Antibiofouling effect of polyphenols on streptococcus biofilms

Varunraj Sendamangalam
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A Thesis

entitled

Antibiofouling Effect of Polyphenols on *Streptococcus* Biofilms

by

Varunraj Sendamangalam

Submitted to the Graduate Faculty as partial fulfillment of the
requirements for a Master of Science in Bioengineering

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The University of Toledo
August 2010
An Abstract of

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Biofouling is a process of surface colonization of microorganisms that adhere to
various surfaces, by producing extracellular polymers (polysaccharides and proteins,
exopolymers hereafter). Dental plaque is one such example. It often causes serious
problems in chemical, medical and pharmaceutical industries. Recently, we demonstrated
that some natural phenolic compounds found in plants have an antibiofouling effect on
Gram-negative bacteria biofilm formation. In this study, we tested the antibiofouling
activities of three natural polyphenols; gallic acid, tannic acid, and quercetin. Ascorbic
acid (vitamin C) and salicylic acid were also tested for comparison. Most natural
polyphenols are antimicrobial and antioxidant also. To distinguish the antibiofouling effect of the polyphenols from their antimicrobial effects, first antimicrobial activities of the polyphenols were investigated in a separate experimental setting. A plate dilution assay was used to assay the antimicrobial effect of the polyphenols. The concentrations of the polyphenols to be used in antibiofouling tests were determined as minimum inhibitory concentrations (MICs) below which the microorganisms didn’t die.

As these polyphenols are well known as strong antioxidants, it was suggested that the antimicrobial effect may be caused by the polyphenols’ antioxidant effect. To verify the contribution of antioxidant capacity to the antimicrobial effect, DMPD and ABTS decolorizing assays were used to evaluate the antioxidant activities. The results indicate that there are relations between the antimicrobial effect and antioxidant capacity of polyphenols.

The antibiofouling activities of polyphenols were measured using a modified micro-titer plate assay. The polyphenols at the concentrations lesser than the minimum inhibitory concentration (MIC) were observed to reduce the biofilm formation. It was hypothesized that the reduction of biofilm in the presence of the polyphenols was due to the inhibition of exopolymers-producing enzymes by the polyphenols. Glucosyltransferase and Fructosyl transferse enzymes produced by Streptococcus mutans are responsible for exopolymers production, and exopolymers are a major component in biofilm formation. Glucosyltransferases and Fructosyltransferases were separated from a culture medium, and then tested for inhibition by the polyphenols. All the polyphenols were observed to inhibit the enzymes, and when they were inhibited, much less biofilms were formed on a solid surface.
The polyphenols investigated in this study killed the *S. mutans* via antioxidant activities, and reduced biofilm formation through inhibition of exopolymers-producing enzymes, glucosyltransferase and fructosyltransferase.

It was hypothesized that the exopolymers-producing enzymes were inhibited by the polyphenols, and as a result, exopolymers were not synthesized and no biofilms were formed. Results suggested that these polyphenols were not only good antimicrobials but also very good antioxidants and antifouling agents.

**Key words.** MIC, antioxidants, antimicrobials, glucosyltransferase, polyphenols, biofilm, exopolymer.
This thesis is dedicated to my wonderful parents and my brother for their love, endless support and encouragement throughout.
Acknowledgements

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Table of Contents

Abstract ........................................................................................................................................ iii
Acknowledgements ................................................................................................................ vii
Table of Contents ...................................................................................................................... viii
List of Tables ............................................................................................................................. xi
List of Figures ............................................................................................................................ xii
List of Abbreviations ................................................................................................................. xiv
List of Symbols ........................................................................................................................ xv
Chapter 1 - Introduction to Biofilms................................................................. 1
1.1 Biofouling and biofouling control ....................................................................................... 1
1.2 Research objectives ............................................................................................................. 4
Chapter 2 - Novel Approaches to Control Dental Plaque Biofilms......................... 5
2.1 Streptococcus mutans ........................................................................................................ 5
   2.1.1 Physiological and biochemical properties ..................................................................... 5
2.2 Dental plaque as a biofilm ............................................................................................... 7
   2.2.1 Mechanism of formation of dental plaque ..................................................................... 7
2.3 Control of biofilms ............................................................................................................ 9
2.4 Polyphenols ....................................................................................................................... 9
   2.4.1 Polyphenols as antimicrobials .................................................................................... 10
   2.4.2 Polyphenols as antioxidants .................................................................................... 13
Chapter 3 - Antibiofouling Effect of Polyphenols against *Streptococcus mutans* ..... 17

3.1 Abstract .......................................................................................................................... 17

3.2 Introduction .................................................................................................................. 18

3.3 Material and methods ................................................................................................. 21

3.3.1 Bacterial strain .......................................................................................................... 21

3.3.2 Materials .................................................................................................................. 21

3.3.3 Antimicrobial assays .............................................................................................. 22

3.3.4 Microtiter-plate test ............................................................................................... 23

3.3.5 Enzyme activity assay ............................................................................................ 24

3.4 Results and discussion ............................................................................................... 25

3.4.1 MIC of polyphenols ............................................................................................... 25

3.4.2 Antibiofouling activity of polyphenols .................................................................. 28

3.4.3 Enzyme inhibition by polyphenols ........................................................................ 31

3.5 Conclusions .................................................................................................................. 33

Chapter 4 - Antimicrobial and Antioxidant Activities of Polyphenols against *Streptococcus mutans* ........................................................................................................ 35

4.1 Abstract ....................................................................................................................... 35

4.2 Introduction .................................................................................................................. 36

4.3 Materials and methods ............................................................................................... 38

4.3.1 Bacterial strain .......................................................................................................... 38

4.3.2 Materials .................................................................................................................. 38

4.3.3 Determination of antimicrobial activity (Plate dilution assay) ............................. 39

4.3.4 Determination of antioxidant activity ..................................................................... 39
4.3.4.1 DMPD/FeCl₃ spectrophotometric method ........................................................................ 39
4.3.4.2 ABTS decolorization assay ....................................................................................... 40

4.4 Results and discussion ........................................................................................................ 41
4.4.1 Antimicrobial activity measurement ........................................................................... 41
4.4.2 Comparison between ABTS⁺ and DMPD⁺ assay ......................................................... 42

4.5 Conclusions ....................................................................................................................... 47

Chapter 5 - Recommendations for future research ............................................................... 50

References .................................................................................................................................... 52
List of Tables

Table 3.1 - The Minimal Inhibitory Concentration (MIC) of polyphenols against *S. mutans* ................................................................. 26

Table 3.2 - Absorbance of the biofilm in the micro-titer plate and the percentage of reduction of absorbance from the control. ................................................................. 30

Table 3.3 - Percentage of inhibition of glucosyl-transferase and fructosyl transferase enzyme activities ......................................................................................... 32

Table 4.1 - % of Inhibition of the DMPD radical in the presence of the different polyphenols. ........................................................................................................... 44

Table 4.2 - % of Inhibition of the ABTS radical in the presence of the different polyphenols. ........................................................................................................... 46

Table 4.3 - Results of DMPD and ABTS assay expressed as Trolox equivalents .......... 47

Table 4.4 - Antimicrobial effects and antioxidant capacities of polyphenols against *S. mutans*. ........................................................................................................... 48
List of Figures

Figure 1.1 - General view of the absorption, attachment, formation and growth of a biofilm over time. .......................................................... 2
Figure 1.2 - Biofilms found on teeth (top left), rocks and pebbles (top right), inside of pipes (bottom left) and human biofilm infection sites (bottom right). ...................... 3
Figure 2.1 - A microscopic gram stained image of *Streptococcus mutans*. .................. 6
Figure 2.2 - Basic steps involved in dental plaque biofilm formation. ......................... 8
Figure 2.3 - Most commonly used antibacterial products in day to day life. ............ 11
Figure 2.4 - Best sources of antioxidants. ................................................................. 14
Figure 3.1 - Overview of *S. mutans* basic metabolic pathways. ............................ 20
Figure 3.2 - The structures of the polyphenolic compounds used in this study. 1. ascorbic acid, 2. gallic acid, 3. salicylic acid, 4. quercetin, 5. tannic acid. ......................... 22
Figure 3.3 - Antimicrobial activities of polyphenols against *S. mutans*; control (♦), ascorbic acid (■), gallic acid (▲), salicylic acid (×) acid, tannic acid (+) and quercetin (●). .................................................. 27
Figure 3.4 - Pictures of *Streptococcus mutans* biofilm taken at 24 and 36 hrs after the addition of dimethyl sulfoxide to the microtiter-plate. ....................................................... 28
Figure 3.5 - Absorbance of the biofilm formed in the presence of various polyphenolic compounds; biofilm formation after 24 hrs (■) and biofilm formation after 36 hrs (□). 29
Figure 3.6 - HPLC chromatogram of sucrose (1), glucose (2) and fructose (3). ....... 31
Figure 3.7 - Effect of glucosyl- and fructosyl-transferase enzymes on the glucose (■) and fructose (□) formation in the presence of natural polyphenols. .................................................. 32

