Anti-cariogenic effect of *Vaccinium macrocarpon* against causative organisms of dental Caries

VISHALI.A

17PBT020

A Thesis submitted to Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore – 641 043

In Partial Fulfillment of the Requirement for the Degree of

Master of Science in Biotechnology

April 2019

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Signature of the

Head of the department

25/4/19

Signature of the

Supervisor

Acknowledgement

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Introduction

1.0 INTRODUCTION

Dental caries, normally referred to as cavities, may be a common dental drawback in the world. Cavity may be a chronic dental illness that damages the hard tissue of teeth and happens due to building up of plaque on teeth surfaces formed by acid-producing microorganism from fermentable carbohydrates. Microorganism interact with carbohydrates which will be fermented after long duration, forming acids thereby lowering pH below essential and leading to demineralization of arduous tissue of teeth (Aneja *et al*., 2015).

Microorganisms related to caries are *Streptococcus mutans, Streptococcus sobrinus, Lactobacillus acidophilus, Staphylococcus aureus, Pseudomonas aeuroginosa, Klebsiella pneumoniae*. These organisms cause plaque *shaped on* the tooth. The prevalence of low pH, lack of fluorine causes initiation of dental caries. The absence of a diet containing the consumption of fruits and vegetables and a high intake of sugared product will simply initiate cavity development. The microorganisms need fermentation of carbohydrates to form a low pH environment to achieve demineralization (Jurikova *et al*.,2019).

Dental caries is caused by several factors, like host factors (teeth and saliva), food substances, microorganisms, and time. Renowned tooth decay microorganisms are *Streptococci* and *Lactobacillus* species(Shaik and Shete,2018). The foremost common cause of cavity is *Streptococcus mutans*. *S. mutans* acts as an initiator of cavity, whereas *Lactobacillus* sp, contributes to the developmental process and also the continuation of tooth decay. However, it is recently been reported that *Veillonella, Bifidobacterium, Propionibacterium, Eubacteria sp*., and *Atopobium sp*. microorganism also play a very important role in the development and continuation of cavity (Mutmainnah and Baktir,2019)

Dental caries may be a quite common problem that affects all age groups. It is a method during which the enamel and also the first statentine are demineralised by acids created by microorganism fermentation of carbohydrates (Gartika *et al*.,2014).It is the foremost common communicable disease affecting human beings.

Streptococcus mutans is generally regarded as a primary microbial agent in the pathogenesis of dental caries although additional acidogenic microorganisms may be involved (Basting, *et al*.,2016). *S. mutans* is an anaerobic bacterium known to produce lactic acid as part of its metabolism. *S. mutans* then binds to tooth surfaces in the presence of

sucrose by the formation of water-insoluble glucans, a polysaccharide that aids in binding the bacterium to the tooth(Waters *et al*.,2014). This bacterium synthesizes extracellular glucans from sucrose using glucosyltransferases (GTFs). Glucans promote the accumulation of cariogenic *streptococci* (and other oral microorganisms) on the tooth surface, and are critical for the formation and structural integrity of biofilms (Philip *et al*., 2019)

Serum concentrations of fluoride and silver on topical application revealed no potential toxicity over the teeth. Silver ions are assumed to be primarily responsible for the antimicrobial action of silver diamine fluoride. Silver ions inhibit the growth of all tested oral bacteria, and denature enzymes that would breakdown collagenous dentin. *Streptococcus mutans*, a primary pathogen in dental caries, is less able to form a biofilm on teeth treated *ex vivo*(Pitts *et al*.,2017). Fluoride promotes deposition of fluoroapatite, which is more resistant to acidic degradation than normal tooth structure. But on the treatment with the silver diamine fluoride was described to have a non-sore, non-irritated spot at the corner of the mouth that "looked like a burn", which is a typical description of the silver precipitation that occurs upon contact with the skin (Nandakumar and Nasim,2018).To reduce the effects of silver fluoride on the teeth or mouth surface alternative source such as natural herbs can be used.

Numerous *in vitro* studies have been performed to investigate the activity of natural plant substances against oral bacteria known to be involved in the etiologic of oral and dental diseases (Casarin *et al*., 2018).India has been traditionally a country known for its treasure of herbs. Plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being (Hu *et al.*, 2015). Recently, herbal medicines have progressively been used to treat several diseases together with many infections. There exists vast literature on the antiviral, anticariogenic, anthelmintic, antibacterial, antifungal, anti-inflammatory and antimolluscal properties of different plants parts (Caufield *et al*., 2015). They are used as remedies for many infectious diseases.Searches for substances with antimicrobial activity in plants are common(Karygianni *et al*., 2016). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Oza *et al*., 2018). Many of the plants have been investigated for development of novel drugs with therapeutic properties.

Vaccinium macrocarpon commonly called as American cranberry is a widely consumed fruit in North America, and has been recognized to have several biological properties which may provide human health benefits (Kharinar *et al*., 2015), including effects on virulence factors of *S.mutans* involved in the pathogenesis of dental caries. Cranberry juice and a high-molecular weight non dialyzable material (NDM) extracted from cranberry inhibit the formation of biofilms and co aggregates of oral bacteria, the activity of GTFs, and the bacterial adherence on apatite surfaces (Duarte *et al*., 2015). Recently,it has been reported that cranberry juice disrupts the accumulation and acidogenicity of *S. mutans* biofilms *in vitro* without killing the organisms (Tamkute *et al*., 2019).Cranberry fruit is a unique and rich source of various classes of potentially bioactive flavonoids (polyphenols). Flavonoids are a large group of polyphenolic natural compounds that are universally distributed in higher plants. Anthocyanins, flavonols and proanthocyanidins are among the most abundant flavonoid classes, and have been associated with the health promoting benefits of cranberry and its products (Gupta *et al*., 2015).

Organisms synthesize exopolysaccharides and it forms a biofilm which will inhibit the growth of the organism in the culture, and it protects us from the unfavorable environmental conditions(Basting *et al*.,2016). The biofilms are attached to the substratum and they are embedded using an extracellular polymeric matrix. Due to the growth of the bacteria the biofilm attaches to the surface and it is hydrated (Aneja *et al*., 2015).Oral biofilm for instance is one of the most prevalent sources of chronic human infectious disease widely spread over all age groups. Although various methods are established and are presently in use to manage oral biofilms, the pursuit for natural and effective antibiofilm agents still continues(Slobodnikova *et al*.,2016).

Phytochemical studies have shown that *Vaccinum macrocarpon* active components include flavanols, anthocyanins, proanthocyanidins are potential anti-caries agents since they inhibit acid production, attachment, and biofilm formation by *Streptococcus mutans(*Sanoner *et al*.,2019). Phytochemicals found in cranberries (flavanols and benzoic and cinnamic acid derivatives) have also attracted a great deal of attention mainly because of their antioxidant, antibacterial, anti-inflammatory and antimutagen properties. Cranberries can play a role in preventing certain infectious diseases, such as urinary tract disorders, dental decay, as well as stomach ulcers and cancers. (Kim *et al.*, 2016).

Generally, antibiotics have an inhibitory activity on pathogenic bacteria. Commercially antibiotics are commonly used to treat the diseases caused by bacteria. Due to multiple-drug resistant pathogens the development of available antibiotics or need to search for new antibacterial agents is a must (Phoolchareon *et al.*,2013). To overcome these problems researchers focused on the antibacterial properties of various plants against antibiotic-resistant bacteria. Medicinal plants may provide new source of antibacterial agents. Plant-derived drug also serve as more effective and less toxic medicines (Philip and walsh, 2019). The compounds obtained from different sources of plant serves as an effective agent and thus could be potential source of low-cost natural antimicrobial agents (Dandekar *et al.*,2017).

This study was designed to analyse the anti cariogenic property of *Vaccinium macrocarpon* extracts against different bacterial strains.

With this background, the present study entitled "Anti-cariogenic effect of *Vaccinium macrocarpon* against causative organisms of dental caries" was designed with the following objectives:

- To evaluate the antibacterial and antibiofilm activity of *Vaccinium macrocarpon* fruit extract.
- To examine the morphological changes in the microbes before and after the addition of cranberry fruit extract using SEM.
- To formulate the juice in the form of a polymer gel to aid topical application.

Review of literature

2.0 REVIEW OF LITERATURE

The review of literature pertaining to the present study "Anti-cariogenic effect of *Vaccinium macrocarpon* against causative organisms of dental caries" is discussed under the following headings

2.1 Dental caries

2.2 Microbial Ecology

2.3 Microorganisms associated with tooth decay

2.4 Etiological Agent of Tooth Decay

2.5 Mutans streptococci (MS)

2.6 Virulence Factors of *S. mutans*

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DENTAL CARIES

Oral cavity harbors a rich and diverse microbial flora because of its ideal humidity and temperature, the frequent passage through it of most nutrients needed by many microbial species and presence of several ecological niche (Sunitha *et al.*,2012). The presence of an abundant microorganisms is a natural part of proper oral health. Oral microbes can adhere to surfaces throughout the oral cavity (Loesche, 1986). These include the tongue, epithelial cells lining roof of the mouth and the cheeks, and enamel of the teeth.

The mouth can be considered an ideal environment for the growth of microorganisms, since it is warm and moist and has a constant influx of nutrients through saliva and food intake. The ecology of the mouth, however, does not just involve interactions among microorganisms. In fact, the host plays a large role in maintaining a uniform ecosystem, especially through the saliva(Farooqi *et al*.,2015). Saliva is a complex mineral- and protein rich solution that delivers nutrients to the many bacterial species within the mouth while also protecting host surfaces. During mastication, increased saliva flow prevents changes in oral pH, because the buffer bicarbonate is present in saliva and acts as an acid sink at a time when acidic products are being introduced into the mouth. Urea and the peptide sialin are both also present in low concentrations in saliva and produce ammonia when hydrolyzed, a basic product capable of raising pH (Loesche,1986). This basicity and buffering counteracts the lactic acid produced by anaerobic bacteria in the mouth during the fermentation that occurs when nutrients are introduced, offsetting decay of the teeth caused by this acid. Saliva also contains glycoproteins that are known to be antibacterial, such as lysozyme and lactoperoxidase. These compounds act independently of the host's immune system, and are able to destroy invasive bacteria without harming the ecological balance of the oral cavity, since indigenous bacteria have evolved resistance (Karpnski and Szkaradkiewicz,2013)

The experience of pain, problem with eating, chewing, smiling and communication due to missing, discolored or spoiled teeth have a foremost impact on people’s everyday life. Globally, there is a great interest in the use of antimicrobial agents for prevention and treatment of dental diseases(Kidd and Fejerskov,2016) The word caries derives from the Latin for rot or rotten. According to Bader, dental caries is a chronic contagious disease caused by a complete interaction of oral microorganism in dental plaque, diet and a broad

array of host factors ranging from societal and environmental factors to genetic and biochemical/immunologic host responses (Yadav and Prakash ,2017).

Types

Primary caries

It can take place on different tooth surfaces. On an approximal surface, the lesion initiates and appears below the contact area between teeth. Caries on an occlusal surface is also a localised phenomenon in pit and fissure. On both occlusal and approximal surfaces, enamel caries is a three-dimensional subsurface demineralisation that spreads along the enamel prisms (yadav *et al*., 2016).

Secondary caries

It is a lesion located at the margin of a dental restoration which characterizes a caries lesion adjacent to the margin with signs of demineralisation (wall lesions) alongside the cavity wall which could be outcome of micro leakage. However, clinical and microbiological studies suggest that this leakage does not lead to active demineralisation below the restoration(Marsh,2012).

Microbial Ecology

The microbial ecology of caries includes the biology of oral bacteria within related habitats i.e.,

The habitat

The hard (enamel and tooth root) and soft (mucosal) tissue surfaces in the mouth provide a variety of microhabitats with distinctly different structural and environmental parameters(Peterson *et al*.,2011). In particular, the non shedding surface of enamel allows the accumulation of a biofilm that provides a protected habitat with a variety of niches that support a wide range of bacterial genera and species.

Microorganisms associated with tooth decay

Two species of the ‘mutans streptococci’ viz. *Streptococcus mutans* and *Streptococcus sobrinus* are the principal agents of enamel caries. *Lactobacillus* and

Actinomyces are also associated with caries. *Actinomyces odontolyticus* colonizes infants before eruption of teeth. Some root caries lesions are dominated by *Actinomyces naeslundii*, *A. israelii* and *A. gerencseriae(*Shafiq *et al*.,2018). The other significant species involved in caries includes *Streptococcus mitis, Bifidobacterium* and, a group of 'low pH' aciduric isolates which have been isolated from white spot lesions in humans. In contrast to bacteria that lower plaque pH including *Veillonella* and *Actinomyces* associated with caries.

Etiological Agent of Tooth Decay

Different microorganisms can exist in single or poly-microbial communities in caries. i.e.

• **Gram positive cocci**: - *Streptococcus mutans, S. mitis, S. salivarus, S. sanguis, S. intermedius, S. vestibularis, Staphylococcus aureus, Atopobium spp, Peptostreptococcus spp, Enterococcus fecalis*.

• **Gram positive rods**: - *Actinomyces odontolyticus, A. naeslundii, A. viscosus, A. israelii, Lactobacillus fermentum, L. acidophillus, Bifiodobacterium dentium, Propionibacterium spp*.
• **Gram negative cocci**: - *Veillonella parvula, Nesseria spp*.

•**Gram negative rods**: - *Bacteriodes denticola, B. melaninogenicus, Fusobacterium necrophorum, F. mortiferum, Escherishia coli, Klebsiella pneumoniae, Enterobacter aerogens, Citrobacter freundi, Pseudomonas fluorescence, Haemophilus spp, Prevotella spp, Leptotrichia spp*.

• **Yeasts**: - *Candida albicans, C. tropicalis, C. glabrata*.

