisha, M., Shailja, Kundra, and Aparna, Y. (2009). Dental caries, gingivitis, periodontitis: a review. Reviews in Clinical medical Biology, 8(1): 75-84.

Nazzaro, M., Mottola, MV., Cara, FL., Monaco, GD., Aquino, RP, and Volpe, MG. (2012). Extraction and characterization of biomolecules from agricultural wastes. Chemical Engineering Transactions, 27: 331-336.

Nelio, V., Daniela, A., Filipa, D., Margarida, P., Ana, V., Liliana, R., Mariana, Silva., Vanessa, M., Francisco, Pereira., Beatriz, V., Joao, Plaza, and Filipa, B. Dental Caries: A Review. Journal of Dental and Oral Health, 2(5): 1-3.

Newman, DJ, and Cragg, GM. (2007). Natural products as sources of new drugs over the last 25 years. Journal of Natural Products, 70: 461–477.

Nickavar, B., Kamalinejad, M, and Izadpanah, H. (2007). *In vitro* free radical scavenging activity of five Salvia species. Pakistan Journal of Pharmaceutical Science, 20(4): 291- 294.

Nicoue, EE., Savard, S, and Belkacemi, K. (2007). Anthocyanins in wild blueberries of Quebec: extraction and identification. Journal of Agriultural and Food Chemistry, 55: 5626-5635.

Nikawa, H., Egusa, H., Makihira, S., Nishimura, M., Ishida, K., Furukawa, M, and Hamada, T. (2003). A novel technique to evaluate the adhesion of *Candida* species to gingival epithelial cells. Mycoses, 46 (10), 384-389.

Nikitina, VS., Kuz'mina, YL., Melent'ev, AI, and Shendel, GV. (2007). Antibacterial activity of polyphenolic compounds isolated from plants of Geraniaceae and Rosaceae families. Applied Biochemistry and Microbiology. 43: 629-634.

Nimmi, OS, and Philomena, G. (2012). Evaluation of the antioxidant potential of a newly developed polyherbal formulation for antiobesity. International Journal of Pharmacy and Pharmaceutical Sciences, 4(3): 505-510.

Nimmi, OS, and Philomena, G. (2012). Evaluation of the Antioxidant Potential of a Newly Developed Polyherbal Formulation for Antiobesity. International Journal of Pharmacy and Pharmaceutical Sciences, 4(3): 505-510.

Nishimura, F., Takahashi, K, and Kurihara, M. (1998). Periodontal disease as a complication of diabetes mellitus. Annals of Periodontology, 3: 25-32

Nobbs, AH., Lamont, RJ, and Jenkinson, HF. (2009). Streptococcus adherence and colonization. Microbiology and Molecular Biology Review, 73: 407-415.

Nunes, PX., Silva, SF., Guedes, RJ, and Almeida, S. (2012). Biological oxidations and antioxidant activity of natural products, Phytochemicals as nutraceuticals- Global Approaches to Their Role in Nutrition and Health.

Nweze, EL., Okafor, JL, and Njoku, O. (2004). Antimicrobial Activityies of Methanolic extracts of *Trume guineesis* (Scchumn and Thorn) and *Morinda lucinda* used in Nigerian Herbal Medicinal practice. Journal of Biological Research and Biotechnology, 2(1): 34-46.

Offenbacher, S., Katz, V, and Fertik, G., (1996). Periodontal infection as a possible risk factor for preterm low birth weight. Journal of Periodontology, 67(10): 1103-1113.

Okada, M., Soda, Y, and Hayashi, F. (2005). Longitudinal studies on dental caries incidence associated with *Streptococcus mutans* and *Streptococcus sobrinus* in pre-school children. Journal of Medical Microbiology, 54: 661–65.

Oktay, M., Gulcin, I, and Kufrevioglu, OI. (2003). Determination of *in-vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. Lebensum.Wiss. U.Technol. 36:263-271.

Olajokun, HRE., Folarin, AA., Olaniran, O, and Umo, AN. (2008). The prevalent bacterial isolates of dental caries in school age children attending the Dental Clinic of Oauthc. African Journal of Clinical and Experimental Microbiology. 9: 103-108.