Figure 4.1 - Change in absorbance of the DMPD radical after the addition of different polyphenols. ...................................................................................................................... 43

Figure 4.2 - Change in absorbance of the ABTS radical after the addition of different polyphenols. ...................................................................................................................... 45

Figure 4.3 - Comparison of antioxidant capacities and antimicrobial activities of the polyphenols. ...................................................................................................................... 49
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>2,2’-azinobis-3-ethylbenzothiazoline-6-sulfonic acid</td>
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<td>DMPD</td>
<td>N,N-dimethyl-p-phenyldiamine dihydrochloride</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-Diphenyl-1-picrylhydrazyl</td>
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<tr>
<td>CFU</td>
<td>Colony forming unit</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNS</td>
<td>Dinitrosalicylic acid</td>
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<tr>
<td>EPS</td>
<td>Exopolymeric substance</td>
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<td>GTF</td>
<td>Glucosyl-transferase</td>
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<tr>
<td>FTF</td>
<td>Fructosyl-transferase</td>
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<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
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<tr>
<td>Nm</td>
<td>Nanometer</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>pH</td>
<td>Potential for hydrogen ion concentration</td>
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<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
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<tr>
<td>UV-vis</td>
<td>Ultraviolet-visible</td>
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<tr>
<td>YN</td>
<td>Yeast nutrient</td>
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**List of Symbols**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Unit</th>
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<tbody>
<tr>
<td>mL</td>
<td>milliliter</td>
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<td>µL</td>
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CHAPTER 1

Introduction to Biofilms

1.1 Biofouling and biofouling control

A microbial biofilm is composed of living, reproducing microorganisms, such as bacteria, and algae, that exist as a colony, or community. It is normally characterized by a complex aggregation of microorganisms on a solid surface. Usually the biofilm is held together and protected by a matrix of excreted polymeric compounds called the exopolymers.

Biofilms have become a “hot” topic in environmental and infectious disease microbiology as well in the popular press. A biofilm forms when bacteria adhere to solid surfaces in aqueous environments and begin to excrete a slimy, glue-like substance that can anchor them to all kinds of material – such as metals, plastics, soil particles, medical implant materials, and tissue. Essentially, biofilms may form on any surface exposed to bacteria and some amount of water. Once anchored to a surface, biofilm microorganisms carry out a variety of harmful or beneficial reactions (by human standards), depending on the surrounding environmental conditions. They are common in nature as different bacteria have different mechanisms for their attachment to the surface and with other bacteria.
Biofilms are the preferred method of growth for many and perhaps most species of bacteria [Roberts et al. 1999]. This method of growth provides a number of advantages to colonizing species. A major advantage is the protection; the biofilm provides colonizing species with protection from competing microorganisms and environmental factors such as host defense mechanisms, and from potentially toxic substances in the environment, such as lethal chemicals or antibiotics. Biofilms also can facilitate processing and uptake of nutrients, removal of potentially harmful metabolic products as well as the development of an appropriate physicochemical environment. A cartoon depicting the general steps involved in biofilm formation is shown in Figure 1.1.

![Figure 1.1 - General view of the absorption, attachment, formation and growth of a biofilm over time. (Source: Online Textbook of Bacteriology, 2008)](image)

Biofilms may be found virtually anywhere and contribute to a range of costly problems in daily life in medical, chemical, pharmaceutical and industries. It ranges from biodeterioration of materials to public health problems. Biofilms are also a persistent source of chronic infection in patients. They colonize a widely diverse set of moist surfaces, including the oral cavity, on the rocks and pebbles at the bottom of most streams and rivers, bottom of boats and docks, the inside of pipes and rocks in streams
(Figure 1.2). In medicine, biofilms spreading along implanted tubes or wires can lead to pernicious infections in patients [Socransky et al. 2005].

![Figure 1.2 - Biofilms found on teeth (top left), rocks and pebbles (top right), inside of pipes (bottom left) and human biofilm infection sites (bottom right). (Source: www.poolcare.net)](image)

Once a biofilm is formed it is very hard to remove, as the microorganisms growing in it are resistant to antimicrobial agents than planktonic cells [Suci et al. 1994; Puverdorj-Gage et al. 2005; Wimpenny et al. 2000; Zhang et al. 1996]. Using high concentrations of antimicrobials is usually not effective because antimicrobials cannot completely kill the cells due to diffusion limitation, and furthermore it can also lead to
antibiotic resistance of the cells. Therefore, it can be said that not all the good antibiotics are effective biofilm agents and that is why most of the antibiotics fail in preventing or removing biofilm contamination in medical devices.

As stated above antimicrobial agents become ineffective over a period of time due to their loss of activity and inability to penetrate the biofilm effectively. There are a lot of methods available that can be used to control the biofilms namely mechanical cleaning, using antimicrobial chemicals, acidic and alkaline solutions, enzymes etc. Although the above methods have had some success in destroying or preventing biofilm formation, they suffer from a lot of disadvantages namely high cost, loss of activity with time, degradation of these antimicrobials over a period of time, and harmful impact on human health and ecology. To address these problems, we have used 4 well-known, stable, cost effective, sustainable natural phenolic compounds in our research against biofouling of *S. mutans*. Ascorbic acid was used as a control for comparison.

### 1.2 Research objectives

The main goals of this thesis are 1) to examine the antimicrobial and antioxidant activity of phenolic compounds on *Streptococcus mutans*, 2) to investigate the effect of the natural polyphenols on biofilm formation of *S. mutans*, and understand the anti-biofouling mechanisms of the natural polyphenols.
CHAPTER 2

Novel Approaches to Control Dental Plaque Biofilms

2.1 Streptococcus mutans:

Clarke first described *S. mutans*, a Gram-positive, facultative anaerobic bacterium in 1924 [Clarke 1924]. Streptococci constitute a major population in the oral cavity, with several different species colonizing the various ecological niches of the mouth. *S. mutans* cells are about 0.5 - 0.75 µm in diameter occurring in pairs or in short - or medium-length chains, without capsules. Under acid conditions in broth and on some solid media, these coccii may form short rods 1.5-3.0 µm in length. The organism can be isolated frequently from humans and rats feces. We chose this strain because of its common occurrence in human health issues. It also grows fast and produces significant amount of exopolymers, which are beneficial to our research.

2.1.1 Physiological and biochemical properties

i. It contains a cell-bound protein, glucosyl-transferase, which serves as an adhesin for attachment to the tooth, and as an enzyme that polymerizes dietary sugars into exopolymeric substances that lead to the formation of plaque biofilm.
ii. It produces lactic acid from the utilization of dietary carbohydrate, which demineralizes the tooth enamel. *S. mutans* produces more lactic acid and is more acid-tolerant than most other streptococci.

iii. It stores exopolymers made from dietary sugars that can be utilized as reserved carbon and energy sources for production of lactic acid.

![Image of Streptococcus mutans](image)

**Figure 2.1 - A microscopic gram stained image of *Streptococcus mutans.***

A microscopic gram stained image of a stationary phase grown culture of *S. mutans* is shown in Figure 2.1. *S. mutans* are known to be important in the initiation of dental caries because its activities lead to colonization of the tooth surfaces, biofilm formation (i.e. plaque), and localized demineralization of tooth enamel. In our research we used a laboratory-prepared broth media designated as yeast nutrient broth (YN) essentially free of sucrose. The broth contained 0.2% sodium chloride, 0.4% potassium phosphate, 0.2% sodium phosphate, 0.1% magnesium sulfate, 0.25% glucose and 1% yeast extract. Glucose was autoclaved separately and combined with the broth aseptically.
2.2 Dental plaque as a biofilm

Dental plaque is a yellowish, sticky biofilm found in the tooth surface consisting of a matrix of polymers and embedded cells. It can be defined as a complex microbial community, with greater than $10^{10}$ bacteria (mainly *S. mutans* and anaerobes) per milligram with the composition varying by location in the mouth. In addition to the bacterial cells, plaque contains a small number of epithelial cells, leukocytes, and macrophages.