***Mutans streptococci* (MS)**

Mutans streptococci are the foremost cariogenic pathogens in tooth decay. They are highly acidogenic, producing short-chain acids which soften hard tissues of teeth(Choi *et al.*2016). Three isozymes of glucosyltransferases catalyze and metabolize sucrose to synthesize insoluble extracellular polysaccharides, which increase their adherence to the tooth surface and persuade biofilm formation. The most significant *mutans streptococci* isolated from tooth caries samples are *S. mutans* and *S. sobrinus*. *S. mutans* is more

cariogenic than *S. sobrinus* because of specific cell-surface proteins, which assist in its primary attachment to the tooth. But, such proteins are deficient in *S. sobrinus*(Marsh,2010).

Streptococcus mutans

Streptococcus mutans has been implicated most of all as the initiator of dental caries. *Streptococcus mutans* is a Gram-positive, facultatively anaerobic bacteria commonly found in the human oral cavity. The natural habitat of *S. mutans* is the human mouth. The organism can be isolated frequently from faeces in human (Philip *et al*., 2018). It was first observed by Clarke who found a small, chained cocco bacillus which was more oval than spherical in shape. He suggested that these microorganisms were mutant *streptococci* and called them *Streptococcus mutans* (Ramadan *et al*.,2019). The cells are spherical or ovoid, 0.5-2.0 µm in diameter, occurring in pairs or chains when grown in liquid media, and stain Gram-positive. The optimum temperature for growth is 37°C, and growth is usually restricted to 25-45°C.

SCIENTIFIC CLASSIFICATION

Kingdom : Bacteria

Phylum : Firmicutes

Class : Cocci

Order : Lactobacillales

Family : Streptococcaceae

Genus : *Streptococcus*

Species : *S. mutans*

Number of sugars and glycosides such as glucose, fructose, sucrose, lactose, galactose, mannose, cellobiose, glucosides, trehalose, maltose and group of sugar alcohols are metabolized by the bacterial action of *S. mutans*. *S. mutans* synthesizes intracellular glycogen like polysaccharides in the presence of extracellular glucose and sucrose (Kumarasamy *et al.*,2014). An important factor in the colonization and establishment of *S. mutans* in the dental biofilm are mutacins (bacteriocins) produced by *S. Mutans*.

Virulence Factors of *S. mutans*

Adhesion, sucrose-independent adhesion, sucrose-dependent adhesion, non-enzymatic glucan binding proteins, carbohydrate metabolism, acidogenicity, acid-tolerance, maintenance of intracellular pH, biofilm formation are the virulence factors of *S.mutans* (Ghasemi *et al.*,2017).

What makes *Streptococcus mutans* such a potent initiator of caries?

A variety of virulence factors unique to the bacterium have been isolated that play an important role in caries formation. First, *S. mutans* is an anaerobic bacterium known to produce lactic acid as part of its metabolism (Chaudhary *et al.*,2012). Then there is the ability of *S. mutans* to bind to tooth surfaces in the presence of sucrose by the formation of water-insoluble glucans, a polysaccharide that aids in binding the bacterium to the tooth. Mutant strains developed to produce water-soluble glucans instead have extremely diminished cariogenicity, especially on the smooth surfaces of the teeth which require greater tenacity for binding to occur (Loesche 1986). Water-insoluble glucan has also been found to lower the calcium and phosphate concentration of saliva, decreasing its ability to repair the tooth decay caused by bacterial lactic acid (Ribeiro *et al.*,2018). The most important virulence factor, however, is the acidophilicity of *Streptococcus mutans*. Unlike the majority of oral microorganisms, *S. mutans* thrives under acidic conditions and becomes the dominant bacterium in cultures with permanently reduced pH. Additionally, unlike many species present in plaque, whose metabolisms slow considerably at such a low pH, the metabolism of *S. mutans* actually improves, as the proton motive system used to transport nutrients through its cell wall in environments of low pH or high glucose concentration is modulated by hydrogen ion content, which increases with acidity (Choi *et al*.,2016). In this way, *S. mutans* can actually continue to lower or maintain the oral pH at an unnaturally acidic value, leading to conditions favorable for its own metabolism and unfavorable for other species it once coexisted with. It is this lowered pH that results in demineralization and cavitation of the teeth, both of which increase with increased rates of *S. mutans*. Under acidic conditions, *S. mutans* succeeds in creating a cycle that is favorable for itself and unfavorable for others involved in the oral ecology – becoming, in effect, a pathogen.

Streptococcus sobrinus

S. sobrinus has been implicated in caries development particularly in instances where caries development appears to be independent of *S. mutans*. *S. sobrinus* exhibits higher acid production and acid tolerance compared to *S. mutans*.

Streptococcus mitis

Streptococcus mitis are commensal bacteria which belong to the viridians *streptococci* group usually arranged in short chains in the shape of cocci. These Gram-positive bacteria are part of human oral flora, usually non pathogenic but commonly cause bacterial endocarditis which colonizes hard surfaces in the oral cavity such as dental hard tissues as well as mucous membranes. The development of this microbial community is reliant on numerous factors including adherence, signalling, nutritional adaption, and host modulation. In addition, environmental conditions such as pH, temperature, oxygen availability, organic metabolites etc. may be involved in *Streptococcus mitis* colonization.

Lactobacilli

Lactobacilli are dominant part of the flora, considered as pioneer microbes in the development of caries particularly in dentin. It inhabits the deep cavities, and their number correlates with the quantity of carbohydrates. The isolation of *Lactobacilli* is from deep caries lesions but rarely just earlier than the development of dental caries and in the early tooth decay.

Staphylococcus aureus

Most of the healthy individuals (30%) are the carrier of *S. aureus*, often carrying the bacterium on their skin, mucous membranes of anterior nares. Such carriers serve as the source of infection to themselves as well as to others.

Actinomycetes

Actinomycetes are not powerfully acidogenic or acid tolerant but are also carbohydrate users which are copious in the human mouth and persuade root surface caries in hamsters and gnotobiotic rats.

Biofilm Formation

Dental plaque is considered as a biofilm. There are many variables coupled with growth in a biofilm including adhesion, nutrient flow, and co-aggregation can persuade growth rate, gene expression and quorum sensing in ways that differ from life in a planktonic environment. gtfB and gtfC genes are directly involved in biofilm development with the variability of gene expression in response to the environment. Evidence advocates that these genes can be both independently transcribed and co-transcribed. Hudson and Curtiss, by using the chloramphenicol acetyltransferase (cat) reporter gene demonstrated increased expression of the gtfB/C genes in response to sucrose or when bound to an artificial tooth pellicle (Chaudhari *et al.*,2012).

Steps of biofilm formation

a. Association – Dental pellicle forms on the tooth & provides bacteria surface to attach.

b. Adhesion – Within hours, bacteria loosely binds to the pellicle.

c. Proliferation – Bacteria spreads throughout the mouth & begins to multiply.

d. Microcolonies – Microcolonies are formed, streptococci secrete protective layer (slime layer).

e. Biofilm formation – Microcolonies from complex groups with metabolic advantages.

f. Maturation – The biofilm develops a primitive circulatory system.

Progression of Dental Biofilm

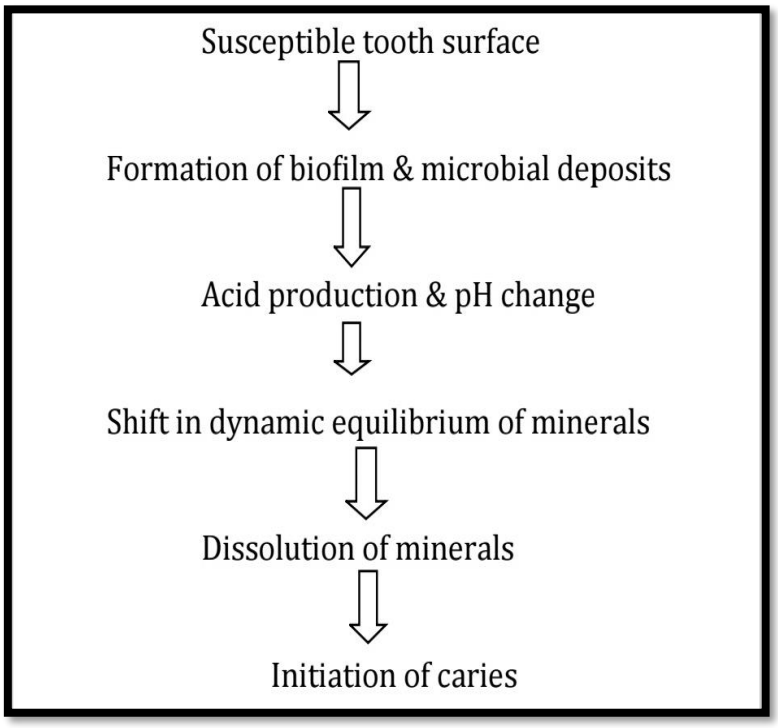
Dental plaque forms through a sequential events resulting in a structurally and functionally organized species rich microbial community (Hanafiah *et al*.,2018). Once plaque forms, its species composition at a site is characterized by degree of stability among the component species. This stability is due to a balance imposed by numerous microbial interactions, such as conventional biochemical interactions where complex host glycoproteins catabolize to develop food chains and cell to cell signaling which leads to coordinated gene expression within the microbial community (Rukayadi *et al*.,2008).

Caries mechanism

The bacterial flora and host defense systems are in the process of being established. Caries development is dependent on the following factors [Flowchart 1]

Susceptible tooth and host

Implantation of *S. mutans* can occur only when teeth are present, because the teeth provide a non-shedding surface for colonisation of the micro-organisms(Garcia *et al.*,2017). The amount of *S. mutans* depends on the number of erupted teeth present. Sometime after eruption, newly exposed enamel surfaces undergo the final stages of post-eruptive maturation and hardening. This period immediately after eruption and prior to final maturation, is when the tooth is most susceptible to caries(Kim *et al*.,2016). The presence of structural developmental defects in enamel may increase the caries risk and may manifest as partial or total loss of enamel (hypoplasia). Irregular surfaces such as pits and grooves lead to plaque retention, increased *S. mutans* and decreased elimination of carbohydrates. Dentin, when exposed, provides little resistance to acid attack.



Flowchart 1: Illustrating Caries Mechanism

Fermentable carbohydrate diet

The formation of dental caries is associated with the carbohydrate component of the diet. Oral micro-organisms, especially *S. mutans*, utilize certain carbohydrates to form a sticky matrix that facilitate them to stick to the teeth(Kolenbrander *et al.*,2000). Organic acids are formed from the carbohydrates, which demineralize the teeth. The frequent consumption of soluble carbohydrate as well as their prolonged contact with tooth surfaces is highly significant risk factors.

Microflora

The micro-organisms are responsible for dental caries. The early colonizers of these micro-organisms are mainly *mutans streptococci*, produce large amounts of acid, especially lactic acid, which are potent in causing tooth demineralization(Kabra *et al*.,2000). Attachment of the *S. mutans* is now thought to be independent of sucrose and mediated by adhesions on the bacterial surfaces interacting directly with the salivary proteins, which form the pellicle on the tooth surface.

Time

Time is a significant factor in the progress of caries in relation to the frequency and amount of exposure of the liquid which will affect both the severity of the lesions and the number of teeth involved. The frequency of contact of the substrate has a major role in cariogenicity during a 24-h period(Tanner *et al*.,2016)

Stages & Symptoms of dental caries

The different five stages of dental caries with symptoms are as follows

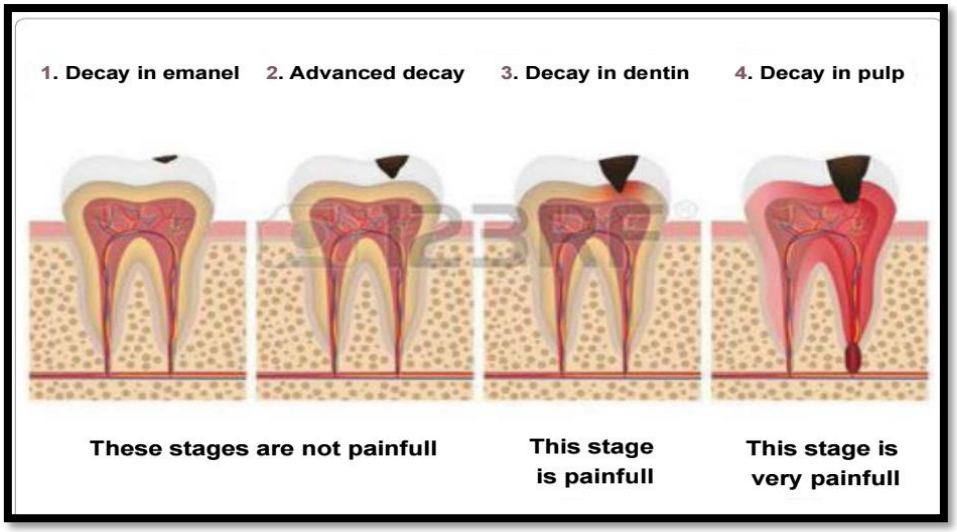
a. **Stage One: white spots**

The appearance of yellowish spots or chalky white areas on the surface of tooth due to the loss of calcium is the first stage of tooth decay which is still reversible with appropriate treatment. There are no subjective symptoms, including pain.

b. Stage Two: enamel decay

The enamel of tooth begins flouting beneath the exterior layer with the outside intact during this stage. The surface of the tooth ruins when decay persists. Such type of damage is irreversible with no pain or sensitivity(Marsh,2010)

Figure 1: Stages Of Tooth Decay



c. Stage Three: dentin decay

In this stage, the decay progresses beyond the enamel into the dentin with pain.

d. Stage Four: involvement of the pulp

The infection of the pulp of the tooth starts in the fourth stage due to the action of bacteria. Blood vessels and nerves in the pulp die due to pus formation.

e. Stage Five: abscess formation

This is the final stage of the infection which reaches to the root tip of the tooth causing severe pain. The bones surrounding the tooth also get infected and visible swelling on the cheeks, along the affected side is observed.