Oleg, J. (1963). Studies on the Mechanism of Action of Chloramphenicol: the conformation of Chloramphenicol in Solution. The Journal of Biological Chemistry, 238(7): 65-71.

Ooshima, T., Minami, T., Aono, W., Izumitani, A., Sobue, S., Fujiwara, T., Kawabata, S, and Hamada, S. (1993). Oolong tea polyphenols inhibit experimental dental caries in SPF rats infected with mutans streptococci. Caries Research, 27:124-129.

Ooshima, T., Osaka, Y., Sasaki, H., Osawa, K., Yasuda, H, and Matsumura, M., (2000) Caries inhibitory activity of cacao bean husk extract in *in-vitro* and animal experiments. Archives in Oral Biology, 45: 639– 45.

Osawa, K., Yasuda, H., Maruyama, T., Morita, H., Takeya, K, and Itokawa, H. (1992). Isoflavanones from the heartwood of *Swartzia polyphylla* and their antibacterial activity against cariogenic bacteria. Chemical Pharma Bulletin, 40: 2970-2974.

Osbourn, AE. (1996). Preformed antimicrobial compounds and plant defense against fungal attack. Plant Cell, 10: 1821-1831.

Ozkan, G., Sagdic, O., Baydar, NG, and Kurumahmutoglu, Z. (2004). Antibacterial activities and total phenolic contents of grape pomace extracts. Journal of the Science of Food and Agriculture. 84: 1807-1811.

Pai, MR., Acharya, LD, and Udupa, N. (2004). Evaluation of antiplaque activity of *Azadirachta indica* leaf extract gel: a 6-week clinical study. Journal of Ethnopharmacol, 90: 99–103.

Pan, X., Niu, G, and Liu, H. (2003). Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. Chemical Engineering and Processing, 42: 129–133.

Papas, AS., Joshi, A., Donald, MSL., Splagounias, ML, Spanedda, PP, and Curro, FA. (1993). Caries prevalence in xerostomic individuals. Canadian Dental Association, 59:171-179.

Paquette, DW. (2004). The periodontal-cardiovascular link. Compend Contin Educ Dent. 25: 681-692.

Park, KM., You, JS., Lee, HY., Baek, NL, and Hwang, JK. (2003). Kuwanon G: an antibacterial agent from the root bark of *Morus alba* against oral pathogens. Journal of Ethnopharmacology. 84(2): 181–185.

Parolo, CC, and Maltz, M. (2006). Microbial contamination of noncavitated caries lesions: a scanning electron microscopic study. Caries Research, 40 (6): 536-541.

Patra, JK., Kim, ES., Kyounghee, O., Kim, HJ., Kim, Y, and Baek, H. (2014). Antibacterial effect of crude extract and metabolites of *Phytolacca americana* on pathogens responsible for periodontal inflammatory diseases and dental caries. BMC Complementary and Alternative Medicine, 14: 1-6.

Payne, GF., Bringi, V., Prince, C, and Shuler, ML. (1991). Plant Cell and Tissue Culture in Liquid Systems, Hanser Publishers.

Pereira AC. (1995): Cáries Dentarias – Etiologia e Prevencao. Lisboa: Edicao Medisa.

Petersen, PE. (2003). "The World Oral Health Report 2003: continuous improvement of oral health in the 21st century— the approach of the WHO Global Oral Health Programme". Community Dentistry and Oral Epidemiology, 31: 3–10.

Petersen, PE., Bourgeois, D., Ogawa, H., Estupinan-Day, S, and Ndiaye, C. (2005). "The global burden of oral diseases and risks to oral health". Bulletin of theWorld Health Organization, 83 (9): 661–669.

Prabhat, A., Navneet, and Avnish, C. (2010). Evaluation of Antimicrobial Activity of Six Medicinal Plants against Dental Pathogens. Report and Opinion, 2(6): 37-42.

Prashith Kekuda, TR., Raghavendra, HL., Mallikarjun, N., Swathi, D., Suchitha, Y., Anil Kumar, HS, and Vinayaka, KS. (2014). Elemental Analysis and Biological Activities of *Chrysophyllum roxburghii G. Don* (Sapotaceae) Leaves. Science, Technology and Arts Research Journal, 3(1): 14-20.