2.2.1 Mechanism of formation of dental plaque

*S. mutans* secrete enzymes known as glucosyl-transferase (GTF) and fructosyl-transferase that convert sucrose to sugary, glue-like substances (exopolymers), which help the bacteria to firmly attach themselves to the tooth surface and form a tough barrier around bacterial colonies [Loesche 1986]. These tough barriers protect the bacterial colonies against environmental assaults, and make them, in some cases, hundreds of times more resistant to antibiotics. Sticky gelatinous matrixes where the bacteria live are known as biofilms, whether they occur on teeth or elsewhere in the body. A schematic of the steps involved in the biofilm formation on teeth by *S. mutans* is shown in Figure 2.2. Glucosyltransferases (GTFs) are a main virulence factor responsible for *S. mutans* biofilm formation, but other pathogens use similar mechanisms to produce exopolymers.

The microorganisms present in dental plaque are all naturally present in the oral cavity, and are normally harmless. However, failure to remove plaque by regular tooth brushing allows them to build up in a thick layer. Because the bacteria in the tooth surface survive in the presence of carbohydrates and those microorganisms nearest the
tooth surface enter the anaerobic respiration state due to the diffusion limitation of oxygen and start producing harmful acids.

![Image of Streptococcus mutans and biochemical reactions]

**Figure 2.2 - Basic steps involved in dental plaque biofilm formation.**

i. Acids released from dental plaque lead to demineralization of the adjacent tooth surface, and consequently lead to dental caries. Saliva is also unable to penetrate the build-up of plaque and thus cannot act to neutralize the acid produced by the bacteria and remineralize the tooth surface.

ii. They also cause irritation of the gums around the teeth that could lead to gingivitis, periodontal disease and tooth loss.

iii. Plaque buildup can also become mineralized and form calculus (tartar).

*S. mutans* has evolved to depend on a biofilm lifestyle for survival and persistence in the oral cavity combined with its role as an opportunistic pathogen; it has become the best-studied example of a biofilm-forming, disease-causing *Streptococcus* [Burne 1998].
Biofilm-like populations of pathogenic streptococci may also reach higher densities in confined areas like heart valves, prosthetic devices, sinuses, tonsillar crypts, terminal respiratory passages, and in infectious skin lesions.

2.3 Control of biofilms

Numerous anti-plaque agents available in the market with varying modes of action have been tested for their ability to intervene dental biofilm formation or metabolism. However, due to several undesirable side effects associated with these agents, the search for alternate agents is in a great need. A suggested approach is using cost effective, stable, novel and natural products as antibiofouling materials in medical, health care and chemical industries. We have investigated these natural products called polyphenols found in abundance in fruits and vegetables in our research.

2.4 Polyphenols

Polyphenols are present in a variety of plants utilized as important components of both human and animal diets [Bravo 1998; Chung et al. 1998; Crozier et al. 2000]. They are characterized by the presence of more than one phenol unit or building block per molecule. Ranging from simple phenolic molecules to highly polymerized compounds with molecular weights of greater than 30,000 Da, the occurrence of this complex group of substances in plant foods is extremely variable [Bravo 1998; Ovaskainen et al. 2008]. Polyphenols have several industrial applications, such as in the production of paints, paper, and cosmetics, as tanning agents, and in the food industry as additives (as natural colorants and preservatives). It is well known that diets containing abundance of fruit and
vegetables are protective against a variety of diseases, particularly cardiovascular disease and cancer. The primary components thought to provide this protection afforded by fruit and vegetables are the antioxidants, free-radical scavengers.

Furthermore, these polyphenols are also well known for their antimicrobial activity [Kubo et al. 2006]. For example, anacardic acid found in cashew nut shell liquid has been investigated for its antimicrobial activities against *Streptococcus mutans* [Himejima et al. 1991] and *Staphylococcus aureus* [Kubo et al. 2003].

As stated above, antioxidant capacity is a well-known attribute of polyphenols. Flavonoids are well known to scavenge the free radicals and are toxic to microorganisms [Georgetti et al. 2003; Nishino et al. 1987]. Gallic acid reported as a free radical scavenger plays an important role in the prevention of malignant transformation and cancer development [Sohi et al. 2003; Taraphdar et al. 2001; Faried et al. 2007].

### 2.4.1 Polyphenols as antimicrobials

Antimicrobials are substances that influence a microorganism in a negative way. Antibiotics are among the most commonly used antimicrobial drugs. For example, 30% or more hospitalized patients are treated with one or more courses of antibiotic therapy.

Antimicrobials can be classified in many ways such as bactericidal, bacteriostatic, antiseptics, disinfectants, and sanitizers. However with antimicrobial resistance on the rise, 70% of bacteria are found resistant to at least one drug. Thus, alternative ways to control bacteria are needed and other options that are not on a high priority are getting a second look. Antimicrobial benefits of polyphenolic compounds span from medical use to cosmetics and food preservations. Like for medical uses, safety is a great concern since cosmetics and food preservations are directly applied to the hair, skin, teeth, and stomach.
Some of the most commonly used antimicrobial products in our day-to-day life are shown in Figure 2.3. Polyphenols as antimicrobials are particularly advantageous to study because of their previous records of use in foods and vast amount of research available. They can be used for food preservation and protection against antimicrobial contaminants, and protecting the skin from free radicals that cause aging and diseases.

Figure 2.3 - Most commonly used antibacterial products in day to day life.
(Source: www.momgoesgreen.com)

Many factors should be considered when determining the antimicrobial activity of polyphenols such as: species/strains of bacteria, concentration and type of polyphenol, microbial load, and synergistic effects of phenols with other antimicrobials, temperature and food additive combination, the food matrix and the phenolic carriers, or the method of addition [Raccach et al. 1982].

However, when it comes to biofilms, antimicrobials are not very effective in killing the cells present in the biofilm. The biofilm matrix provides a chemical resistant barrier
to antibiotics allowing the survival of bacteria in a biofilm [Suci et al. 1994] and the varied micro environments within the biofilm renders several layers of defense by depleting the antibiotic before it reaches the actual target [Puverdorj-Gage et al. 2005; Zhang et al. 1996; Dessen et al. 2001].

High concentrations of antibiotics often result in antibiotic resistance by the bacteria. Antibiotic resistance occurs due to many reasons: high concentration of cells, genetic processes of mutation, fast growth rates and the ability to exchange genes with other bacteria [Dessen et al. 2001]. We have used the minimum inhibitory concentration (MIC) values of polyphenols in our research, which is the lowest concentration of polyphenols that prevents visible growth of the microorganism. The MIC is measured by exposing a bacterial population (the inoculum) to a range of concentrations of the polyphenol in question, under the conditions that are suitable for rapid planktonic growth. MICs may be determined in liquid culture media (broth dilution) or on solid media (agar dilution). Following a period of incubation that is sufficient to allow visible growth in antimicrobial free conditions, the culture medium is inspected. The lowest concentration of antimicrobial agent at which no bacterial growth is visible is considered the MIC. MIC’s are important in diagnostic laboratories to confirm the resistance of microorganisms to an antimicrobial agent and also monitor the activity to new antimicrobial agents [Andrews 2001]. In our study, the polyphenols were used to prevent biofouling. In order to find out the specific effect of polyphenols on bacterial production of exopolymers while they are alive, we used the polyphenols below their MICs.
2.4.2 Polyphenols as antioxidants

Antioxidants are generally compounds that can delay or inhibit the oxidation of lipids or other molecules by removing the free-radicals that initiate or propagate oxidizing chain reactions [Chipault 1962]. Various compounds found in all kinds of plants, vegetables and fruits have been observed to have antioxidant effect. Figure 2.4 shows some of the best sources of antioxidants. Among the numerous constituents of foods, principally of plant origin, that have antioxidant activity are the polyphenols. The polyphenol compounds are primary antioxidants that act as free radical acceptors and chain breakers. This activity is due to the ability of donating an H atom from an aromatic hydroxyl group to a free radical, and the major ability of an aromatic structure to support an unpaired electron.

Antioxidants in foods can be further defined as any substance that is added to fats to retard oxidation and prolong wholesomeness and stability. An antioxidant should be able to stop oxidation by scavenging oxidative free-radicals; however it must also keep food quality and wholesomeness. Antioxidants are introduced to a food system to react with the free radicals before they react naturally with the fats in the food matrix causing rancidity [Dziezek 1986]. In some cases the antioxidants can also act as prooxidants producing their own free radicals. For an antioxidant to be ideal [Fung et al. 1985; Gailani et al. 1984; Lindsay 1996], the following qualities need to be met: no harmful physiological effects, no contribution to off flavors, odor, and color to food, effective in small concentrations, must be fat soluble, readily available, cost efficient and last through processing to provide protection to food.
Antioxidants are commonly used as food additives to prevent the lipid peroxidation of the food products, thereby extending shelf life of the food products. It is well known that diets containing abundance of fruit and vegetables are protective against a variety of diseases, particularly cardiovascular disease and cancer. The primary nutrients thought to provide the protection afforded by fruit and vegetables are the antioxidants. Natural antioxidants are in demand for preparing functional foods and supplements because of their possible health benefits and consumer preference of chemical free products. We used 4 different sustainable, natural and cost effective polyphenols in our research that are well-known for antioxidant capacity. Ascorbic acid was used as a standard antimicrobial and antioxidant agent for comparison.