Problems Associated with Tooth Decay

The complications of tooth decay are toothache, pulpitis, tooth loss, tooth discoloration, oral cancer, endocarditis, cavernous sinus thrombosis and Ludwig angina can be fatal (Pitts *et al*.,2017).

Dental Caries Risk Assessment

Risk factors are either "causal" or "associated" is one of the most important queries. Initially, the consumption of sugar, plaque, hygiene regimen and the host were considered as local caries process were the associated risk factors for caries. Burt in 2005 highlights that these factors should be comprehensive and stated that "Social determinants of health and population health are also associated with caries"(Peterson *et al*.,2017). Major three types of factor related to tooth decay risk assessment have been defined:

Risk factor or caries-promoting factor

The exposure that plays an essential role in caries development is known as risk factor.

a. Sugar consumption

The growth rate of many oral bacteria increases and changes the composition of the microflora in a caries-promotion with sucroserich diet. Acid-producing micro-organisms, such as the *mutans streptococci* in dental plaque, play a crucial role in the caries development.

b. Tooth location

Molars and premolars are more susceptible towards tooth decay as these teeth have lots of grooves, pits and crannies that can accumulate food particles. As a consequence, they're difficult to maintain hygienic and clean. Plaque can fabricate and bacteria can increase between back teeth, producing the acid that demolishes tooth enamel (Farooqi *et al*.,2015)

Risk indicator

The exposure that co-exists with an increased probability of developing a disease is known as risk indicator.

a. Socio-economic factors

Low socio-economic status and immigrant background that ultimately influence oral hygiene standards and attitudes to tooth care in children and adolescents are social risk indicators.

b. Age

In the United States, cavities are more frequent in children and teenagers and older adults also are at elevated jeopardy. Teeth can wear down and gums may recede, making teeth more vulnerable to root decay by overtime. Older adults can possibly use supplementary medications that decrease saliva flow, escalating the risk of dental caries.

c. Dry mouth

Dry or dehydrated mouth is caused by a deficiency of saliva. Substances found in saliva also assist to oppose the acid produced by bacteria and can help in restore early caries. Certain medications, some medical conditions, radiation to head or neck, or certain chemotherapy drugs can augment risk of cavities by reducing saliva production (Garcia *et al*.,2017).

d. Worn fillings or dental devices

Dental fillings can weaken, begin to break down or develop rough edges over the years which allow plaque to build up more simply and make it harder to remove. Dental devices can also stop fitting well, allowing decay to begin beneath them.

e. Eating disorder

Significant tooth erosion and cavities can also be due to anorexia and bulimia. Stomach acid from repeated vomiting (purging) cleanses over the teeth and instigates melting the enamel. Eating disorders can also hinder with saliva production.

f. Heartburn

Gastroesophageal reflux disease (GERD) or heart burn can cause stomach acid to flow into mouth (reflux), wearing away the enamel of teeth leads to significant tooth damage.

g. Genetic factors

The presence of bacteria and carbohydrates are necessary for caries to develop. Whether this actually happens depends upon the inherited or acquired resistance of the teeth (Caufield *et al*.,2015).

h. Malnutrition

The children who are malnourished pre-, peri- or post-natally and/or who are of low birth weight are likely to have hypomineralised or hypoplastic primary teeth. These teeth have a higher risk of becoming carious and are more susceptible to *mutans streptococci* colonization.

Risk Inhibitor

Risk inhibitor is an exposure that prevents with the probability of developing a disease (Asadi *et al*.,2019).

a. Proper tooth brushing and use of fluoride toothpaste

The declination of caries can occur with the use of fluoride toothpaste with daily tooth brushing. Fluoride has significant effect in caries prevention which is mostly due the topical effect of different fluoride vehicles after tooth eruption.

b. Antibacterial therapy

Products such as chlorhexidine rinses are effective.

c. Fermentable carbohydrates

Reduce the amount and frequency of ingestion.

d. Salivary flow

Salivary flow can be increased by chewing sugarless gum, for example, those with a non-sugar sweetener such as xylitol.

Diagnosis

• Examination of all visible tooth surfaces using a good light source, dental mirror and explorer for a small chalky area or cavity.

• Early, uncavitated caries is often diagnosed by blowing air across the suspect surface.

• X-rays of tooth are used for less visible areas of teeth in particular caries between the teeth.

• Lasers without ionizing radiation also now used for detection of interproximal decay.

HERBAL EXTRACTS: SCOPE AND SIGNIFICANCE AS THERAPEUTIC AGENTS

oral diseases continues to be a major health problem worldwide. In mainstream medicine, new medical treatments are assumed to be ineffective, until they are proved to be useful. In addition, the adverse effects associated with mainstream medicine makes their use less desirable and less reliable by the population. Traditional medicine is a socio economic and socio cultural heritage, serving approximately 80% of the population of developing countries. The global need for alternative treatment of oral diseases that are safe, effective and economical arises from the rise in disease incidence, increased resistance of pathogens to currently used antibiotics and chemotherapeutics, opportunistic infections in immune compromised individuals and financial considerations(Hakeen *et al.*,2019)

Although many agents are being commercially used for treatment of oral microbiota, their undesirable side effects make them less successful in safety aspects (Vinod *et al*.,2018). Some of the chemical antibacterial agents such as cetylpyridinium chloride, chlorhexidine, and amine fluorides have shown to exhibit some kind of toxicity with staining of teeth, leading to oral cancer. Therefore, the search for alternative substances is in great demand and bioactive compounds from plants are being used in traditional medicines as complementary medicine (Yim *et al*. 2013).

Numerous in vitro studies have been performed to investigate the activity of natural plant substances against oral bacteria known to be involved in the etiology of oral and

dental diseases. Substances exhibiting such activity comprises of plant extracts such as cinnamic aldehyde, cinnamon bark oil, clove bud oil and papua-mace extracts etc. Herbal preparations against this species of cariogenic microflora can be derived from the root, leaves, seeds, and flowers(Rane *et al*.,2013).

Herbs comprising medicinal properties are a valuable and effective source for treatment of various diseases (Alviano *et al.*,2018). These herbal extracts have been consistently used in maintaining oral health by tooth cleaning and as antimicrobial plaque agents (Bobido *et al*.,2018).Various medicinal herbs are having applications in maintenance of oral hygiene by suppressing various oral microbes and by other curative ways (Eskandarian *et al*. 2017).

Vaccinium macrocarpon

The *Vaccinium macrocarpon* is one of three native North American fruits,the others being concord grapes and blueberries,that grow in the wild from the Carolinasto Canada. The fruit of the cranberry is widely consumed in various food 25 products, including fresh and dried fruits, sauces, and juices, as well as in powder form in capsules and tablets. Cranberry extracts are a rich source of flavonoids and especially of the flavonols myricetin and quercetin, and possess biological properties that may provide human health benefits(Gill *et al.*,2016).

Cranberries are healthy fruit that contribute color, flavor, nutritional value, and functionality. They are one of only three fruits native to America(Rane *et al.*,2013). Over the past decade, public interest for the North American cranberry (*Vaccinium macrocarpon*) has been rising with reports of their potential health benefits linked to the numerous phytochemicals present in the fruit— the anthocyanins, the flavonols, the flavan-3-ols, the proanthocyanidins, and the phenolic acid derivatives(Labrecque *et al.*,2006). The presence of these phytochemicals appears to be responsible for the cranberry property of preventing many diseases and infections, including cardiovascular diseases, various cancers, and infections involving the urinary tract, dental health, and Helicobacter pylori-induced stomach ulcers and cancers. Also they exhibit the antioxidant, radical scavenging, antibacterial, antimutagen, and anticarcinogen properties(Catunescu *et al*.,2019)

***Vaccinium macrocarpon* extract**

In the past decade, cranberry extracts have been attracting ever-growing attention by dental researchers.A non-dialysable cranberry fraction enriched in high molecular weight polyphenols has very promising properties with respect to cariogenic and periodontopathogenic bacteria, as well as to the host inflammatory response and enzymes that degrade the extracellular matrix. Cranberry components are potential anti-caries agents since they inhibit acid production, attachment, and biofilm formation by *Streptococcus mutans*. Glucan-binding proteins, extracellular enzymes, carbohydrate production, and bacterial hydrophobicity, are all affected by cranberry components(Polak *et al*., 2013) Regarding periodontal diseases, the same cranberry fraction inhibits host inflammatory responses, production ,and activity of enzymes that cause the destruction of the extracellular matrix, biofilm formation, and adherence of *Porphyromonas gingivalis*, and proteolytic activities and coaggregation of periodontopathogens. The above-listed effects suggest that cranberry components, especially those with high molecular weight, could serve as bioactive molecules for the prevention and/or treatment of oral diseases(Sanoner *et al*.,2019)

Biological Properties Of Cranberry Bioactive Compounds

Vaccinium macrocarpon have been identified with beneficial properties toward bacterial infections involving the urinary tract disorders, dental decay, as well as stomach ulcers and cancers (Cote *et al*.,2011). Although berry phenolics are potent in vitro

antioxidants, they exert in vivo biological activities beyond antioxidation and can have complementary and overlapping mechanisms of action (Al-zobaidy *et al.*,2019). In vitro experimental systems showed that berry phenolics possess antioxidant and free radical-scavenging activities, but also metal chelation, antiproliferative, anticarcinogenic, antibacterial, anti-inflammatory, antiallergenic, and antiviral properties (Steinberg *et al*.,2004). This would explain why they exhibit such strong physiological activities against infections, cancerous mutations and cancers, as well as against allergies, inflammation, virus, hypertension, arthritis, and AIDS (Gupta and Jain,2015).

Antimicrobial Properties

Juice of the American cranberry (*Vaccinium macrocarpon*) has long been consumed for the prevention of urinary tract infections. Its ability to protect the urinary tract from adherence of urinopathogenic bacteria such as Escherichia coli and other pathogens, has led doctors to recommend drinking cranberry juice as a treatment for various urinary tract infections and prostatitis. For more than a decade, the tannic components of cranberry have been proposed to inhibit bacterial adherence to the epithelial cells by competing for the bindings of both these fimbriae. In a 2015 Cochrane clinical study review, the authors(Kim et al.,2015) concluded that there was some evidence that cranberry juice may decrease the number of symptomatic urinary tract infections over a 12 month period, particularly for women with recurrent urinary tract infections. All this knowledge has led to the first ever health claim on berry phenolics issued by the French Food Safety Authority in April 2004: "cranberry proanthocyanidins, in a daily dose of 36 mg, help reducing the adhesion of certain E. coli bacteria to the urinary tract"(Babu *et al*.,2015). Cranberry has also showed potential inhibitory effect against bacteria involved in dental caries and periodontal diseases.

Evaluation of cytotoxicity

The cytotoxicity of the various compounds was tested using cell culture assay on cellular systems. *In vitro* methods have shown a significant potential for assessing the toxicity of both environmentally and occupationally occurring compounds. These include methods for testing the integrity of the cell membrane, as measured by enzymatic activity released by damaged cells eg., LDH and the metabolic activity of viable cells(Loon *et al*.,2018).

The cell viability and cytotoxicity effects are mainly based on various cell functions such as enzyme activity,cell membrane permeability,cell adherence , ATP production, co-enzyme production, and nucleotide uptake activity. The various established methods for the assessment of cytotoxicity such as colony formation, crystal violet method, Tritium-Labeled Thymidium uptake, MTT, and WST, are used for counting the number of live cells(Fani and Kohante,2012).

From all these methods, the suspended cells in the culture will not adhere to the bottom of the well. So this may cause loss in the number of cells. To overcome this problem the MTT assay technique is used. The assay is mainly recommended for examining the cytotoxic effect of xenobiotics and also for analysing the cell activity(Berber *et al*.,2013).

Antimutagen and Anticarcinogen Properties

Current evidence available from cell culture experiments suggests that many of the biological effects of cranberry phenolic compounds are related to their ability to modulate cell signalling pathways. Studies reporting *in vitro* and *in vivo* antitumor and antiproliferative activity of cranberry have implicated flavonoidrich extracts as contributing to these activities (Islam *et al*., 2007). Fareen and geetha,(2018) reported that total cranberry extract (200 µg/mL) could inhibit the proliferation of human oral, colon, and prostate tumor cells *in vitro*. Similar effects were also observed with some purified cranberry fractions, including the total phenolic fraction (200 µg/mL), the anthocyanin fraction (7.1 µg/mL), and the proanthocyanidin fraction (6.5 µg/mL). Quercetin (40.90 ± 1.12 µM) and Cranberry anthocyanins could play a role in the fight against cancer through their displayed anti-carcinogenic properties.

The methodology adopted for present study is presented in the next chapter.

Methodology

3.0 METHODOLOGY

Dental caries is one of the most prevalent chronic diseases worldwide. Tooth get decayed because of a mixture of reasons which includes unhealthy oral hygiene, stagnation of food on or around the teeth, presence of plaque on the tooth structure and also due to the presence of caries causing microorganisms(Singh *et al*.,2010). The accumulation of bacteria like *Streptococcus mutans* in the tooth surface produces acid which demineralises the enamel and forms dental caries. This study is aimed at investigating the antimicrobial and antibiofilm activity of *Vaccinium macrocarpon* extract against oral pathogens as well as the potential health benefits of the extract. Cranberry constituent suppressed the adhesion of cariogenic bacteria on the tooth surface. This was because of the anti-adhesion activity of cranberry constituent (Giradof *et al*.,2014).

The aim of the present study was to investigate the antimicrobial activities like MIC, MBC, antibiofilm, time kill assay and MTT activity of cranberry fruit extract and later formulation of polymer gel to be used against dental caries causing microbes both in the presence and absence of standard antibiotic and also to examine the morphological changes in the microbes before and after the addition of cranberry fruit extract using FESEM. As a model for *in vitro* studies *Streptococcus mutans, Klebsiella pneumonia, Staphylococcus aureus, Pseudomonas aeuroginosa* were used for all experiments. In this chapter the methodology of present study entitled with "Anti-cariogenic effect of *Vaccinium macrocarpon* against causative organisms of dental caries is discussed under the following headings.