Puupponen, PR., Nohynek, L., Alakomi, H, and Oksman, CK. (2008). The action of berry phenolics against human intestinal pathogens. Biofactors, 23(4): 243-251.

Rachael, C., Seethalakshmi, M., Sankoli., Shubharani, R., Sivaram, V., Ganti SM, and Ruben MC. (2013). Evaluation of phytochemicals and in vitro antioxidant studies of *Toddalia asiatica* leaf. Medicinal Plants, 5(4): 1-4.

Raghu, CH., Ramamurthy, S., Godavarthi, A., Santoshkumar, HD., Hitesh, VJ, and Pottekkad, V. (2014). Investigation of the Antioxidant and Hepatoprotective Potential of *Hypericum mysorense*. Antioxidants, 3: 526-543.

Rahim, ZH, and Khan, HB. (2006). Comparative studies on the effect of crude aqueous (CA) and solvent (CM) extracts of clove on the cariogenic properties of *Streptococcus mutans*. Journal Oral Science, 48: 117–23.

Raja, M., Hannan, A, and Ali, K. (2010). Association of oral candidal carriage with dental caries in children. Caries Research, 44: 272-276.

Rasooli, I., Shayegh, S., Taghizadeh, M, and Astaneh, SD. (2008). Phytotherapeutic prevention of dental biofilm formation. Phytotherapy Research, 22: 1162–1167.

Rawee, T., Pajaree, K.,, Nuntiya, P, and Jindaporn, P. (2016). Screening for antibacterial and antibiofilm activity in Thai medicinal plant extracts against oral microorganisms. Journal of Traditional and Complementary Medicine, 1-6.

Razak, FA., Othman, RY, and Rahim, ZH. (2006). The effect of *Piper betle* and *Psidium guajava* extracts on the cell-surface hydrophobicity of selected early settlers of dental plaque. Journal of oral science, 48(2): 71–75.

Revilla, E., Ryan, JM, and Martin, OG. (1998). Comparison of several procedures used for the extraction of anthocyanins from red grapes. Journal of Agricultural and Food Chemistry, 46: 4592-4597.

Rice Evans, CA., Miller, NJ, and Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radicals, 20: 933-956.

Rice Evans, CA., Miller, NJ., Bolwell, PG., Bramley, PM, and Pridham, JB. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radicals Research, 22(4): 375-383.

Richa R. (2012).Antimicrobial Susceptibility of Viridans Group Streptococci Isolated from Blood of Hospitalized Patients. The Bioscan, 7(2): 302-307, 2012.

Ridker, PM., Hennekens, CH., Buring, JE, and Rifai, N. (2000). C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. New English Journal of Medicine, 342: 836-843.

Rìos, JL, and Recio, MC. (2005). Medicinal plants and antimicrobial activity. Journal of Ethnorpharmacology, 80-84.

Riva, TD, and Corvan, L. (2003). Sugars and dental caries. American Journal of Clincal Nutrition, 78: 881–892.

Rogosa, M., Mitchell, JA, and Wiseman, RF. (1951). A selective medium for the isolation and enumeration of oral lactobacilli. Journal of Dental Research. 30 (5): 682-689.

Roland, L, and Patrice, C. (2002). Resistance to Macrolides and Related Antibiotics in *Streptococcus pneumonia*. Antimicrobial agents and Chemotherapy, 2727–2734.

Rosella, CC., Gabriela, UU., Dyanne, MF., Stefany, CG., Frank, MT, and Juana, VM. (2016). Antibacterial activity of *Myrciaria dubia* (Camu camu) against *Streptococcus mutans and Streptococcus sanguinis*. Asian Pacific Journal of Tropical Biomedicine, 6(9): 740–744.

Ruchi P., Sumitra, C., Rajashekhar, I, and Meonis, P. (2015). Ascertaining Anti-Inflammatory and Analgesic Activity in Aqueous Leaf Extract of *Anisomeles indica* (L.) Kuntze. Advances in Bioresearch, 6 (1): 1-5.