**Tannic Acid:** A basic ingredient in the chemical staining of wood can be found in chestnut, red wine, tea, mangrove and oak. Its structure is based mainly on glucose esters of Gallic acid. In dental practice tannic acid is a valuable agent for local use in the
treatment of diseases such as mercurial stomatitis, ulceration of the gums and mucous membrane of the mouth [Ferdinand 1901].

**Ascorbic acid:** A naturally occurring form of Vitamin C is a sugar acid with antioxidant properties. Green peppers, citrus fruits, strawberries and tomatoes are some of the sources. Ascorbic acid and its sodium, potassium, and calcium salts are commonly used as antioxidant food additives. It is a very good antioxidant [Frei et al. 1989] found at high levels in a variety of tissues in comparison to plasma levels. Ascorbic acid is not categorized as a polyphenol, but was used in our research as a control to compare the antimicrobial and antioxidant effects with the natural polyphenols.

**Salicylic Acid:** Found in leaves of wintergreen and willow bark. Heating phenol with carbon dioxide can also synthetically produce it. It is a key ingredient in many skin-care products for the treatment of acne, psoriasis, calluses, corns and warts [Grimes 1999; Roberts 2004]. Because of its effect on skin cells, salicylic acid is used in several shampoos used to treat dandruff. Salicylic acid is also used as an active ingredient in gels, which remove warts. Use of concentrated solutions of salicylic acid may cause hyperpigmentation on unprotected skin for those with darker skin types, as well as with the lack of use of a broad-spectrum sun block [Zanta et al. 2009].

**Gallic Acid:** Predominant phenolic acid in Tea. Pure gallic acid is a colorless crystalline organic powder found in gallnuts, sumach, tea leaves, oak bark, and many other plants. Esters of gallic acid have a diverse range of industrial uses, as antioxidants in food, in cosmetics and in the pharmaceutical industry.
**Quercetin**: A well-known flavonoid for its strong antioxidant activities can be derived from many sources in the human diet including fruits, vegetables, red wine and tea. Researchers previously demonstrated that the flavonoids: quercetin and myricetin in cranberry extract prevented adhesion of the bacteria *Streptococcus mutans*, an agent for dental caries, to teeth [Iio et al. 1984].

Antioxidant capacities of these polyphenols are hypothesized to play a main role in preventing bacteria from forming biofilms. The effects of antioxidant capacity of these polyphenols were investigated to better understand the polyphenols’ antibiofouling mechanisms.
CHAPTER 3

Antibiofouling Effect of Polyphenols against *Streptococcus mutans*

3.1 Abstract

Biofouling is a process of surface colonization by microorganisms through cell adhesion and production of extracellular polymers (polysaccharides and proteins). It often causes serious problems in chemical, medical and pharmaceutical industries. Recently, we have demonstrated that some natural phenolic compounds found in plants and vegetables have an anti-biofouling effect reducing formation of biofilm by Gram-negative bacteria [Jagani et al. 2009]. In this study, *S. mutans*, a Gram-positive bacterium was investigated for the antibiofouling effect of polyphenols. We hypothesized that the two enzymes, glucosyltransferase and fructosyltransferase, produced by *S. mutans* would be inhibited by the natural phenolic compounds. When these two enzymes were inhibited: less or no biofilms were formed. Enzymes were separated from a *S. mutans* culture medium, and their activities were measured with 4 different polyphenols using microtiter-plates and HPLC method. The results of minimum inhibitory concentration (MIC) were used to determine the enzyme inhibition effect of polyphenols on biofilm formation without killing the cells. Most of the polyphenols we used showed considerable reduction of biofilm formation. Gallic acid and tannic acid showed significant enzyme inhibition effect under the MIC.
Keywords: biofouling; biofilm; enzyme activity; polyphenols; *Streptococcus mutans*.

### 3.2 Introduction

A biofilm consists of a gelatinous matrix formed on a solid surface by adhesion of microbes to the surfaces with production of extracellular polymers. This process is referred to as biofouling. Biofouling causes serious problems in the chemical, medical and pharmaceutical industries. Dental plaque is an example of a biofilm, and it is generally recalcitrant to the action of antimicrobial agents. Formation of dental caries is caused by the colonization and accumulation of oral microorganisms on the teeth; cell adhesion is the first step in the colonization process and in the second step cells produces extracellular polymers to form biofilms [Gibbons 1984]. Streptococci especially *Streptococcus mutans* (*S. mutans*) have been implicated as a primary causative organism of dental caries [Hamada et al. 1980]. Since *S. mutans* has evolved to depend on a biofilm lifestyle for survival and persistence in the oral cavity combined with its role as an opportunistic pathogen, it has become the best-studied example of a biofilm-forming, disease-causing *Streptococcus* [Burne 1998].

Not only in the dental carries, but also pathogenic streptococci may also reach a higher level of density in a biofilm developed in confined areas such as in heart valves, prosthetic devices, sinuses, crypts, terminal respiratory passages, and infectious skin lesions [Cvitkovitch et al. 2003]. Numerous anti-plaque agents available in the market with various modes of action have been acknowledged for their ability to interfere with dental biofilm formation, or metabolism [Park et al. 1998; Yanagida et al. 2000; Koo et al. 2003]. However, due to several undesirable side effects and high prices associated
with these agents, the search for alternative agents continues [Schae 1989]. A suggested approach is to find inexpensive, effective, and natural materials as antibiofouling agents. Natural polyphenols found in plants, fruits, and vegetables, have recently received much attention as sustainable antimicrobial agents to control biofilm formation on dental caries [Raccach et al. 1982; Yanagida et al. 2000]. Even though many natural polyphenol products are well known for antimicrobial activities [Kubo et al. 1995; Davison 1983; Kozubek et al. 2001; Krinsky 1992], few studies have been done about antibiofouling effects [Jagani et al. 2009; Chelikani et el. 2009]. We have studied antimicrobial and antibiofouling effects of natural polyphenols against many problematic microbes from natural products [Kim et al. 2002; Choi et al. 1998; Choi et al. 2002; Chelikani et al. 2009; Yoon et al. 2009; Jagani et al. 2009] and recently, we have demonstrated that some natural phenolic compounds found abundant in plants have an anti-biofouling effect on biofilm formation of Gram-negative bacteria [Chelikani et al. 2009; Jagani et al. 2009].

*Streptococcus mutans*, Gram-positive strain, has been implicated as a primary etiological agent of dental caries in animals and humans, and it is also involved in plaque formation and accumulation [Loesche 1986]. *S. mutans* produces enzymes, glucosyltransferase and fructosyltransferase, and synthesizes water insoluble glucan and fructan with sucrose. These polysaccharides are known to mediate the adhesion and accumulation of the bacteria on the tooth surface [Leme et al. 2006; Koo et al. 2003]. This process is outlined in Figure 3.1 below. We hypothesized that inhibition of enzymes (glucosyl- and fructosyl-transferases) with polyphenols would lead to lesser biofilm formation. In this study, we investigated the antibiofouling effect of the polyphenols, and the relation of these phenols’ enzyme inhibition effects and biofilm formation. Four well-known natural
phenolic compounds, tannic acid, gallic acid, quercetin and salicylic acid, were selected and tested against *S. mutans* using microtiter plate assays and HPLC analysis. Before the enzyme inhibition assays and antibiofouling tests, the minimum inhibition concentrations (MIC’s) of the polyphenols were determined. Above the MIC, the polyphenols kill the cells. In order to determine the effect of polyphenols’ enzyme inhibition on biofilm formation, the concentrations of polyphenols used in the enzyme inhibition assay were below the minimum inhibition concentration.

Figure 3.1 - Overview of *S. mutans* basic metabolic pathways.
(Source: www.freewebs.com)
3.3 Materials and methods

3.3.1 Bacterial strain

The strain *S. mutans* ATCC 25175, Gram-positive bacterium (American Type Culture Collection, Manassas, VA, USA) was used. *S. mutans* was chosen because of its ability to produce large amounts of extracellular polymers rapidly and to form biofilms (dental plaque) causing dental caries.