3.1.Extraction Of Cranberry(*Vaccinium macrocarpon)*

3.1.1 Organic Extraction

3.1.2 Formulation Of Cranberry(*Vaccinium macrocarpon)* Extract Into Gel

3.2.Antimicrobial Activity

3.2.1.Determination Of Minimum Inhibitory Concentration(MIC)

3.2.2.Time Kill Assay

3.3.Antibiofilm Assay

3.4.MTT Assay

3.5.Scanning Electron Microscopy

3.6 Formulation Of Cranberry Extract Into Gel

MATERIALS AND METHODS

PLANT MATERIAL

Cranberry (*Vaccinium macrocarpon*) is used as fresh fruit, juice, sauce and also as medicine. It contains polyphenols, vitamins, proteins, flavonoids and alternative rare phytochemicals. It has antimicrobial, anti-inflammatory and anti tumour activities. Cranberry plantation is historically done in certain colder countries at high altitude. It is widespread throughout the cool temperate northern hemisphere, including northern Europe, northern Asia and North America. Fresh cranberry was collected and used in the extraction purposes.

BOTANICAL CLASSIFICATION

Kingdom : Plantae
Clade : Angiosperms
Clade : Eudicots
Clade : Asterid
Order : Ericales
Family : Ericaceae
Genus : Vaccinium
Subgenus : Oxycoccus
Species : *Vaccinium macrocarpon*

3.1 EXTRACTION OF CRANBERRY

3.1.1 ORGANIC EXTRACTION

Methanol extraction of the cranberry was carried out by using methanol according to method described by (Ulrey *et al*., 2014). This was obtained by using 10 g of dried fruit placed in 100 ml of methanol in a conical flask, and then kept on a rotary shaker at 190 - 220 rpm for 24 h. After 24 h, then filtered and centrifuged at 4500 rpm for 15 min. The supernatant was collected and the solvent was evaporated by using rotary evaporator to get rid of methanol and then stored at 4°C in airtight bottles.

3.2. MICROORGANISM

Bacterial strains of four different species (*Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeuroginosa, Klebsiella pneumoniae*) which are known for enhancing activity in caries formation were selected. All tested strains were obtained from the microbiological laboratories and were subcultured into Mueller-Hinton Agar slants and were maintained at 4°C.

3.2.ANTIBACTERIAL TEST

3.2.1.DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION(MIC)

The minimum inhibitory concentration (MIC) was the lowest concentration of the juice that prevented visible growth of bacteria. The antibacterial assay was performed using the broth macrodilution method. The absence of microbial growth was interpreted as an antibacterial activity. The procedure is outlined in the Appendix-1.

3.2.2.TIME KILL ASSAY

Bactericidal activity of cranberry extract was examined using a time kill assay. It is the most appropriate method for determining the bactericidal effect. It is a strong tool for obtaining dynamic information between the anti-microbial agent and the microbial strain. The time kill test reveals a time dependent or a concentration dependent antimicrobial effect (Kidd and Fejerskov,2016).The detailed procedure for the time kill assay is outlined in the Appendix-2.

3.3.ANTIBIOFILM ACTIVITY

3.3.1.INHIBITION OF BIOFILM FORMATION

The effect of extract on biofilm formation of each representative strain, (*Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeuroginosa, Klebsiella pneumoniae*) was examined using the modified microdilution method of Sanchez *et al*,2016. The procedure is outlined in Appendix-3.

3.4 MTT ASSAY

MTT assay is the most sensitive method for measuring cytotoxicity. The cytotoxicity of the cranberry (*Vaccinium macrocarpon)* was assessed using (*Streptococcus mutans , Staphylococcus aureus, Pseudomonas aeuroginosa, Klebsiella pneumoniae*). The 2-(4,4-dimethyle-2-tetrazoyl)2-diphenyle-2 tetrazolium salt (MTT) was converted into formazan derivative by living cells. The amount of formazan formed is measured from the number of surviving cells. After solubilising of formazan in a suitable solvent the cell viability was measured in micro titre plate reader (Caufield *et al.*,2015). The methodology adopted for this study is given in Appendix- 4.

3.5.SCANNING ELECTRON MICROSCOPY

A scanning electron microscopy (SEM) was performed to examine the morphological changes of each representative strain after treatment with 10 % DMSO or 0.1 mg/ml of cranberry extract as the control and treated sample, respectively (Teanpaisan *et al*.,2013). The methodology adopted for this method is given in Appendix-5

Once the anticariogenic effect of *Vaccinium macrocarpon* extract was confirmed we decided to formulate it in the form of a gel.

3.6 FORMULATION OF CRANBERRY EXTRACT INTO GEL

The evaluation of antimicrobial potency was studied prior to gel formulation to compare the changes in activity after incorporation in polymer gel. The topical formulations were developed using different concentrations of polymers. The method used for formulation of cranberry extract into gel is outlined in Appendix-6.

The results obtained in the present study are discussed in the next chapter.

Results and Discussion

4.0 RESULTS AND DISCUSSIONS

Dental caries, also known as tooth decay is an epidemic, microbiological contagious disease of the teeth that ends in localized dissolution and damage of the calcified structure of the teeth. This disease occurs due to multiple factors such as interactions within the plaque community, host physiology, diet, fluoride, pH and the nature of the tooth enamel, and dominance of microbial flora. The time factor is significant for the commencement and development of caries in teeth. The oral cavity contains a wide variety of oral bacteria, but only a few specific species of bacteria are believed to cause dental caries namely *Streptococcus mutans, Lactobacillus acidophilus, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* (Yadav and Prakash,2017).These microbes can be controlled using certain drugs but due to their side effects an alternative source of natural plant extracts is preferred.

Medicinal plants have been found useful in the cure of a number of diseases including bacterial diseases owing to a rich source of antimicrobial agents. Plant extracts are abundant sources of biologically active compounds, making them effective alternatives to routinely used drugs (Tran *et al.,*2019). The increased bacterial resistance to natural herbal extracts currently used in dentistry has a great importance for the prevention of oral bacterial growth, adhesion and colonization. Various medicinal herbs are being exploited for the maintenance of oral hygiene by suppressing various oral microbes and by other curative ways (Jose *et al*., 2014).

The *Vaccinium macrocarpon* is one of three native North American fruits. *Vaccinium macrocarpon* extracts are a rich source of polyphenols and flavonoids, especially of the flavonols myricetin and quercetin, and possess biological properties that may provide human health benefits. *Vaccinium macrocarpon* has many medicinal properties which have been reported to have anticarcinogenic, antibacterial, antiviral, antifungal, and antioxidant properties. Because of its antiadhesive property, it is used against periodontal disease, dental caries, and oral squamous cell carcinoma (Zepon *et al*., 2019).

In the present study, the methanolic extract of *Vaccinium macrocarpon* was prepared and it is used against the dental caries causing microbes to investigate the antimicrobial activities like agar well diffusion, MIC, antibiofilm, time kill assay and MTT activity and the extract was incorporated into gel. Also to examine the morphological changes in the microbes before and after the addition of *Vaccinium macrocarpon* extract, SEM analysis was done.

4.1 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION(MIC)

The *Vaccinium macrocarpon* can be considered as antimicrobial agent and also they are potential agents to help manage and prevent infections. The minimum inhibitory concentration (MIC) of *Vaccinium macrocarpon* was determined in triplicate by using the microdilution broth method in 96-well microplates. MIC is the lowest concentration of an antibacterial agent necessary to inhibit visible growth of the bacteria. The antimicrobial activity of *Vaccinium macrocarpon* was investigated against the dental caries causing microbes namely *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae.*

Table 1

Determination of minimum inhibitory concentration using *Vaccinium macrocarpon* extract against dental caries causing microorganisms.

Test organisms	**MIC (mg/ml of *Vaccinium macrocarpon)* – values are expressed in mean of three independent experiments**
Streptococcus mutans	60
Staphylococcus aureus	40
Pseudomonas aeruginosa	40
Klebsiella pneumonia	20

Table 1 lists the results obtained for the assays against causative agents of dental caries. *Vaccinium macrocarpon* afforded MIC value of 60 mg/ mL against *Streptococcus mutans*, which characterized a bactericidal effect. *Vaccinium macrocarpon* also exhibited bacteriocidal effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with MIC value of 40 mg/mL, respectively. For *Klebsiella pneumoniae*, MIC value was equal to 20 mg/mL, respectively.

Aneja *et al*.,(2015) examined the acetone, methanol and ethanol extracts of *Piper cubeba* against both two Streptococcal species namely, *S. aureus* and the caries causing *S.mutans* with an MIC of 50 mg/ mL.

Antibacterial agent kuwanon G isolated from root bark of *Moras alba* has showed action against *Streptococcus mutans* at an MIC of 8.0 μg/ml. The bactericidal test showed that kuwanon G completely inactivated *S. mutans* at the concentration 20 μg/ml in 1 min was reported by Park *et al*.,(2003).

Banso,(2009) reported the *Acacia nilotica* stem bark extracts contain alkaloids, saponins, cardiac glycosides, tannins, flavonoids and anthraquinones which have high inhibitory activity against *Streptococcus mutans* with a MIC in the range of 9.75-313μg/ml.

MIC of *Cocos nucifera* husk against *S.mutans* has been estimated to range mainly between 50 mg/ml and 100 mg/ml which was reported by Jose *et al*.,(2014).

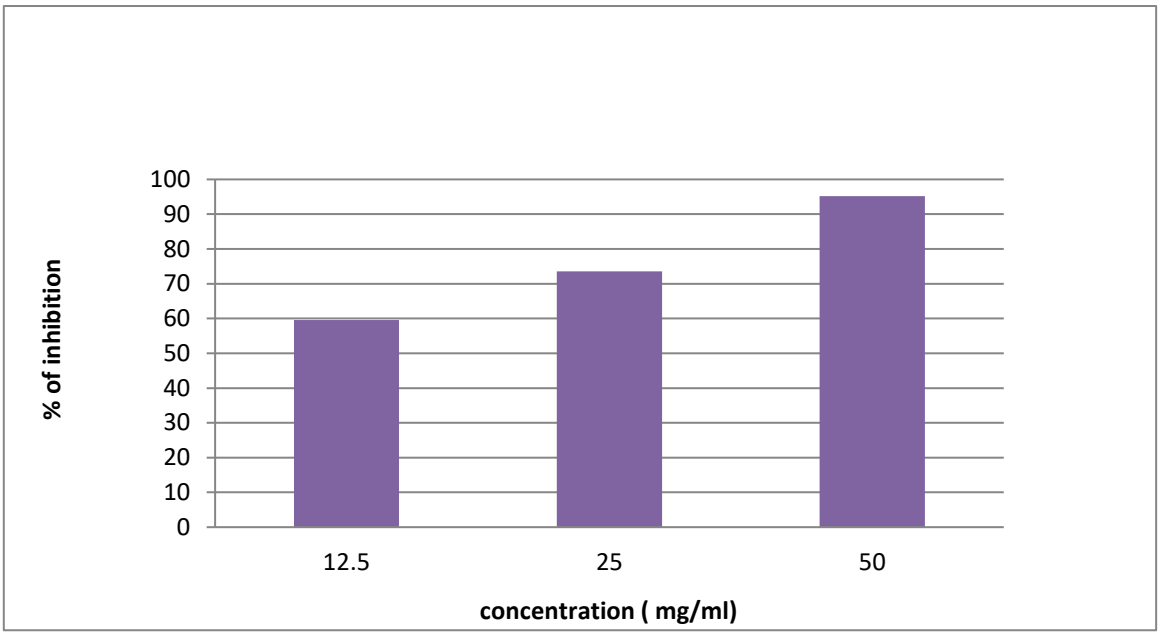
Kumarasamy *et al*.,(2014) study confirms that a crude aqueous extract (1000μg/ ml) of ripe *Morinda citrifolia* fruits effectively inhibited the growth of *S. mutans* and *S. mitis* with an MIC of 125 μg and 62.5 μg, respectively.

4.2 ANTIBIOFILM ASSAY

To investigate whether *Vaccinium macrocarpon* extract reduced biofilm formation, antibiofilm assay was done. The microorganisms are ubiquitous in nature which attach to the surfaces and produce extracellular polysaccharides. Dental plaque actually consists of hundreds of different bacterial taxa. Most of these bacteria exist on the surface of teeth in heterogeneous communities called plaque or biofilms. The mouth thus acts as a reservoir for these bacteria and are pathogenic and can cause tooth decay. Biofilm eradication is very difficult to achieve because host are strong and antibiotic resistance are also increasing. There are several mechanisms used to explain the resistance of biofilm to antimicrobial agents which makes it difficult to predict the behavior of biofilm cells. In the present study, antibiofilm activity of *Vaccinium macrocarpon* using the bacterial strains *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae* was evaluated after 24 hours and percentage biofilm inhibition was observed.