Rukayadi, YY., Kim, KH, and Hwang, JK. (2008). *In vitro* anti-biofilm activity of macelignan isolated from *Myristica fragrans Houtt.* against oral primary colonizer bacteria. Phytotherapy Research, 22 (3): 308–312.

Russell, MW, and Lehner, T. (1978). Characterization of antigens extracted from cells and culture fluids of *Streptococcus mutans* serotype C. Archives of Oral Biology, 23(1): 7–15.

Ryan, ME., Carnu, O, and Kamer, A. (2003). The influence of diabetes on the periodontal tissues. American Dental Association, 134: 34S-40S.

Sadangi, N., Padhy, RN, and Sahu, RK. (2005). A contribution to medico-ethnobotany of Kalahandi district, Orissa on ear and mouth disease. Ancient Science of Life, 160-63.

Sadao, M., Hiroshi, O., Yoshiyuki, N., Yoji, N, and Michinori, T. (1977). Resistance Mechanism of Chloramphenicol in *Streptococcus haemolyticus*, *Streptococcus pneumoniae* and *Streptococcus faecalis*. Microbiology and Immunology. 21(2): 69-76.

Saeeda, A., Halima, SQ, and Anser, M. ().Dental caries status in 6-9 years old children. Pakistan Oral & Dental Journal, 28(1) : 107-111.

Saeki, Y., Kato, T., Naito, Y., Takazoe, I, and Okuda, K. (1996). Inhibitory effects of funoran on the adherence and colonization of mutans streptococci. Caries Research. 30: 119–125.

Sakanaka, S., Kim, M., Taniguchi, M, and Yamamoto, T. (1989). Antibacterial substances in Japanese green tea extract against *Streptococcus mutans,* a cariogenic bacterium. Agricultural Bioloical Chemistry, 53: 2307–2311.

Saldana, MDA., Mohamed, RS., Baer, MG, and Mazzafera, P. (1999). Extraction of purine alkaloids from mate (*Ilex paraguariensis*) using supercritical CO_2. Journal of Agricultural and Food Chemistry. 47: 3804–3808.

Salete, MF., Bersan., Livia, CC., Galvão., Vivian, FF., Goes., Adilson, S., Glyn, M., Vera, LGR., Severino, MA, Renata, MTD., Pedro, LR, and Marta, CTD. (2014). Action of essential oils from Brazilian native and exotic medicinal species on oral biofilms. BMC Complementary and Alternative Medicine, 14:451:1-9.

Salvi, GE., Yalda, B, and Collins, JD. (1997). Inflammatory mediator response as a potential risk marker for periodontal diseases in insulin-dependent diabetes mellitus patients. Journal of Periodontology, 68: 127-135.

Sandhya Rani, M., Pippalla, RS., Krishna, MG, and Gangaraju, M. (2012). Anti-diabetic activity of methanolic and ethyl acetate extracts of W*rightia tinctoria* r.br. Fruit. International Journal of Pharmaceutical Science and Research, 3(10): 3861-3866.

Sanni, O., Obidoa O, and Omale J. (2014). Toxicity, anti-lipid peroxidation, *in-vitro* and *in-vivo* evaluation of antioxidant activity of *Annona muricata* ethanol stem bark extract. American Journal of Life Sciences, 2(5): 271-277.

Sarabjot, Kaur, and Poonam, M. (2014). Study of Total Phenolic and Flavonoid Content, Antioxidant Activity and Antimicrobial Properties of Medicinal Plants. Journal of Microbiology & Experimentation, 1(1): 1-6.

Sarker, SD, and Nahar, L. (2007). Chemistry for Pharmacy Students General, Organic and Natural Product Chemistry. England: John Wiley and Sons, 283-359.

Sasaki, H., Matsumoto, M, and Tanaka, T. (2004). Antibacterial activity of polyphenol components in oolong tea extract against *Streptococcus mutans*. Caries Research, 38(1): 2–8.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, KM, and Latha, LY. (2011). Extraction, isolation and characterization of bioactive compounds from plant's extracts. African Journal of Traditional, Complementary and Aternative Medicine, 8: 1-10.