3.3.2 Materials

Upon arrival from ATCC, *S. mutans* was cultured in a yeast nutrient broth (Fischer Scientific, Fair lawn, NY, USA) extracted essentially free of sucrose. The broth contained 0.2% sodium chloride, 0.4% potassium phosphate, 0.2% sodium phosphate, 0.1% magnesium sulfate, 0.25% glucose and 1% of yeast extract. Separately, 1.25 g of glucose was mixed with 500 mL of DI water to make a 0.013 M stock solution. Glucose solution was autoclaved and, then combined with the broth aseptically by adding 10 mL of the stock solution into 90 mL of broth. The final concentration of glucose was 0.0013 M (0.23 mg/mL). *S. mutans* was cultured in an anaerobic environment using a temperature controlled shaker (New Brunswick Scientific, Edison, NJ, USA).

Quercetin was obtained from MP Biomedicals (Aurora, OH, USA). Other polyphenols were purchased from Sigma (St. Louis, MO, USA). Gentian violet (2% w/v aqueous solution, Ricca Chemical Company, Arlington, TX, USA) was used to stain the biofilm. Methanol was used to fix the attached bacteria. Dimethyl sulfoxide (DMSO, Fisher Scientific, Fair Lawn, NY, USA) was used to resolubilize the dye bound to the adherent cells. All other chemicals were of analytical-grade purity. All materials were
autoclaved for 20 min at 120 °C before use. The structures of the polyphenol compounds used are shown in Figure 3.2.

Figure 3.2 - The structures of the polyphenolic compounds used in this study. 1. Ascorbic acid, 2. gallic acid, 3. salicylic acid, 4. quercetin, 5. tannic acid.

3.3.3 Antimicrobial assays

The MIC of polyphenols was assessed using a broth dilution method [Yoshino et al. 1995; Kubo 2003; Kubo et al. 1993; Muroi et al. 1996; Smullen et al. 2007; Kubo et al. 1995]. Briefly, S. mutans was grown overnight in an YN culture medium with glucose. A 0.1 mL sample was taken from the culture when the stationary growth phase was reached
after 16 hrs. The sample was transferred to culture tubes that contained 15 mL of the culture medium.

The polyphenol solutions were prepared as follows: The natural polyphenols were dissolved in DMSO at different concentrations resulting in a 1% DMSO solution when added to the broth. The solution of 1% DMSO and polyphenols of various concentrations were tested for their effects on the growth of *S. mutans*.

0.1 mL of the polyphenol solutions of different concentrations were taken and added to the culture tubes. At a regular interval, 0.1 mL of the solution from each culture tube was serial-diluted and plated on the yeast nutrient agar plates. The plates were incubated at 37°C and colonies were counted after 24 hrs. The MIC assay was done in triplicates for all the samples, and the averages of the results were taken.

### 3.3.4 Microtiter-plate test

For the microtiter-plate test, the YN medium supplemented with sucrose was used for growing *S. mutans* to simulate dental biofilm formation on table sugar (sucrose). First, as in the MIC assay, the strain was grown in a test tube until the culture reached the stationary phase. Then, the effect of the polyphenolic compounds on *S. mutans* biofilm formation was investigated using a modified microtiter plate method [Srdjan et al. 2000; Jagani et al. 2009; O’Toole et al. 1999]. 200 µL of the bacterial suspension were filled in each of the six wells. 0.1 mL of 5 DMSO solutions, each of which containing the different polyphenolic compounds at its MIC was added to each well. The plate was covered with a lid and incubated anaerobically for 24 and 36 hrs at 37°C. Then the content of each well was aspirated. The wells were washed three times with 250 µL of
sterile physiological phosphate buffered saline. The plate was shaken well so that non-adherent bacteria were removed. The bacteria attached to the walls were then fixed with 200 µL of 99% methanol per well, and then, after 15 minutes the methanol was decanted and the well was dried. 200 µL of 2% gentian violet was added to the wells to stain the biofilm cells for 5 min. The plate was placed under the running tap water to rinse off the excess violet dye and then the plates were air-dried. The dye bound to the adherent cells was re-dissolved with 200 µL of DMSO per well. The optical density (OD) of the each well was measured at 570 nm by using a plate reader (Titertek Multiscan Plus, ICN, Costa Mesa, CA USA). The microtiter-plate tests were done in triplicates, and the averages were taken.

3.3.5 Enzyme activity assay

First *S. mutans* was grown in a 20 mL broth for 16 hrs with constant shaking at 37°C until the culture reached the stationary phase. The culture supernatant was obtained by centrifugation (16,300 × g, 15 min, 4 °C). The culture supernatant was used as crude glucosyl- and fructosyl-transferase enzymes.

After the addition of the enzyme to a 10 mL sucrose (60 mM) solution, 1 mL of the polyphenols was then added and the mixture was incubated for 24 hrs. A 0.22 µm Millipore filter was used to separate the extracellular polymers from the mixture and a HPLC assay was used to quantify the activity of the enzyme. HPLC analysis was carried out by Agilent 1200 series HPLC system (Palo Alto, CA, USA) with a refractive index detector. Polysaccharide analysis was performed on a Shodex SPO 810 column (8 mm × 30 cm, Showa Denko, Tokyo) at 35 °C. Ultrapure water was used as mobile phase at a
constant flow rate of 0.8 mL/min at 80 °C. All injections were 30 μL in volume. Standards stock solutions of sucrose, glucose, and fructose, were prepared and diluted to appropriate concentrations for calibration. Five concentrations of three different sugars were injected in duplicate, and then the calibration curves were constructed by plotting the peak areas against the concentration of each analyte. The enzyme activity was calculated by the difference in the concentration of the products, glucose and fructose as reducing sugars in the presence of polyphenols when compared with the control.

3.4 Results and discussion

3.4.1 MIC of polyphenols

Various concentrations of the polyphenols were used and the minimum concentration of polyphenols that was required to kill the cells was determined as the MIC. Table 3.1 shows the log (CFU/mL) results for the cells measured soon after the addition of polyphenols. The control in Table 3.1 shows the number of cells in the growth medium without the polyphenols. The other data were taken when the concentration of each polyphenol was at the MIC. These results demonstrate that the CFU, i.e. cell viability in the presence of the polyphenols was not significantly affected when the polyphenols concentration was at the MIC.
Table 3.1 - The Minimal Inhibitory Concentration (MIC) of polyphenols against *S. mutans*

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>MIC (mg/mL)</th>
<th>Log (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>10.36 (±0.208)</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>2</td>
<td>10.34 (±0.208)</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>1.2</td>
<td>10.33 (±0.555)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.5</td>
<td>10.35 (±0.781)</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>3.8</td>
<td>10.35 (±0.381)</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>0.4</td>
<td>10.35 (±0.361)</td>
</tr>
</tbody>
</table>

Tannic acid, a gallic acid derivative, which is present in many foods including tea, cocoa beans, grape, strawberry, persimmon etc., showed the lowest MIC among the tested polyphenols, which indicates that tannic acid, is the strongest antimicrobial that kills the cells at a low concentration compared to other polyphenols tested. In our study, tannic acid inhibited the growth of *S. mutans* with an MIC of 0.4 mg/mL, while other polyphenols were less effective with the MIC values in the range of 1.5 mg/mL - 3.8 mg/mL. The strong antimicrobial activity of tannic acid against *S. mutans* may be due to the presence of the ester linkage between gallic acid and glucose in its structure [Yamamoto et al. 2002]. Salicylic acid, a phenol derivative with no alkyl side chain exhibited the weakest antimicrobial activity amongst the phenolic compounds tested in our study with an MIC of 3.8 mg/mL.
Figure 3.3 - Antimicrobial activities of polyphenols against *S. mutans*; Control (●), ascorbic acid (■), gallic acid (▲), salicylic acid (×) acid, tannic acid (+) and quercetin (●).

Figure 3.3 compares the cell counts of *S. mutans* in the presence of the different polyphenols at the MICs. The arrow in the Figure 3 represents the time at which the polyphenols were added to the culture. As discussed above, MIC is the minimum concentration of polyphenols that kills the cells. As seen in Figure 3.3, it can be clearly observed that the bacterial culture grew exponentially until the beginning of stationary phase and started to die once the polyphenols were added. The polyphenols were added after 16 hrs of inoculation. The MIC values of gallic acid, salicylic acid and tannic acid against *S. mutans* were close to the values reported by other researchers [Himejima et al. 1991; Yoshino et al. 1995; Yamamoto et al. 2002].
3.4.2 Antibiofouling activity of polyphenols

The image of the micro-titer plates after staining with crystal violet, and the dye bound to the adherent cells was resolubilized after 24 and 36 hrs is shown in Figure 3.4. The assay was done in triplicate. Starting from left to right, the wells contain control (no natural phenolic compounds), ascorbic acid, gallic acid, quercetin, salicylic acid and tannic acid.