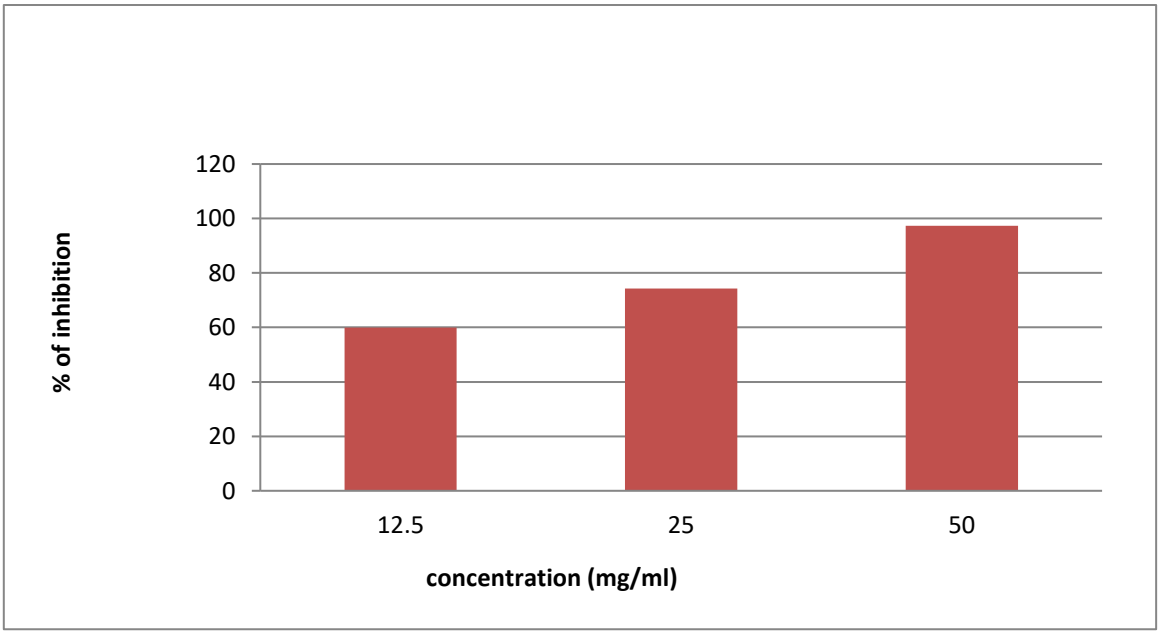
Antibiofilm activity of *Vaccinium macrocarpon* against *Streptococcus mutans*

Figure.2



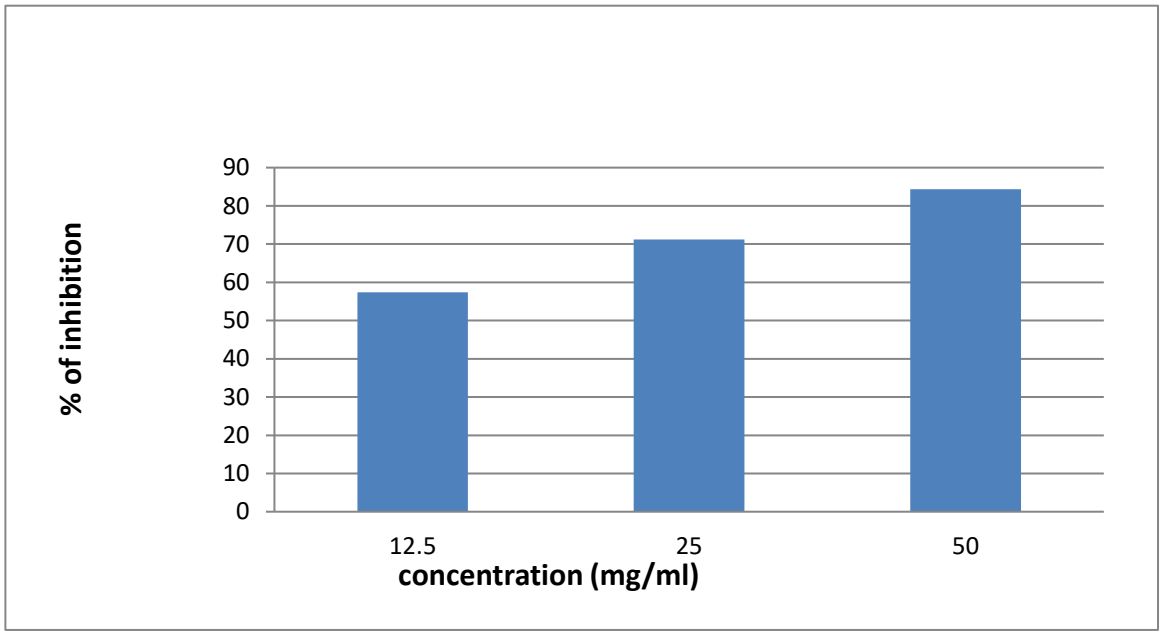
Antibiofilm activity of *Vaccinium macrocarpon* against *Pseudomonas aeruginosa*

Figure.3



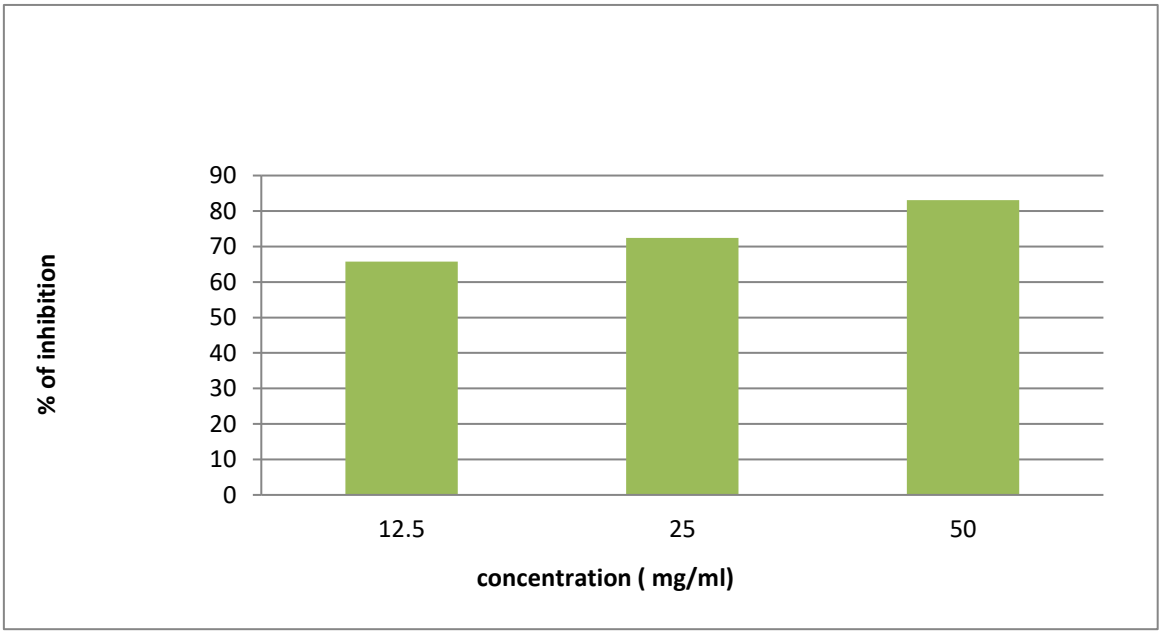
Antibiofilm activity of Vaccinium macrocarpon against *Klebsiella pneumoniae*

Figure.4



Antibiofilm activity of Vaccinium macrocarpon against Staphylococcus aureus

Figure.5



Vaccinium macrocarpon was found to exhibit antibiofilm effect against *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae.* The results depicted in the above figures, shows that *Vaccinium macrocarpon* exhibited antibiofilm activity, by inhibiting the growth of microorganisms. Higher concentration of the extract 50mg/ml showed maximum percentage of inhibition for all the four bacterial cultures *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae* at 95.2%, 83.07%, 97.3%, 84.35% after 24 hours. It confirmed that the phytochemicals of *Vaccinium macrocarpon* caused bacterial cell death and cell damage of dental caries causing microbes by binding to bacterial cell membrane. Thus, *Vaccinium macrocarpon* acted as a potential antimicrobial agent.

Subramenium *et al.(*2015) reported the biofilm inhibition assay with increasing concentrations of limonene against *Streptococcus pyogenes*. Crystal-violet stained biofilm biomass was quantified. The results unveiled a concentration-dependent increase in antibiofilm activity of limonene with significant reduction observed at concentrations above 350 mg/ml . The concentration (400 mg/ml) which showed 83% inhibition was considered as MBIC. Since clinical isolates are more potent in biofilm formation, the biofilm antagonistic activity of limonene at 400 mg/ml was evaluated against five clinical isolates of *Streptococcus pyogenes*. Limonene showed a remarkable antibiofilm activity (75– 95 %) against all the clinical isolates tested at 400 mg/ml. To delineate the antibiofilm efficacy of limonene against other species of the genus *Streptococcus*, its effect on the biofilm formation of *Streptococcus mutans* and *Streptococcus mitis* was also assessed. It was evident from the results that limonene indeed possesses a broad-spectrum antibiofilm activity against different species of *Streptococcus*.

Hanafiah *et al*.,(2018) reported the stem bark acetone extract of *Melastoma malabathricum* was reported previously to possess antibiofilm and antiadherence activity against *Streptococcus mutans*. They found that the biofilm formation of *S. mutans* reduced significantly in concentration dependent manner when it was treated with *Melastoma malabathricum*. Treatment with 80 mg/mL of extract inhibited 70% biofilm formation compared to only 20% at MIC value (10 mg/mL). DMSO did not inhibit biofilm formation of *S. mutans,* while penicillin reduced 85% of biofilm formation against non-treated bacteria.

Hu *et al.*,(2013) reported the curcumin can also inhibit sortase A in *S. mutans* cells. When 15 in μmol/l curcumin was added to culture media and incubated for 18 h, Pac was released into the supernatant. Furthermore, 15 in μmol/l curcumin significantly reduced the biofilm formation of *S. mutans*, and the reduced biofilm formation could not account for any differential rates of cell death. However, the mechanism may be driven by reduction of the *S. mutans* surface protein Pac. The potential anti-adhesion effect of curcumin on *S. mutans* in the formation of extracellular matrices and tooth surfaces, and the anti-adhesion effect occurred without influencing the growth of *S. mutans*.

Liu *et al*.,(2017) evaluated the effect of *Bergenia crassifolia* leave extracts on biofilms. *S. mutans* was grown in 96-well polystyrene plates for 24 h. The viability of *S. mutans* within biofilms decreased significantly upon treatment with all extracts/sub-extracts but with different sensitivities. Compared with control groups, the percentages of remaining bacteria in the biofilms were initially reduced in proportion to the concentration of extracts and then entered a stable phase at a higher level. In addition, the maximum inhibition was not significantly different among the test samples.These results indicated that *B. crassifolia* leaves could effectively inhibit the viability of biofilms of *S.mutans*.

Teanpaisan *et al*.(2014) reported the higher concentrations of *Artocarpus lakoocha* extract are required to significantly inhibit existing biofilm cells. This is an expected result since bacteria in the biofilm are strongly protected and less susceptible to antimicrobial agents than in planktonic form. It was shown that *A. lakoocha* extract was able to eradicate oral biofilm in a dose and time dependent manner. *A. lakoocha* extract acted as a potent antibiofilm agent that has dual actions preventing biofilm formation and eradication of existing biofilm.

These results are in line with our findings observed in our study.

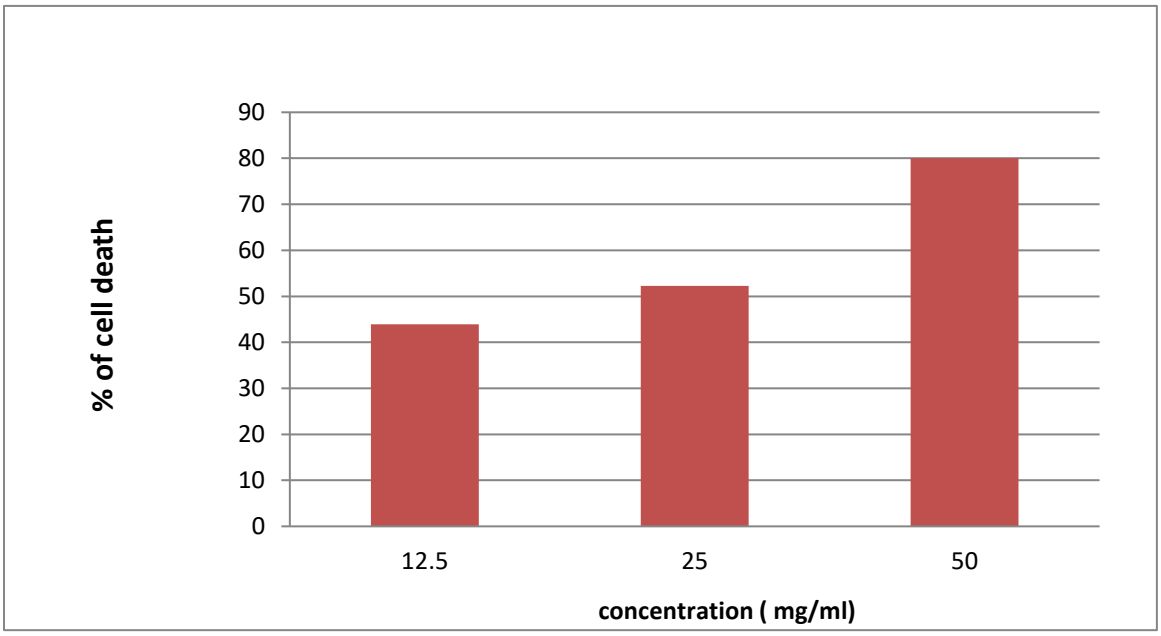
Inorder to be used in formulations,the extract should not be cytotoxic. So cytotoxicity was texted in *Vaccinium macrocarpon*.

4.3 Assessment of cytotoxicity of *Vaccinium macrocarpon*

Cell cytotoxicity refers to the ability of mediator cells to destroy living cells. By using a cytotoxic compound, healthy living cells can either be induced to undergo necrosis or apoptosis. The ability to accurately measure cytotoxicity can prove to be a valuable tool in identifying compounds that might pose certain health issues in living organisms. For evaluating the intracellular activity of the cell and for determining the cell viability the MTT assay is carried out. The assay is carried out using the bacterial strains *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae.* From the present study it was clear that when the concentration of the extract increased, the percentage of dead cells were also increased.