Sato, M., Fujiwara, S, and Tsuchiya, H. (1996). Flavones with antibacterial activity against cariogenic bacteria. Journal of Ethnopharmacology, 54(2): 171–176.

Sato, M., Tanaka, H., Fujiwara, S., Hirata, M., Yamaguchi, R., Etoh, H, and Tokuda, C. (2003). Antibacterial property of isoflavonoids isolated from *Erythrina variegata* against cariogenic oral bacteria. Phytomedicine, 10: 427-433.

Scannapieco, FA., Bush, RB, and Paju, S. (2003). Periodontal disease as a risk factor for adverse pregnancy outcomes: a systematic review. Annals of Periodontology, 8: 70-78.

Schmidt, S., Niklova, I., Pokorny, J., Farkaš, P, and Sekretar, S. (2003). Antioxidant activity of evening primrose phenolics in sunflower and rapeseed oils. *European* Journal of Lipid Science and Technology. 105: 427-435.

Seiji, O., Tetsuro, M, and Tetsuro, M. (2005). Mechanisms of Resistance to Imipenem and Ampicillin in *Enterococcus faecalis*. Antimicrobial agents and Chemotherapy, 2954–2958.

Selwitz, RH., Isamail, AI, and Pitts, NB. (2007). Dental Caries. Lancet, 369 (9555): 51- 59.

Serkedjieva, J, and Manolova, N. (1992). Anti-influenza virus effect of some propolis constituents and their analogues (esters of substituted cinnamic acids). Journal of Natural Products, 55: 294-297.

Shahidi, F., and Wanasundara, PK. (1992). Phenolic antioxidants. Critical Reviews in food science and Nutrition, 32: 67- 103.

Shaik, AT, and Balakumar, BS. (2014). Studies on Phytochemical and Antioxidant potential of certain medicinal plants from Udayagiri Hill Range, Andhra Pradesh, India. Scholars Academic Journal of Biosciences, 2(7): 432-436.

Sharon, N, and Ofek, I. (2002). Fighting infectious diseases with inhibitors of microbial adhesion to host tissues. Critical Reviews in Food Science and Nutrition, 42:267-72.

Shazi, S., Rosina, K., Raffaele, Z, and Asad, UK. (2008). Aminoglycosides versus bacteria – a description of the action, resistance mechanism, and nosocomial battleground. Journal of Biomedical Science, 15: 5–14.

Shimada, T. (2006): Salivary Proteins as a Defence against Dietary Tannins. Journal of Chemistry and Ecology. 32(6): 1149-1163.

Shklair, IL, and Keene, HJ. (1974). A biochemical scheme for the separation of type five varieties of *Streptococcus mutans*. Archives of Oral Biology. 19(11):1079-81.

Shouji, N., Takada, K., Fukushima, K, and Hirasawa, M. (2000). Anticaries effect of a component from shiitake (an edible mushroom). Caries Research, 34: 94–98.

Shrikhande, AJ. (2000). Wine byproducts with health benefits. Food Research International, 33: 469–474.

Shukla, M., Singh, U., Singh, P, and Singh, RL. (2009). Nutraceutical properties of agrowaste part of some citrus plants. Journal of Ecophysiology and Occupational Health, 9: 97-103.

Shuvho, CB, Khandakar, MBH., Mortuza, MA., Khadiza, B., Tanvir, A., Shirajum, MM, and Alimul, MI. (2015). Isolation, identification and antibiogram profile of bacteria isolated from dental caries patients of Mymensingh district of Bangladesh. Asian Journal of Medical and Biological Research, 1 (2): 244-253.

Shyla, J., Jayasurya,, K., Emmanuel, S., Sathish, and Devapriya, D. (2011). Antimicrobial Activity of Few Medicinal Plants against Clinically Isolated Human Cariogenic Pathogens—An *In Vitro* Study. International Scholarly Research Network (ISRN) Dentistry, 1-6.

Silva, NCC, and Fernandes, JA. (2010). Biological properties of medicinal plants: a review of their antimicrobial activity. The Journal of Venomous Animals and Toxins including Tropical Diseases, 16 (3): 402-413.