![Streptococcus mutans biofilm](image)

**Figure 3.4 - Pictures of *Streptococcus mutans* biofilm taken at 24 and 36 hrs after the addition of dimethyl sulfoxide to the microtiter-plate.**

Each bar graph in Figure 3.5 represents the absorbance of the crystal violet dye bound to the biofilm cells. A large absorbance value indicates more biofilm formation. All polyphenolic compounds showed a significant reduction in biofilm formation by *S. mutans* compared with the control. Gallic acid was the strongest anti-biofouling agent against *S. mutans* when compared to ascorbic acid, quercetin, salicylic acid and tannic acid. Salicylic acid, which has a carboxyl group and possesses strong redox properties [Robert et al. 2006], showed some reduction of biofilm formation. However, it was the weakest anti-biofouling agent when compared to the other polyphenols used in our study. Therefore it is thought that the carboxyl group add no or very weak effects on the
antibiofouling activity [Jagani et al. 2009]. Ascorbic acid, an organic acid with good antioxidant properties, was used as a reference antibiofouling agent for comparison, reduced the biofilm formation to the same extent as quercetin. Quercetin along with tannic acid belong to the flavonoid family are well known to possess strong redox properties [Khan et al. 2000] showed good antibiofouling effects after gallic acid against \textit{S. mutans}.

![Figure 3.5 - Absorbance of the biofilm formed in the presence of various polyphenolic compounds; biofilm formation after 24 hrs (■) and biofilm formation after 36 hrs (□).](image)

Tannic acid, though having strong redox potential due to the presence of hydroxyl groups in its structure, did not show antibiofouling activity as good as gallic acid. It appears that the presence of both hydroxyl and carboxyl groups enables the polyphenol to reduce biofouling activity of the cells. By comparing different redox active phenolic compounds (i.e., antioxidant capacity) for their antifouling effects, we concluded that antioxidant activities of these phenolic compounds play a role in preventing the formation of the Gram-positive bacterial biofilm.

29
Table 3.2 - Absorbance of the biofilm in the micro-titer plate and the percentage of reduction of absorbance from the control.

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>Absorbance</th>
<th>% Reduction of biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.109 (±0.322)</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.269 (±0.069)</td>
<td>75.7</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.199 (±0.015)</td>
<td>82.1</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.276 (±0.046)</td>
<td>75.1</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>0.413 (±0.097)</td>
<td>62.8</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>0.372 (±0.034)</td>
<td>66.5</td>
</tr>
</tbody>
</table>

Table 3.2 summarizes the difference in the percentage in the absorbance between polyphenolic compounds and the control for biofilm formation after 36 hrs. The 36 hrs biofilm was considered because of higher absorbance values for all the wells. For example, in the presence of gallic acid, biofilm was formed 82.1% less than the control where no polyphenol was present. Overall biofilm formation of *S. mutans* was suppressed by 62-82% at the MICs of each polyphenol. Again, the prevention of biofilm formation in the presence of each polyphenol was not caused by the antimicrobial activity of the polyphenols because they were used at below MIC’s. At the concentration level of polyphenols used in our experiments, all the compounds did not kill the bacteria, and showed significant antibiofouling effects against *S. mutans*. Considering that all these compounds have high redox potential, it can be postulated that the enzymes, glucosyl- and fructosyl-transferases, produced by *S. mutans* involved in biofilm formation may be inhibited by natural phenolic compounds through redox reactions.
3.4.3 Enzyme inhibition by polyphenols

The glucosyltransferase and the fructosyltransferase enzymes play a major role in converting sucrose to glucose and fructose and also in polymerizing glucose to glucans and fructose to fructans. Glucans and fructans are extracellular polymers that help the bacteria to glue to the tooth surface [Hamada et al.1984]. To verify the hypothesis that the enzymes produced by *S. mutans* may be inhibited by the polyphenols, and thereby resulting in lesser biofilm formation, the activity of the enzymes in the presence of the different polyphenols was determined by measuring the product (glucose and fructose) concentrations. We observed a 25% conversion of 60 mM sucrose to glucose and fructose by the enzyme from *S. mutans* without the polyphenols. Figure 3.6 shows the retention time and the areas of sucrose, glucose and fructose. The average retention time of sucrose was 12 min, 13 min for glucose, and 19 min for fructose.

![Figure 3.6 - HPLC chromatogram of sucrose (1), glucose (2) and fructose (3).](image)

Figure 3.6 - HPLC chromatogram of sucrose (1), glucose (2) and fructose (3).
Figure 3.7 - Effect of glucosyl- and fructosyl-transferase enzymes on the glucose (■) and fructose (□) formation in the presence of natural polyphenols.

Table 3.3 represents the percentage of inhibition of the glucosyl- and fructosyl-transferase enzymes in the presence of the polyphenols. The results revealed that quercetin and ascorbic acid had the highest % of inhibition (45%) of the enzymes.

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>Reducing sugars concentration (mM)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.56</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>7.45</td>
<td>45.05</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>11.5</td>
<td>15.19</td>
</tr>
<tr>
<td>Quercetin</td>
<td>7.39</td>
<td>45.5</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>12.2</td>
<td>10.02</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>9.23</td>
<td>31.93</td>
</tr>
</tbody>
</table>

As reported by Iio et al. (1984) quercetin, which is well known for strong antioxidant capacity, showed a high percentage of inhibition of the enzymes close to 45%
in our study. It reduced 75% of the *S. mutans* biofilm. Therefore it can be used as an anti-plaque agent to reduce dental plaque. Quercetin is also reported to inhibit the acidic conditions and stop the bacteria from attaching to the tooth surface [Prabhu et al. 2006]. Ascorbic acid was used for comparison and showed similar enzyme inhibition effect and antibiofouling activity to quercetin.

Yanagida et al. (2000) previously reported that gallic acid had very weak enzyme inhibition activities. We observed the same trend in our results as shown in Figure 3.6. However gallic acid showed very high antibiofouling activities (82.1% reduction), but had very less enzyme inhibition results (~15% inhibition). This means that gallic acid reduces bacterial biofilm formation through different mechanisms than enzyme inhibition. Tannic acid, an ester of gallic acid, which is a mixture of polygalloylglucose showed significant enzyme inhibition effects (~32%) and antibiofouling activities (~66%) against *S. mutans* biofilm. Salicylic acid showed a weak antimicrobial effect and was the weakest antibiofouling agent (~63%) with very weak enzyme inhibition effects (10%) in our study.

3.5 Conclusions

The polyphenolic compounds inhibited the biofilm formation of *S. mutans* in vitro at the concentrations that did not kill the cells. They may be useful for controlling dental caries. The reduction trend of biofilm in the presence of polyphenols was almost similar to each other, and thereby proving our hypothesis. Based on the results, we concluded that all these polyphenols decrease the activities of glucosyl- and fructosyl-transferases. It is suggested that these polyphenols interfere with glucan synthesis mechanism by
inhibiting the enzymes that synthesize extracellular polymers. Tannic acid, gallic acid and quercetin could inhibit the biofilm formation of *S. mutans* at the concentrations employed in this study, and may be useful for controlling dental caries. We conclude that our hypothesis was proved correct as inhibition of the glucosyl- and fructosyl-transferase enzymes led to lesser biofilm formation. The observed reduction in biofilms in the presence of polyphenolic compounds (except gallic acid) was possibly caused by inhibition of the enzymes.
CHAPTER 4

Antimicrobial and Antioxidant Activities of Polyphenols against *Streptococcus mutans*

4.1 Abstract

Four natural sustainable polyphenols, gallic acid, tannic acid, quercetin and salicylic acid, were investigated for their antimicrobial and antioxidant activities against *Streptococcus mutans*. Ascorbic acid, well known for its strong antimicrobial and antioxidant activity, was used to compare the results. First antimicrobial effect of the polyphenols was assessed using the plate dilution assay. Minimum inhibitory concentration (MIC) of each polyphenol has been determined. Salicylic acid showed the highest MIC, 3.8 mg/mL, and tannic acid showed the lowest, 0.4 mg/mL, indicating the weakest and strongest polyphenols respectively tested in our study. Antioxidant capacities were evaluated using the DMPD and ABTS decolorizing assays. Antioxidant capacity of each polyphenol was measured at its MIC concentration to see if there is a relationship between antimicrobial activity and antioxidant capacity. Results suggest that these polyphenols were good antimicrobials and also very good antioxidants. Although some conflicting results were observed between DMPD and ABTS methods, polyphenols with high antioxidant capacities also showed considerable antimicrobial activities.
suggesting that antioxidant capacity contributes to the antimicrobial effect of polyphenols.