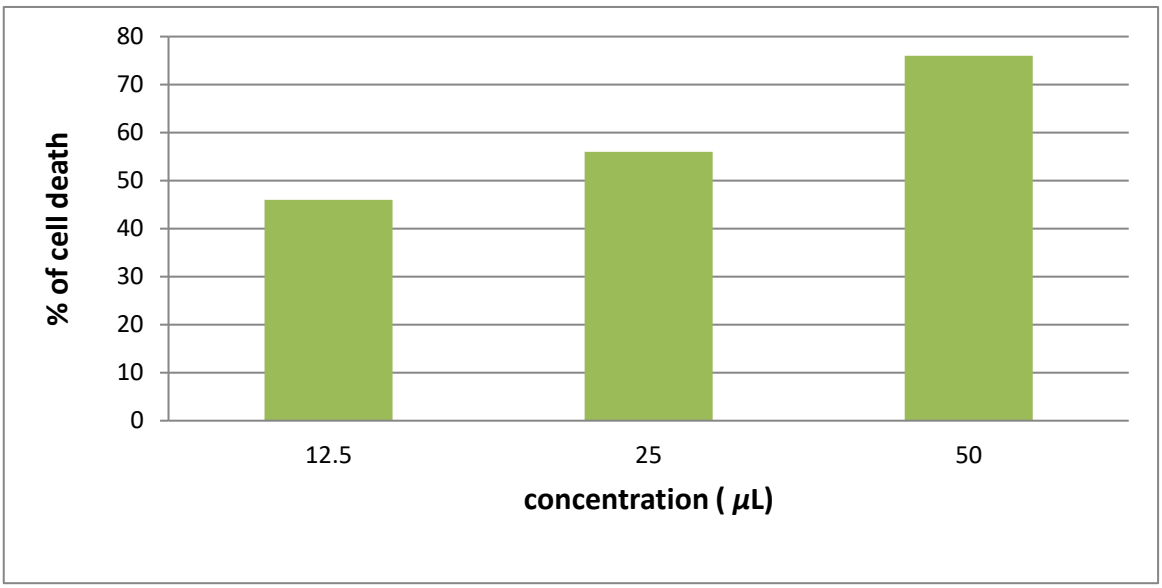
Antimicrobial activity of *Vaccinium macrocarpon* against *Pseudomonas aeruginosa* by MTT cytotoxicity assay

Figure.6



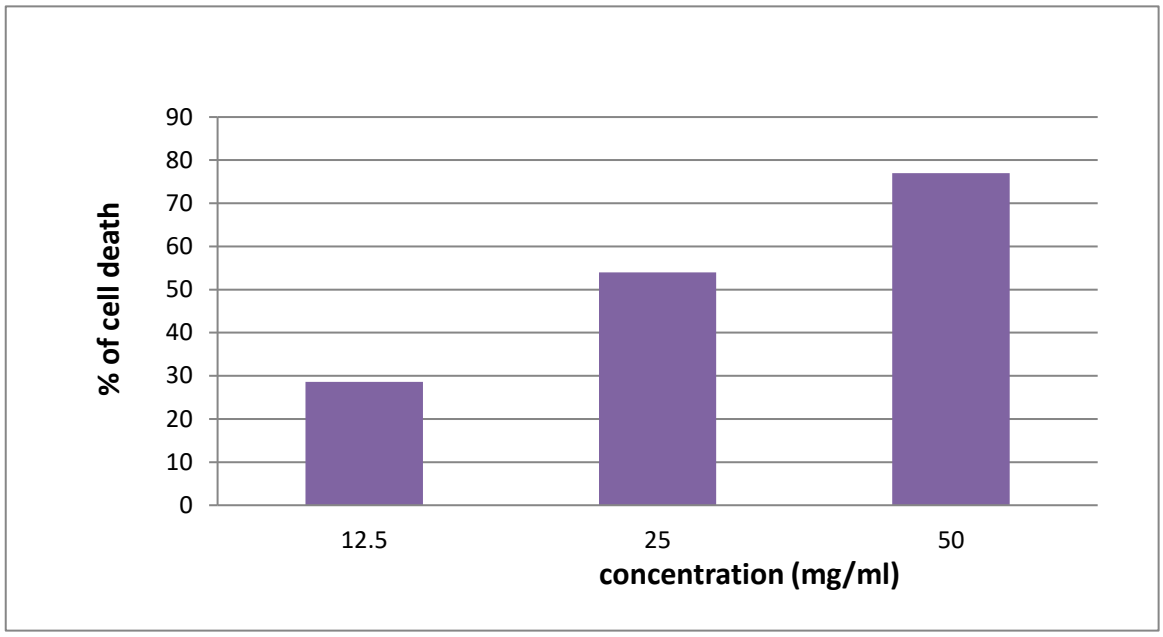
Antimicrobial activity of *Vaccinium macrocarpon* against *Staphylococcus aureus* by MTT cytotoxicity assay

Figure.7



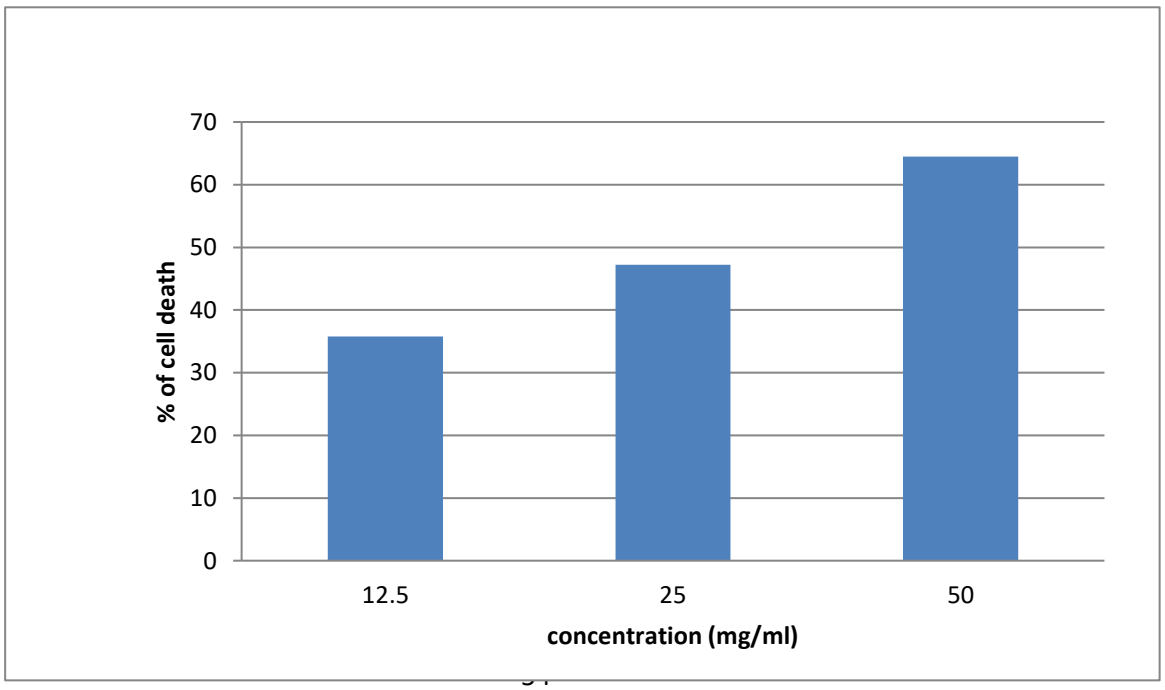
Antimicrobial activity of *Vaccinium macrocarpon* against *Streptococcus mutans* by MTT cytotoxicity assay

Figure.8



Antimicrobial activity of *Vaccinium macrocarpon* against *Klebsiella pneumonia* *by MTT cytotoxicity assay*

Figure.9



The results of MTT cell viability assay as shown in above figures clearly shows that the methanolic extract of *Vaccinium macrocarpon* at a concentration of 20mg/ml shows less cytotoxic effect. But as the concentration increased the cytotoxicity of the methanolic extract of cranberry also increased. When the concentration is 50mg/ml the percentage of the bacterial cell death of the microbes *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae* observed was 76%, 70%, 80%, 64%.The percentage of dead cells increased at the 60mg/ml and still the percentage can reach upto 100% by increasing the concentration of the methanolic extract of *Vaccinium macrocarpon.*

Anand *et al*.(2015) reported the microculture tetrazolium assay is a sensitive, quantitative, and reliable method to assess the cellular metabolic activity,The percentage survival rate of Human gingival fibroblast cells and V79 Chinese hamster lung fibroblast cells treated with cashew and mango was found to be significantly more than CHX-based and PI-based mouth rinses. This indicates less toxicity and long time usage of active components of these plants as an alternative to commercial mouth rinses.

Veiga Junior *et al.*,(2017) assessed CRO cytotoxicity in mouse peritoneal macrophage cultures by the MTT (3-[4,5- dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Oleoresin concentrations of 5, 50, and 500 mg/mL did not alter the viability of the macrophages.

Kreling *et al*.,(2016) examined the percentage metabolism of HaCat and OBA-9 cells. After exposure for 24 h, D6-17 did not affect the cell metabolism of either epithelial line at the concentrations tested. D1-23 showed toxicity at concentrations > 0.2 mM for both epithelial cells. LL-37 and CHX were the most cytotoxic peptides, demonstrating toxicity at concentrations > 0.02 mM for both cells.

Dental plaque in the oral cavity is recognized as the most complex oral biofilm. Microorganism cause a wide range of oral infection such as dental caries,periodental diseases and peri-complant diseases due to the formation of biofilm. The structural organisation of cells and thick exopolysaccharide matrix reduces the efficiency of topically applied agents and leads to the multidrug resistant microbes.(Fiboche *et al*., 2010) this has shifted the focus of oral health care to plant-based products.

4.4 TIME KILL ASSAY

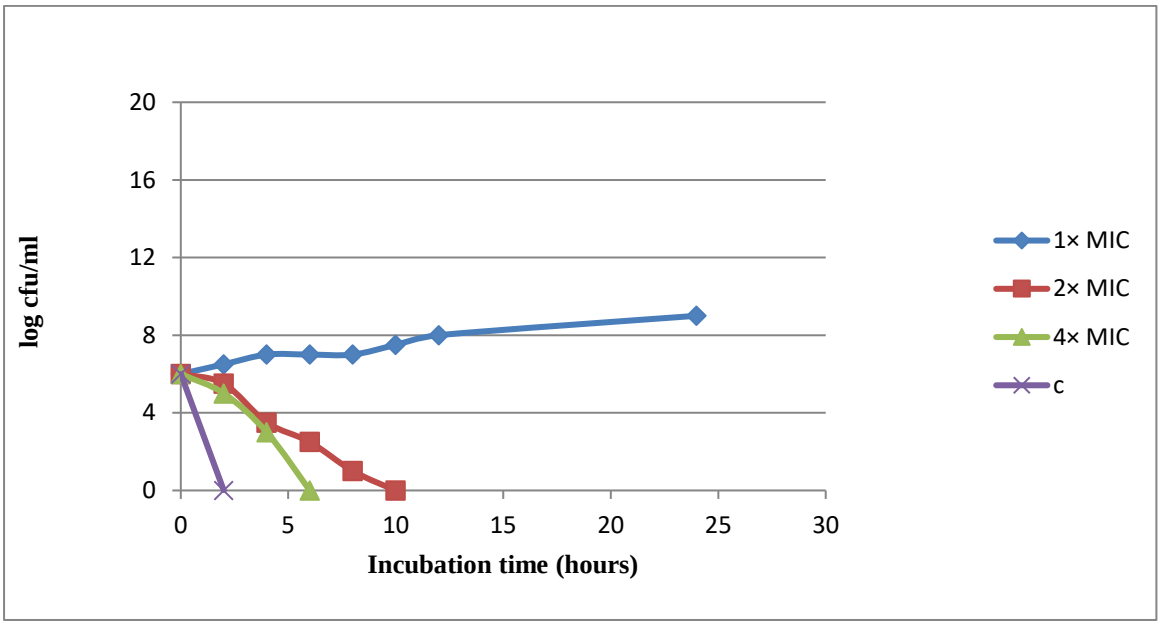
Bactericidal activity of *Vaccinium macrocarpon* was examined using time kill assay. Time Kill Test is a basic microbiology method for assessment of antimicrobial activity. The Time-Kill Kinetic study reveals the rate at which a microorganism is killed by a product at enough exposure time points such that a graph can be constructed modeling the decline in population over time to a point of extinction. In the present study,the time kill test was carried out to evaluate the methanolic extract of *Vaccinium macrocarpon* to reduce the microbial population ability and to assess its efficiency against selected dental bacterial strains. We expected that if the *Vaccinium macrocarpon* extract is having antimicrobial activity they would reuce the microbial population.

Time kill curves were constructed for all the four pathogens namely *Streptococcus mutans, Lactobacillus acidophilus, Staphylococcus aureus, Pseudomonas aeuroginosa, Klebsiella pneumoniae* was studied. The results showed the methanolic extract of cranberry was more effective in reducing the microbial count and the reduction was in a concentration and time dependent manner.

A graph was plotted with incubation time against log cfu/ml.