Singh, J., Kumar, A., Budhiraja, S, and Hooda, A. (2007). Ethnomedicine: Use in dental caries. Brazillian Journal of Oral Science, 6:21.

Siqueira, JJF., and Sen, BH. (2004). Fungi in endodontic infections. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics, 97(5): 632-641.

Smolinski, MS., Hamburg, MA, and Lederberg, J. (2003). Microbial threats to health: Emergence, detection, and response. Washington, DC: Institute of Medicine, National Academies Press. 203-210.

Solomon, WGO., Ugoh, SC, and Mohammed, B. (2014). Phytochemical Screening and Antimicrobial activities of *Annona muricata (L)* leaf extract. American Journal of Biological, Chemical and Pharmaceutical Sciences, 2 (1): 1-7.

Sonia, S., Simran, K., Airambano, K, and Nanda, H. (2015). Evaluation of antimicrobial and antioxidant Activity of leaf extracts of medicinal plants. World Journal of Pharmacy And Pharmaceutical Sciences, 4(5): 1403-1413.

Soong, YY, and Barlow, PJ. (2004). Antioxidant activity and phenolic content of selected fruit seeds. Food Chemistry, 88: 411–417.

Spak, CJ., Johnson, G, and Ekstrand, J. (1994). Caries incidence, salivary flow rate and efficacy of fluoride gel treatment in irradiated patients. Caries Research, 28:388-393.

Sreeja, R., Minal, C, and Madhuri, G. (2013). Enumeration of Salivary Streptococci and Lactobacilli in Children with Differing Caries Experiences in a Rural Indian Population. ISRN Plastic Surgery, 1- 6.

Steinberg, D., Feldman, M., Ofek, I, and Weiss, EI. (2004). Effect of a high-molecular-weight component of cranberry on constituents of dental biofilm. Journal of Antimicrobial Chemotherapy, 54: 86–89.

Sundararaj T. (2003). Microbiology Laboratory Manual. University of Madras, 48-117.

Svensater, G., Sjogreen, B, and Hamilton, IR. (2000). Multiple stress responses in *S. mutans* and the induction of general and stress-specifi c proteins. Microbiology, 146: 107–17.

Syed J., Dileep, N., Rakesh, KN., Prashith Kekuda, TR, and Raghavendra, HL. (2013). Contents of total phenolics and flavonoids, radical scavenging and anticaries activity of leaf and seed extract of *Anisomeles indica Linn*. International Journal of Drug Development and Research, 5(4): 286-292.

Syed, SA, and Loesche, WJ. (1972). Survival of human dental plaque flora in various transport media. Applied Microbiology, 24 (4): 638-44.

Sziegoleit, F., Sziegoleit, A, and Wetzel, WE. (1999). Effect of dental treatment and/or local application of amphotericin B to carious teeth on oral colonization by Candida. Medical Mycology, 37: 345-50.

Takahashi, N, and Yamada, T. (1996). Catabolic pathway for aerobic degradation of lactate by *Actinomyces naeslundii.* Oral Microbiology and Immunology, 11:193-198.

Takahashi, N, and Yamada, T. (1999). Glucose and lactate metabolism by *Actinomyces naeslundii.* Critical Review in oral biology and medicine, 10: 504-518.

Takahashi, N., Kalfas, S, and Yamada, T. (1995). Phosphorylating enzymes involved in glucose fermentation of *Actinomyces naeslundii.* Journal of bacteriology, 177:5806-5811.

Takarada, K., Kimizuka, R., Takahashi, N., Honma, K., Okuda, K, and Kato, T. A comparison of the antibacterial efficacies of essential oils against oral pathogens. Oral Microbiology and Immunology, 19: 61–64.

Tanzer, JM., Krichevsky, MI, and Keyes, PH. (1963). The metabolic fate of glucose catabolised by a washed stationary phase caries-conducive *Streptococcus.* Caries Research, 3: 167–77.

Tanzer, JM., Livingston, J, and Thompson, AM. (2001). The microbiology of primary dental caries in humans. Journal of Dental Education, 65(10):1028-1037.