**Keywords:** MIC, antioxidants, antimicrobials, polyphenols.

### 4.2 Introduction

Plant polyphenols have received great attention for their biochemical and microbial effects over the past years. These secondary plant metabolites are naturally present in most edible fruits and vegetables and therefore are common in the everyday diet of many people. It is well known that diets containing abundance of fruit and vegetables are protective against a variety of diseases, particularly cardiovascular disease and cancer. The primary components thought to provide the protection afforded by fruit and vegetables are the antioxidants [Eastwood 1999].

These polyphenols are also well known for their antimicrobial activity [Kubo et al. 2006]. For example, anacardic acid found in cashew nut shell liquid has been investigated for its antimicrobial activities against *Streptococcus mutans* [Himejima et al. 1991] and *Staphylococcus aureus* [Kubo et al. 2003].

As stated above, antioxidant capacity is another well-known attribute of polyphenols. Flavonoids are well known to scavenge the free radicals and are toxic to microorganisms [Georgetti et al. 2003; Nishino et al. 1987]. Gallic acid reported as a free radical scavenger plays an important role in the prevention of malignant transformation and development of cancer [Sohi et al. 2003; Taraphdar et al. 2001; Faried et al. 2007].

Polyphenols that have both antioxidant and antimicrobial effects are particularly useful to study their effects and mechanisms. One of the hypotheses in this paper is that
polyphenols’ antimicrobial effect is caused or facilitated by the significant electrical charge change, e.g. redox potential, through the free radical scavenging activity of the antioxidants [Schulze-Osthoff et al. 1992; Wu et al. 2005; Yakovleva et al. 2007]. Furthermore, these polyphenols provide antimicrobial/antioxidant research with better opportunities because of their previous records for use in food and vast amount of research results available. One example of the benefits of using polyphenols as antioxidants and antimicrobials is in cosmetics. They can be used for preservation and protection against antimicrobial contaminants, while simultaneously protecting the skin from free radicals that accelerate aging and cause diseases.

To determine the antimicrobial and antioxidant activities of these polyphenols, first the antimicrobial effect of polyphenols was assessed against *S. mutans* by the minimum inhibitory concentration (MIC) assay using the plate dilution technique. *S. mutans*, Gram-positive bacteria, was chosen because the antimicrobial effect of natural polyphenols on it was seldom performed in antimicrobial research compared to on other Gram-negative bacteria such as *Pseudomonas aeruginosa*. *S. mutans* is a dental plaque-forming bacterium, and antimicrobial effect of dietary polyphenols and its relation to antioxidant activity have not been studied much. Antioxidant capacities of the polyphenols were determined by the DMPD (N,N-dimethyl-p-phenyldiamine dihydrochloride) method and the ABTS (2,2’-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) decolorizing assay.

This paper includes comparisons of the antioxidant capacities along with the antimicrobial effect of polyphenols. Quercetin, gallic acid, tannic acid, and salicylic acid were selected as model polyphenols because they are commercially available in food and pharmaceutical industries and they have been investigated for their antioxidant and
antimicrobial activities intensively. Ascorbic acid was used as a control as it is a strong
antimicrobial and antioxidant itself. A possible relationship or trend between the
antimicrobial and antioxidant activities was sought based on the results.

4.3 Materials and methods

4.3.1 Bacterial strain

The strain used was *Streptococcus mutans* ATCC 25175, a Gram-positive
bacterium (American Type Culture Collection, Manassas, VA, USA).

4.3.2 Materials

*S. mutans* was cultured in yeast nutrient broth (YN) essentially free of sucrose. The
broth contained 0.2% sodium chloride, 0.4% potassium phosphate, 0.2% sodium
phosphate, 0.1% magnesium sulfate, 0.25% glucose and 1% yeast extract. Glucose was
autoclaved separately and combined with the broth aseptically. All materials were
autoclaved for 20 min at 120 °C before use. Quercetin was obtained from MP
Biomedicals (Aurora, OH, USA). Other polyphenols, DMPD, ABTS, Trolox and ferric
chloride were obtained from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO,
Fisher Scientific, Fair Lawn, NY, USA) was used as a solvent for the polyphenols.
Spectrophotometric measurements were recorded by using an UV-vis Shimadzu 1700
(Japan) apparatus. Chemical structures of the phenolic compounds used are illustrated in
Figure 3.2.
4.3.3 Determination of antimicrobial activity (Plate dilution assay)

A broth dilution method was performed to assess the MIC’s [Kubo et al. 2003; Yoshino et al. 1995; Kubo et al. 1993; Muroi et al. 1996; Smullen et al. 2007; Kubo et al. 1995]. Briefly, *S. mutans* was grown overnight in YN with glucose. After 16 hours 0.1 mL of the stationary phase culture from the broth was transferred to a culture tube that contains 15 mL of the broth. The natural polyphenols were dissolved in DMSO at different concentrations resulting in 1% DMSO when added to the sterile medium. The solution of 1% DMSO concentration and polyphenols in the desired concentrations were tested for their effects on the growth of *S. mutans*.

When the growth in the culture tubes reached the stationary phase, 0.1 mL of the various polyphenols with different concentrations were added to different culture tubes. At regular interval, 0.1 mL of the solution from each culture tube was serial-diluted and plated on the yeast nutrient agar plates. The plates were incubated at 37 °C and colonies were counted after 24-48 hrs. The antimicrobial assay was done in triplicates for all the samples, and the averages of the results were taken.

4.3.4 Determination of antioxidant activity

4.3.4.1 DMPD/FeCl$_3$ spectrophotometric method. We used the DMPD method as it guarantees a very stable end point in contrast to the ABTS method [Gulcin 2005]. The standard protocol developed by Fogliano et al 1999 was followed. Briefly, a 100 mM DMPD$^-$ solution was prepared by dissolving 0.209 g of DMPD in 10 mL of deionized water; 1 mL of this mixture was added to 100 mL of 0.1 M acetate buffer, at pH 5.25; the purple colored radical cation was then created by the addition of 0.2 mL of a 0.05 M
ferric chloride solution to the acetate buffer. One mL of the final DMPD$^+$ solution was placed in a quartz cuvette, and the absorbance was taken at 505 nm.

When the stable DMPD$^+$ solution was achieved, with optical density in the proper range, 0.1 mL of various antioxidant compounds was added to the quartz cuvette. After 10 minutes the absorbance was taken again, to measure the color change observed after the addition of the antioxidant compound. The resultant change in absorption was recorded as the percentage inhibition of the radical cation solution. In the presence of an antioxidant that can donate a hydrogen atom to the DMPD$^+$ molecule, the DMPD$^+$ solution changes from purple to a clear solution.

Antioxidant capacities of the polyphenols were expressed as TEAC (Trolox equivalent antioxidant capacity) using a calibration curve plotted with different concentrations of Trolox [Miller et al. 1993].

4.3.4.2 ABTS decolorization assay. Measurements of the antioxidant capacities of polyphenols were carried out as described by Jiménez et al. [2008]. The ABTS$^+$ radical cation was generated by reacting 7mM ABTS stock solution with 2.45 mM potassium persulphate and by allowing the mixture to remain in the dark at room temperature for 12-16 h before use. The ABTS$^+$ solution (2 days stable) was diluted with methanol to an absorbance of 0.70 ± 0.02 at 658 nm.

The effect of each antioxidant on the ABTS$^+$ radicals was estimated according to the procedure in Brand Williams et al. 1995 described for the DPPH radicals and modified by Sánchez-Moreno et al. [2002]. An aliquot of DMSO (0.1 mL) solution containing the different polyphenols was added to 3.9 mL of ABTS$^+$ in methanol
prepared daily. Absorbance at 658 nm was measured using a spectrophotometer. The reaction was monitored for 6 min. The absorbance was taken and the percentage of inhibition was calculated for each polyphenol. Methanoic solutions of the known Trolox concentrations were used for calibration. The equation for inhibition percentage is the same as the previous equation except for the wavelength.

4.4 Results and discussion

4.4.1 Antimicrobial activity measurement

We conducted a plate dilution assay in order to assess the antimicrobial activity of polyphenols against *S. mutans*. Various concentrations of the polyphenols were used and the minimum concentration of polyphenols that was required to kill the cells was determined as the MIC. Table 3.1 shows the log (CFU/mL) results for the cells measured after 16 hrs soon after the addition of polyphenols. The control in Table 3.1 shows the number of cells in the growth medium without any polyphenols in the DMSO solution. The other data in Table 3.1 were taken when the concentration of the polyphenols used were at their MIC values. These results demonstrate that the CFU, i.e. cell viability in the presence of the polyphenols was not significantly affected when the polyphenols concentration was at the MIC.