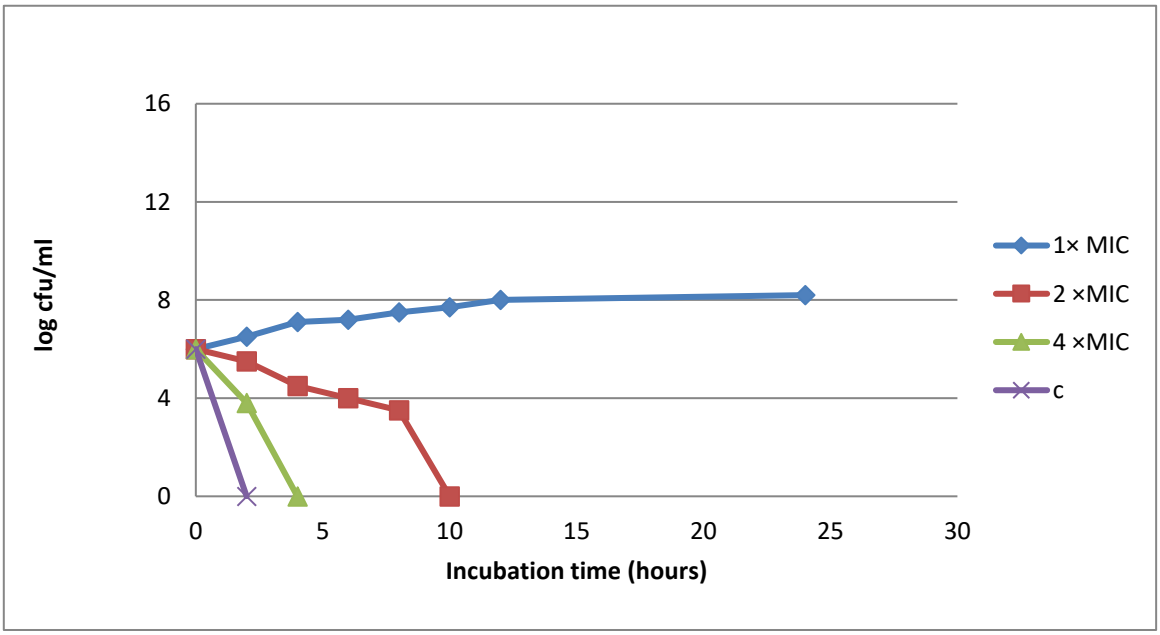
Time kill kinetics of *Vaccinium macrocarpon* against *Pseudomonas aeruginosa*

Figure.10



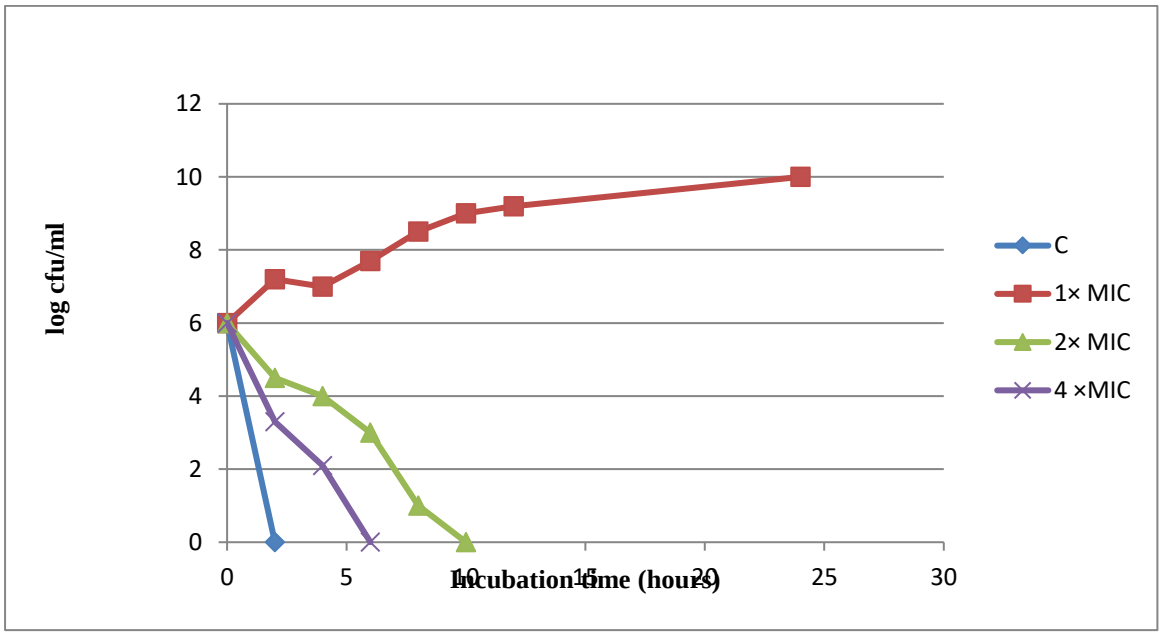
Time kill kinetics of *Vaccinium macrocarpon* against *Klebsiella pneumoniae*

Figure.11



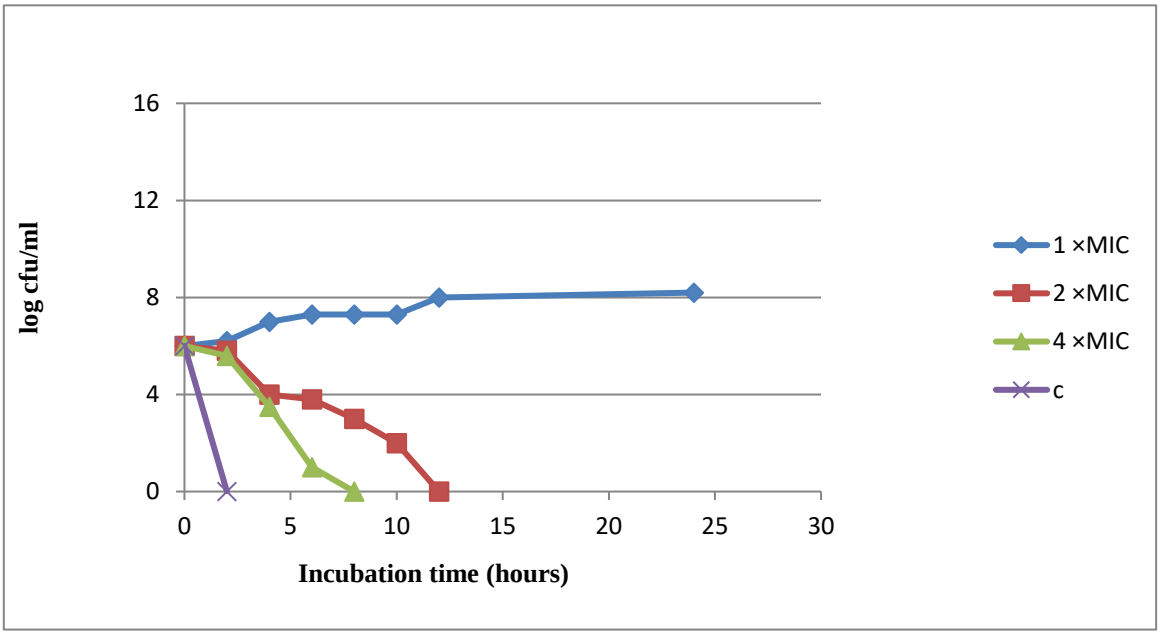
Time kill kinetics of *Vaccinium macrocarpon* against *Streptococcus mutans*

Figure.12



Time kill kinetics of *Vaccinium macrocarpon* against *Staphylococcus aureus*

Figure.13



The results depicted in the above figures 10,11,12,13 shows that the methanolic extract of *Vaccinium macrocarpon* reduces the number of microbial count on exposure to the 1×MIC, 2×MIC, 4×MIC at different time intervals. Ciprofloxacin antibiotic was used as control. Generally 1×MIC could reduce the number of CFU by approximately 50% although complete sterility could not be achieved. The time kill curve against the oral pathogens *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa*, *Klebsiella pneumoniae* at 1×MIC showed only the 50% of the microbial count reduction, whereas the control ciprofloxacin antibiotic showed the minimal microbial count within 30mins. At 4× MIC and 2× MIC, *S. mutans* was killed after 6 and 10 h, while *Staphylococcus aureus* was killed after 8 and 12 h, *Pseudomonas aeruginosa* was killed after 6 and 10h,and *Klebsiella pneumoniae* was killed after 4 and 10h.

Hanafiah *et al*.,(2018) reported the time-kill assay curve of SF12 against *S. mutans*. SF12 displayed bacteriostatic activity by reducing colony number of <3 log10 of CFU from the initial inoculum following incubation for 10 h with SF12 at 10–40 mg/mL concentrations. No growth inhibition was determined at 1.25–5 mg/mL of SF12. Initial exposure to SF12 at 2.5 mg/mL and 5 mg/mL caused no *S. mutans* growth but, re-growth was detected after 4h of treatment.

Teanpaisan *et al.,(*2014) reported the Time-kill curves were performed for 2 representative oral pathogens (*S. mutans* and *A. actinomycetemcomitans*); the killing activity depended on time and concentrations of A. lakoocha extract. Generally, 1× MIC could reduced the number of the CFU by approximately 50 %, although complete sterility was not achieved. At 4× MIC and 2× MIC, *S. mutans* was killed after 8 and 12 h, while *A. actinomycetemcomitans* was killed after 6 and 8 h, respectively (Figure 1). The killing of the positive control (CHX) was observed within 30 min.

Dziedzic *et al*.,(2015) reported the time kill kinetics study,where they observed a sudden decrease in the number of oral microorganisms compared to growth control and related to the high BECl concentration above 256 µg/mL. After 24 h of experiment, up to the BECl concentrations of 128 µg/mL, a total growth inhibition was recorded for all tested strains, and no remarkable change of OD values was observed. The reduction of oral *Streptococci* proliferation was observed mainly for higher BECl concentrations at the end of

experiment and longer incubation time (12–24 h), while lower concentrations up to128 µg/mL seemed not to affect noticeably the growth of some strains. It was demonstrated that a longer treatment with berberine had a deleterious effect on oral bacteria viability as natural products are supposed to have beneficial effect after recurrent exposure frequently at low concentrations.

Taweechaisupapong *et al*.,(2018) reported the results of time-kill assay showed that 1% lemongrass oil killed all 105 CFU/ml of *C. albicans* ATCC 10231 within 1 min. Therefore, 1% lemongrass gel was developed and tested for its therapeutic effect in a rat model of oral candidiasis. The dose based on the active constituent (citral) in 1% lemongrass gel was 0.83%. The results revealed that administration of lemongrass gel for two weeks significantly reduced the *C. albicans* CFU in oral tissues compared with gel base group ($P < 0.001$), but the difference between lemongrass gel and Daktarin ®-treated rats was not statistically significant.

Mossa *et al*.,(2013) reported the synergistic effect arising from the combination of β-lactams and FC111 in checkerboard assays was explored in greater details by using kill kinetic studies. Combination of the cranberry fraction FC111 at its MIC (1 mg/mL) and 1/8 × MIC of amoxicillin induced a strong bactericidal effect (>3 Log10 CFU/mL) against a bovine mastitis strain *S. aureus* Newbould, compared to the bactericidal effect observed with FC111 or amoxicillin alone. Moreover, the combination of FC111 at its MIC and oxacillin (1/512 × MIC) also showed strong bactericidal effect against *S. aureus*.

In our present study too,*Vaccinium macrocarpon* extract was found to inhibit the causative organisms.

4.5 Formulation of *Vaccinium macrocarpon* into gel

Local delivery of the drugs to the tissues of the oral cavity has a number of applications in the treatment of periodontal diseases like dental caries and root caries.Gels are typically semi-solid formulations having a liquid phase that has been thickened with other components. Uses of topical gel preparations along with the herbal extracts are for skin application or percutaneous penetration of medicament or local action to certain mucosal surfaces. The methanolic extract of the *Vaccinium macrocarpon* exhibited effective antibacterial activity. The oral gel formulation of *Vaccinium macrocarpon* extract was developed with carbapol 934.Formulation composition is given in table 1. The formulated gel was pinkish white in colour and the homogeneity of the gel was good. The pH of the gel was within 6-7, the normal pH range of the buccal cavity which substantiates that the prepared gels will be irritation free.

INGREDIENTS	QUANTITY
***Vaccinium macrocarpon* extract**	**100µl**
Carbapol 934	**0.3g**
Propylene glycol	**15 ml**
Glycerin	**5 ml**
Methyl paraben	**0.18g**
Propyl paraben	**0.02g**
Aspartame	**0.4g**
Distilled water	**Required quantity**

Table 1: Composition of gel formulation

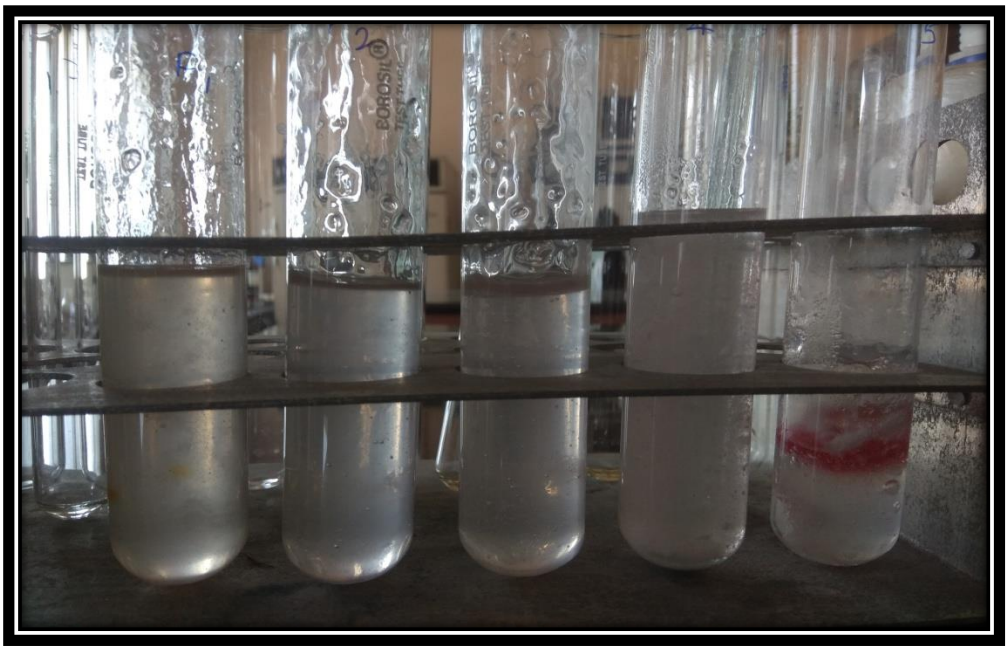


Plate 1: Formulated *Vaccinium macrocarpon* gel with different concentrations of carbapol.

Rajeshwari *et al.*,(2017) reported the formulation of thermoreversible gel with a novel thermosensitive polymer P407 (copolymer of polypropylene glycol and polyethylene glycol) which has the potential to form gel when exposed to higher temperature. This thermoreversible property of poloxamer was manipulated and concentration was optimized so as to deliver the CJC in periodontal pocket with temperature in the range of 33.4 - 36.1°C.It was observed that poloxamer at a concentration of 19%w/v and carbopol in the concentration of 0.2%w/v demonstrated desirable gelation temperature which is considered as the prime factor for optimization of gels.