Tardrew, PL., Mao, JCH, and Kenney, D. (1969). Antibacterial Activity of 2'-Esters of Erythromycin. Applied Microbiology, 159-165.

Thitilertdecha, N., and Teerawutgulrag, A., Kilburn, JD, and Rakariyatham, N. (2010). Identification of major phenolic compounds from *Nephelium lappaceum l.* and their antioxidant activities. Molecules, 15: 1453-1465.

Torres, JL, and Bobet, R. (2001). New flavanol derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. Journal of Agricultural and Food Chemistry, 49: 4627–4634.

Touyz, LZ, and Amsel, R. (2001). Anticariogenic effects of black tea *(Camellia sinensis)* in caries-prone rats. Quintessence International, 32: 647–650.

Tsuchiya, H., Sato, M., Iinuma. (1994). Inhibition of the growth of cariogenic bacteria *in vitro* by plant flavanones. Experientia, 50(9): 846–849.

Tsui, VWK., Wong, RWK, and Rabie, ABM. (2008). The inhibitory effects of naringin on the growth of periodontal pathogens *in vitro.* Phytotherapy Research, 22(3): 401–406.

Tuchila, C., Jianu, I., Rujescu, CI., Butur, M., Ahmadi, KM, and Negrea, I. (2008). Evaluation of the antimicrobial activity of some plant extracts used as food additives. Journal of Food Agriculture and Environment, 6: 68–70.

Ulhe, SK, and Narkhede, SD. (2013). Histological and Phytochemical studies on aromatic plant, *Anisomeles indica* (L.) of family Lamiaceae (M.S.) India. International Journal of Life Science, 1(4): 270-272.

Van Acker, SA., Van Den Berg, DJ, Tromp, MN., Griffioen, DH.,Van Bennekom, WP., Van der Vijgh, and WJ., Bast, A. (1996). Structural aspects of antioxidant activity of flavanoids. Free Radicals. Journal of Biology Medicine, 20 (3): 331-342.

Van den Bogaard, AE, and Stobberingh, EE. (2000). Epidemiology of resistance to antibiotics: Links between animals and humans. International Journal of Antimicrobial Agents, 14:327-335.

Van der Reijden, WA., van der Kwaak, JS., Veerman, ECI, and Nieuw, AAV. (1996). Analysis of the concentration and output of whole salivary constituents in patients with Sjogren's syndrome. European Journal of Oral Science, 104:335-340.

Van Houte, J., de Moor, C, and Jansen, H. (1970). Synthesis of iodophilic polysaccharide by human oral streptococci. Archives of Oral Biology, 15: 263–66.

Van Wyk, BE, and Wink, M. (2004). Medicinal plants of the world: An Illustrated Scientific Guide to Important Medicinal Plants and Their Uses. Timber Press: Portland, OR, USA: 480.

Van Wyk, and Michael, W. (2004). Medicinal Plants of the World: An Illustrated Scientific Guide to Important Medicinal Plants and Their Uses, Hardcover – Import, 15 Feb 2004.

Velioglu, YS., Mazza, G., Gao, L, and Oomah, BD. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products, Journal of Agricultural Food Chemistry, 46: 4113-17.

Venugopal, TM., Mallikarjun, N., Somasundar, K., Nagaraj, K, and and Bhargavi, P. (2016). Antioxidant and Antibacterial activity of Methanol Extract of *Malphigia glabra* against viridans Group of Streptococci involved in Dental Caries. Advances in Bioressearch, 7(1): 173-180.

Venugopal, TM., Swathi, D., Suchitha, Y., Prashith Kekuda, TR., Mallikarjun, N., Soundarya, S., Eyasu, E, and Raghavendra, HL. (2012). Mineral Composition, Cytotoxic and Anticariogenic Activity of *Scleropyrum pentandrum (Dennst.) Mabb*. International Journal of Drug Development and Research, 4(1).

Vercauteren, J. (2009). Compositions of stilbenic polyphenolic derivatives, their preparation, and their use in the treatment of disease and aging. Fr. Demande.

Vijay Laxmi, AK., Bhatia., Anjana, G., Nitin, W, and Anubhuti, S. (2015). Phytochemicals Screening and Analysis using HPLC to determine the Antimicrobial efficacy of *Cassia fistula* extract. Advances in Bioresearch, 6(3):21-25.

Vijayameena, G., Subhashini, M., Loganayagi, and Ramesh, B. (2013). Phytochemical screening and assessment of antibacterial activity for the bioactive compounds in *Annona muricata*. International Journal of Current Microbiology and Applied Sciences, 2(1): 1-8.

Voravuthikunchai, SP, and Kitpipit, L. (2003). Activities of crude extracts of Thai medicinal plants on methicillin- resistant *Staphylococcus aureus*. Journal of Clinical Microbiology and Infection, 9: 236.

Wen, D., Liu, Y., Li, W, and Liu, H. (2004). Separation methods for antibacterial and antirheumatism agents in plant medicines. Journal of Chromatography, 812: 101–117.

Wescombe, PA., Heng, NC., Burton, JP., Chilcott, CN, and Tagg, JR. (2009). Streptococcal bacteriocins and the case for *Streptococcus salivarius* as model oral probiotics. Future Microbiol, 4: 819– 835.

Whittaker, CJ., Klier, CM, and Kolenbrander, PE. (1996). Mechanisms of adhesion by oral bacteria, Annu. Rev. Microbiol. 50: 513–552.

Winslow, LC, and Kroll DJ. (1998). Herbs as medicines. Archives of International Medicine, 158(20): 2192–2199.

Wiseman, H, and Halliwell, B. (1996). Damage to DNA by reactive oxygen and nitrogen species: Role of inflammatory disease and progression to cancer. Journal of Biochemistry. 313: 17–29.

Wojdylo, A., Oszmianski, J, and Czemerys, R. (2007). Antioxidant activity and phenolic compounds in 32 selected herbs, Food Chemistry, 105 (3): 940-949.

Wolfe, KL, and Liu, RH. (2003). Apple peels as a value-added food ingredient. Journal of Agricultural and Food Chemistry, 51: 1676–1683.

Wolinsky, LE., Mania, S., Nachnani, S, and Ling, S. (1996). The inhibiting effect of aqueous *Azadirachta indica* (neem) extract upon bacterial properties influencing *in vitro* plaque formation, Journal of Dental Research, 75: 816–822.

Yamanaka, A., Kimizuka, R., Kato, T, and Okuda, K. (2004). Inhibitory effects of cranberry juice on attachment of oral streptococci and biofilm formation. Oral Microbiology and Immunology, 19: 150– 154.

Yang, J.H., Lin, H.C, and Mau, JL. (2002). Antioxidant properties of several commercial Mushrooms. Food Chemistry, 77: 229-235.

Yanti., Rukayadi, Y., Kim, KH, and Hwang, JK. (2008). *In vitro* anti-biofilm activity of macelignan isolated from *Myristica fragrans Houtt.* against oral primary colonizer bacteria. Phytotherapy Research, 22: 308-312.

Yong, Z., Tong, Li., Xiong ,Fu., Margaret, B., Arshad, MA., Bisheng, Z, and Rui HL. The use of an enzymatic extraction procedure for the enhancement of highland barley (*Hordeum vulgare* L.) phenolic and antioxidant compound. International Journal of Food Science & Technology, 51: 1916–1924.

Yoo, SO., Park, SJ., Jeong, DK., Kim, K., Lim, S., Lee, S., Choe, S., Chang, Y, and Park, I., Kook, J. (2007). Isolation and Characterization of the *Mutans Streptococci* from the Dental Plaques in Koreans. The Journal of Microbiology, 45(3): 246-255.

Zhang, L., Kong, Y., Wu, D., Zhang, H., Wu, J., Chen, J., Ding, J., Hu, L., Jiang, H, and Shen, X. (2008). Three flavonoids targeting the β- hydroxyacyl-acyl carrier protein dehydratase from *Helicobacter pylori:* Crystal structure characterization with enzymatic inhibition assay. Protein Science, 17:1971-1978.

Zulema, P., Almudena, M., Maria, JS, and Miguel, P. (2016). Ultrasound-Assisted Extraction of Stilbenes from Grape Canes. Molecules, 21(6): 784-791.