Shende *et al.*,(2017) reported the tooth gel was formulated using the *Aloe vera* leaves extract and small amount of synthetic agent. The tooth gel was yellowish brown in colour, translucent in appearance and showed good homogeneity with absence of lumps.

Nasra *et al.*,(2017) reported the gel preparation using the cold method to prevent lumps formation in the case of hot process. Formulations G1 and G2 prepared by dispersing curcumin powder in cold carbopol– poloxamer solution, upon storage in refrigerator, only

partial curcumin precipitation was observed. For comparison, formulations G3, G4 and G5 containing solubilized curcumin were prepared using two different solvents, PEG400 and ethanol.

Khushali *et al.,(*2018) reported the formulation of papain and clove oil based chemo-mechanical caries removing gel. In this study, gel formulation was prepared using pectin as polymer, propylene glycol and triethanolamine as viscosity modifier, tween 80 as surfactant and preservatives and buffer components.Various formulations of papain-clove gel were tried by varying the concentration of other excipients such as viscosity modifier and surfactant.

Thombre *et al*.,(2018) reported the formulation of guava leaves extract into gel.All the three batches of developed formulation showed antifungal activity against *Aspargilious aureus* which are main microorganism responsible for mouth ulcer and formulations that can be used to treat mouth ulcer infection.

The gel prepared using *Vaccinium macrocarpon* extract was found to be good in appearance and stability. The antimicrobial activity of the gel needs to be assessed,and the pH of the gel formulation was compatible with normal pH range.

The present study reported the antibiofilm efficacy of *Vaccinium macrocarpon* extracts against *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia*. The results of cytotoxicity assay also revealed it to be effective against the organisms tested. The time kill assay also confirmed the bacteriostatic action of the extract. For its use, it was formulated into a gel to be applied to treat dental caries.

The results of the present study are summarized in the next chapter.

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Summary and Conclusion

5.0 SUMMARY AND CONCLUSION

Dental caries also known as cavities, is a breakdown of teeth due to acids made by bacteria. It is an epidemic, microbiological contagious disease of the teeth that ends in localized dissolution and damage of the calcified structure of the teeth. This disease occurs due to multiple factors such as interactions within the plaque community, host physiology, diet, fluoride, pH and the nature of the tooth enamel, and dominance of *Streptococcus mutans*. The time factor is significant for the commencement and development of caries in teeth.

The main instigation and progress of dental caries involves acidogenic and aciduric activity of Gram-positive bacteria and Gram negative bacteria such as *Streptococcus, Lactobacillus, Staphylococcus, Pseudomonas, Klebsiella and Actinomycetes* colonizing the supragingival biofilm which impede with usual nutrition intake, verbal communication, self-worth and daily habitual behavior. Complications may include inflammation of the tissue around the tooth, tooth loss, and infection or abscess formation. It can be treated with antibiotics which leads to side effects so an alternative use of natural herbal extracts is don.

Vaccinium macrocarpon extracts have been attracting ever-growing attention by dental researchers. A non-dialysable *Vaccinium macrocarpon* fraction enriched in high molecular weight polyphenols has very promising properties with respect to cariogenic and periodonto pathogenic bacteria. *Vaccinium macrocarpon* components are potential anti-caries agents since they inhibit acid production, attachment, and biofilm formation by *Streptococcus mutans*. Glucan-binding proteins, extracellular enzymes, carbohydrate production, and bacterial hydrophobicity, are all affected by cranberry components. The above-listed effects suggest that *Vaccinium macrocarpon* components, especially those with high molecular weight, could serve as bioactive molecules for the prevention and treatment of oral diseases.

The cytotoxic activity of methanolic extract of *Vaccinium macrocarpon* was analysed by using MTT cells cytotoxicity assay. The assay were carried out to check the cytotoxity of the *Vaccinium macrocarpon*. Different concentration of *Vaccinium macrocarpon* was used at varying time intervals. The results showed that if the concentration increased the percentage of the dead cells also increased. *Streptococcus mutans, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae* were used for checking cytotoxicity.

Since the *Vaccinium macrocarpon* exhibited cytotoxic activity, it indicates that it may have antibiofilm activity. When tested, the biofilm formed by the pathogenic microorganisms

were inhibited by the methanolic extract of *Vaccinium macrocarpon*. The extract exhibited strong antibiofilm inhibition to the growth of *Streptococcus mutans, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae*. It was a dose and time dependent activity. So its clear *Vaccinium macrocarpon* was cytotoxic to the microbial cells.

The time kill test is a basic microbiology test used in the assessment of antbacterial activity. In the present study the time kill test was carried out to evaluate the methanolic extract of *Vaccinium macrocarpon* to reduce the microbial population ability and to assess its efficiency against selected bacterial strains. The methanolic extract against the four bacterial strains showed effective reduction of the microbial count at different time intervals. Whereas at 1× MIC concentration showed only 50%reduction of the microbial count although complete sterility could not be achieved. But at 2× MIC and 4× MIC complete reduction of the microbial count at specific hours was observed.

The methanolic extract of the *Vaccinium macrocarpon* exhibited effective antibacterial activity. So,the *Vaccinium macrocarpon* extract is formulated into gel which can be used for the topical applications. Local delivery of the drugs to the tissues of the oral cavity has a number of applications including in the treatment of periodontal diseases like dental caries and root caries. Gels are typically semi-solid formulations having a liquid phase that has been thickened with other components. Uses of topical gel preparations along with the herbal extracts are for skin application or percutaneous penetration of medicament or local action to certain mucosal surfaces. The oral gel formulation of *Vaccinium macrocarpon* extract was developed with carbapol 934. The formulated gel was pinkish white in colour with good homogeneity and the pH of the gel lie within 6-7, which lie within the normal pH range of the buccal cavity so the prepared gels will be irritation free.

Scanning electron microscopy was done in order to examine the morphology changes of each representative bacterial strain after treating with DMSO which served as control and when treated with *Vaccinium macrocarpon* it act as the treated sample.

The results of the present study showed that the *Vaccinium macrocarpon* components are potential anti-caries agents since they inhibit acid production, attachment, and biofilm formation by *Streptococcus mutans, Pseudomonas aeruginosa, Klebsiella pneumoniae and Staphylococcus aureus.* Also it was clear that *Vaccinium macrocarpon* was cytotoxic to the microbial cells which was assessed by cytotoxicity assay. The time kill assay depict the reduction of microbial count at the different time intervals. Scanning electron microscopy

showed the morphological changes of the bacteria on treating with the extract. With the above activities it is more effective to prepare topical gel which can be used for treating dental caries.

Recommendations for future research

1. The anticariogenic activity of the gel can be further analysed.
2. The mechanism of action can be probed.
3. Other extracts of *Vaccinium macrocarpon* can be investigated and active compound can be isolated for future studies.

Appendices

APPENDIX-1

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION

(Mutmainnah *et al.,*2019)

PRINCIPLE

The minimum inhibitory concentration (MIC) was the lowest concentration of the juice that prevented visible growth of bacteria. The antibacterial assay was performed using the broth micro dilution method. The absence of microbial growth was interpreted as an antibacterial activity.

REAGENTS

1. Muller Hinton Agar Medium
2. Muller Hinton broth
3. Bacterial strain

PROCEDURE

The minimum inhibitory concentration (MIC) was the lowest concentration of the juice that prevented visible growth of bacteria. The antibacterial assay was performed using the broth micro dilution method. A twofold serial dilution of the juice was prepared in Mueller-Hinton Broth (MHB). For every experiment a negative control (distilled water, medium, and inoculums) was included. The negative control consisted of broth and bacterial cell suspension without the agent, and the blank control contained only the medium. 100 μL of Mueller-Hinton Broth plus different concentrations of cranberry ***(****Vaccinium macrocarpon*) extracts was prepared and transferred to each micro plate well to obtain dilutions of the active extract, ranging from 1.0 to 25 mg/mL. Then, 10 μL of a fresh culture of test organisms was added. Micro plates were incubated at 37°C for 24 h. The MIC end-point was defined as the lowest concentration of the test agent that completely inhibited growth or produced at least 90 % reduction of absorbance in comparison with the negative control. All experiments were performed in triplicate and the average values were reported as MIC. Microbial growth in each well was determined by observing and comparing the well with the negative control. The absence of microbial growth was interpreted as an antibacterial activity.

APPENDIX-2

TIME KILL ASSAY

(Teanpaisan *et al.*,2014)

PRINCIPLE

Bactericidal activity of cranberry extract was examined using a time kill assay. It is the most appropriate method for determining the bactericidal effect. It is a strong tool for obtaining information about the dynamic information between the anti-microbial agent and the microbial strain. The time kill test reveals a time dependent or a concentration dependent antimicrobial effect.

REAGENTS

1. Bacterial strain
2. ciprofloxacin

PROCEDURE

Growing cultures of each representative strain,(*Streptococcus mutans, Klebsiella pneumonia, Pseudomonas aeuroginosa, Staphylococcus aureus*) were added to appropriate medium and exposed to 1× 2× and 4× the MIC of *vaccinium macrocarpon* extracts. Samples were taken for colony counts at 0^{th}, 30^{th} min and, 2, 4, 6, 8, 10, 12 and 24 h. The viable counts were determined after appropriate incubation and each experiment was performed in triplicates. Ciprofloxacin (0.1 %) and extract free medium were used as the positive and negative controls, respectively.

APPENDIX-3

ANTIBIOFILM ASSAY

(Sanchez *et al.*,2016)

PRINCIPLE

The ability of the *vaccinium macrocarpon* extracts to inhibit the biofilms assayed by antibiofilm assay. Biofilms provide a reservoir for microbial cells. Its dispersion enhances the chronic and persistent infections. It also promotes the reinjection of colonized sites

REAGENTS

1. Trypticase soy broth
2. Bacterial strains
3. 1% crystal violet
4. 95% ethanol

PROCEDURE

Overnight culture of *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeuroginosa, Klebsiella pneumonia* in trypticase soy broth was diluted in the ratio 1:100 in respective fresh medium and allowed to grow for another hour.100µl of the diluted strains were added to 96 well titre plate and the different concentrations of *vaccinium macrocarpon* extracts were added and incubated at 37ºC for overnight. After the incubation the medium was removed and 100 µl of crystal violet solution was added and incubated at room temperature for 30 minutes. The dye was removed after staining and the wells were washed thoroughly with distilled water and finally incubated with 95% ethanol for 15 minutes and read in spectrophotometer at 595nm. Inhibition mediated reduction of biofilm formation was calculated by the following formula

$$\% \text{ of inhibition} = \frac{\text{OD of control} - \text{OD of treatment}}{\text{OD of control}} \times 100$$

APPENDIX-4

MTT ASSAY

(Subaramenium *et al.*,2015)

PRINCIPLE

The 2-(4,4-dimethyle-2-tetrazoyl) 2-diphenyle-2 tetrazolium salt (MTT) is converted into formazan derivative by living cells. The amount of formazan formed is measure of the number of survival cells. After solubilizing the formazan in a suitable solvent the cell viability can be measured in microtitre plate reader.

REAGENTS

1. Bacterial strains
2. Acid propanol
3. MTT salt

PROCEDURE

Overnight culture of *Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeuroginosa, Klebsiella pneumonia* was prepared in nutrient broth and 100 µl of the overnight culture were added to 96 well titre plate and the different concentrations of *vaccinium macrocarpon* extracts were added. The cells were incubated for one hour. And added 50 µl of MTT to 100 µl of treated cells of strain and incubated at 37ºC for 3 hours. Then 200 µl of acid-propanol was added to it after incubation and left overnight in dark room. The cell viability was noted at 650nm in a microtitre reader by fixing the control group as 100%viability and the percentage of viable cells were calculated relative to other treatment groups.

$$\% \text{ of cell viability} = \frac{\text{OD of control} - \text{OD of treatment}}{\text{OD of control}} \times 100$$

APPENDIX-5

FIELD EMISSION SCANNING ELECTRON MICROSCOPY

(Hanafiah *et al*., 2018)

PRINCIPLE

Field Emission Scanning Electron Microscopy (FESEM) is often used for imaging and characterization purposes. It is a type of electron microscope that images a sample by scanning it with a high – energy beam of electrons. The electrons interact with the atoms that make up the sample producing signals that contain information about the samples surface topography, composition and other properties such as electrical conductivity. FESEM can produce very high – resolution images of a sample surface revealing details about less than 1 to 5 nm in size.

PROCEDURE

A scanning electron microscopy (SEM) was performed to examine the morphology changes of each representative strain in *S. mutans* after treatment with 10 % DMSO or 0.1 mg/ml of *Vaccinium macrocarpon* extract as the control and treated sample, respectively. After 8 h incubation at 37 °C in an appropriate condition, the bacterial pellet was collected and washed twice with phosphate buffered saline with pH 7 by centrifugation at 1000 rpm at 4 °C for 5 min. The bacterial pellet was fixed overnight in 2.5% glutaraldehyde and 0.1 M cacodylate buffer at cool temperature, and then dehydrated in a graded series of ethanol solutions for 30 min. The samples were subsequently dried by a critical point drying method and coated with gold. The microbial morphology was observed with a field emission SEM.

APPENDIX-6

FORMULATION OF *Vaccinium macrocarpon* EXTRACT INTO GEL

(Vinita *et al*.,2013)

Carbopol 934 gels were prepared by soaking carbopol in water and by neutralizing with triethanolamine to pH 6.4. Weighed amount of methyl and propyl paraben were added to water prior to the addition of carbopol 934. In another beaker the required quantity of propylene glycol was taken in another test tube to which accurately measured amount of cranberry fruit extract corresponding to its MIC was incorporated and finally this mixture was added to the beaker containing carbopol with stirring. Sweetening agent aspartame was also added to the polymer dispersion and stirred continuously till it forms a homogeneous product. The volume was made up with distilled water and stirring was done vigorously. All the prepared gels were then subjected to evaluation tests in order to select the best formulation.