Tannic acid, a gallic acid derivative, which is present in many foods including tea, cocoa beans, grape, strawberry, persimmon etc. showed the lowest MIC among the tested polyphenols. In our study, tannic acid inhibited *S. mutans* with a MIC of 0.4 mg/mL, while other polyphenols were less effective with the MIC values in the range of 1.5 mg/mL - 3.8 mg/mL. The strong antimicrobial activity of tannic acid against *S. mutans*
may be due to the presence of the ester linkage between gallic acid and glucose in its structure [Chung et al. 1993]. Salicylic acid, a phenol derivative with no alkyl side chain exhibited the weakest antimicrobial activity amongst the natural phenolic compounds tested in our study with a MIC of 3.8 mg/mL.

Figure 3.3 compares the cell counts of *S. mutans* in the presence of the different polyphenols. The arrow in the Figure 3.3 represents the time at which the polyphenols were added to the culture. As discussed above, MIC is the minimum concentration of polyphenols that kills the cells. As we see in the figure, it can be clearly observed that the bacterial culture grew exponentially until the beginning of stationary phase and started to die once the polyphenols are added. The polyphenols were added after 16 hours of inoculation. The MIC values of gallic acid, salicylic acid and tannic acid against *S. mutans* were close to the values reported by other researchers [Himejima et al. 1991; Yoshino et al. 1995; Yamamoto et al. 2002].

### 4.4.2 Comparison between ABTS$^{+}$ and DMPD$^{-}$ Assay

The antioxidant capacities of polyphenols are reported to be attributed to various mechanisms; among these are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging [Heinoman et al. 1998]. Polyphenolic compounds are very important plant constituents because of their scavenging ability that is due to their hydroxyl groups [Georgi Gao et al. 1999]. Antioxidants, under proper conditions, may protect important molecules from free radicals’ attack and consequently reduce the risks of health problems such as accelerated aging, cancer and heart disease [Ames et al. 1993;
As mentioned earlier, we used the MIC’s of polyphenols as a basis of comparison because the polyphenols’ effect, mass, concentration and structure play an important role in their antimicrobial and antioxidant activities. Moreover we used minimum concentrations of polyphenols that kills the least number of cells. As a lot of polyphenols are known to act as pro-oxidants when high concentrations are used, we believe that, at the minimum inhibitory concentrations, these polyphenols do not become prooxidants and exhibit representative antioxidant activities. To more accurately measure the antioxidant capacities of the polyphenols, two different methods were used in our study, ABTS and DMPD methods.

As the ABTS$^+$ method is one of the most widely used assays by the food industry and agricultural researchers to measure the antioxidant capacities of foods [Huang et al. 2005], We compared the results obtained by this assay and the DMPD$^-$ assay for the same samples, to see whether the antioxidants show a similar trend towards these two radicals, or a quite different one.

![Figure 4.1 - Change in absorbance of the DMPD radical after the addition of different polyphenols.](image-url)
In Figure 4.1, each bar graph represents the color of the DMPD radical cation in the presence of the different polyphenols. A high % of inhibition represents high antioxidant capacity. As shown in Table 4.1, ascorbic acid was the strongest antioxidant with a 99% inhibition of the DMPD free radicals among the polyphenols used in our study. These results clearly indicate that ascorbic acid is a very good source of natural antioxidants. Gallic acid, tannic acid and salicylic acid exhibited significant radical scavenging effects with 62%, 49% and 44% inhibitions of the free radicals when compared to quercetin (27%). Normally upon the addition of an antioxidant compound, the color of the DMPD\(^+\) solution changes from purple to a clear solution. However, in our experiment for quercetin, the color of the DMPD solution changed to a yellow solution, which disqualified justifiable comparison with other polyphenols. Due to the interfering color change of quercetin in the DMPD solution, the antioxidant capacity of quercetin with the DMPD method was not used for comparison with other results.

**Table 4.1 - % of Inhibition of the DMPD radical in the presence of the different polyphenols.**

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>Absorbance</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.823</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.008</td>
<td>99.03</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.315</td>
<td>62</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.605</td>
<td>26.60</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>0.46</td>
<td>44.20</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>0.42</td>
<td>49.10</td>
</tr>
</tbody>
</table>
The ABTS assay is based on the inhibition by antioxidants of the absorbance of the radical cation (ABTS\(^+\)). As shown in Figure 4.2, ascorbic acid, gallic acid, tannic acid and quercetin were all very strong antioxidants when compared to salicylic acid. It was very difficult to rank the antioxidant capacities of all these polyphenols because the % of inhibition results was very close to each other. Ascorbic acid, a well-known antioxidant was used as a standard to compare the antioxidant activity of polyphenols.

As shown in Table 4.2, Gallic acid, quercetin and ascorbic acid showed close to 99% inhibition and tannic acid showed 96% inhibition of the ABTS radical. According to the ABTS assay, salicylic acid turned out to be the weakest antioxidant in comparison with the other polyphenols as it was able to scavenge only 3.5% of the ABTS free radicals.

![Graph showing absorbance at 658 nm for different polyphenols](image-url)

**Figure 4.2 - Change in absorbance of the ABTS radical after the addition of different polyphenols.**
### Table 4.2 - % of Inhibition of the ABTS radical in the presence of the different polyphenols.

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>Absorbance</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.715</td>
<td>-</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.008</td>
<td>99.03</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.007</td>
<td>99</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.005</td>
<td>99.25</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>0.693</td>
<td>3.40</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>0.024</td>
<td>96.70</td>
</tr>
</tbody>
</table>

The results for both the DMPD and ABTS assays were expressed in Trolox equivalents [Miller et al. 1997] as shown in Table 4.3. Trolox equivalent antioxidant capacity (TEAC) measures the antioxidant capacity of a given substance, as compared to the standard, Trolox. Results revealed that ascorbic acid had the highest antioxidant capacity with the highest Trolox concentrations in both the assays. Gallic acid came next to ascorbic acid in the both methods. The trends were similar for ascorbic acid, gallic acid and tannic acid in the both assays. Again, however, antioxidant capacity of quercetin was inconclusive because it had color interference on the DMPD radical giving a totally contradictory result when compared with the ABTS assay result. For comparison, antioxidant capacities of polyphenols by the ABTS method and antimicrobial effect expressed by MIC’s are plotted in Figure 4.3. Antimicrobial activities of the polyphenols are represented in terms of the inverse of MIC, and plotted on the left-side y-axis. The higher the bar, the higher is the antimicrobial activity. Antioxidant capacities are plotted on the right-side y-axis. Although the order of the antioxidant capacities does not exactly
match with that of the antimicrobial effects of the polyphenols, it is clear that all the strong antimicrobials showed high antioxidant capacities.

### Table 4.3 - Results of DMPD and ABTS assay expressed as Trolox equivalents.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TEAC (DMPD Method)</th>
<th>TEAC (ABTS Method)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trolox Equivalent</td>
<td>Trolox Equivalent</td>
</tr>
<tr>
<td></td>
<td>Conc., µM</td>
<td>Conc., µM</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>887.89</td>
<td>2394.5</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>476.44</td>
<td>2394.25</td>
</tr>
<tr>
<td>Quercetin</td>
<td>83.11</td>
<td>2400</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>278.67</td>
<td>3.75</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>333.11</td>
<td>2336.25</td>
</tr>
</tbody>
</table>

### 4.5 Conclusions

As shown in Table 4.4 and Figure 4.3, a clear trend was observed between the antimicrobial and antioxidant activities of polyphenols. We used the inverse of MIC as the antimicrobial activity of polyphenols in Figure 4.3. The inverse of MIC was used as strength of antimicrobial activity because a high MIC represents low antimicrobial activity. The polyphenols showed antioxidant activities, but however we think that as the concentration of polyphenols are increased above the minimim inhibitory concentrations, these antioxidants can become prooxidants and the final antioxidant activity can significantly change at these concentrations. Tannic acid, gallic acid and quercetin showed very good antimicrobial activities as well as good antioxidant activities. However salicylic acid was the weakest polyphenol used in our study with very weak antimicrobial and antioxidant activities. This was a clear indication that the antioxidant capability of
polyphenols may be involved in antimicrobial mechanisms. Quercetin, however, could not be properly measured for its antioxidant capacity by DMPD method. Tannic acid can be used as an effective antimicrobial agent (MIC: 0.4 mg/mL). Tannic acid, due to its strong antimicrobial activity, also has been commonly used to treat burns caused by incendiary bombs, mustard gas or lewisite [Halkes et al. 2001]. We conclude by saying that there was no clear relationship between the antimicrobial and antioxidant activities of polyphenols. However, there may be some contribution of the antioxidant capacities of polyphenols on their antimicrobial activities. Further experimentation would have to be done to confirm our hypothesis.

Table 4.4 - Antimicrobial effects and antioxidant capacities of polyphenols against *S. mutans.*

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC Conc., µM</th>
<th>TEAC (DMPD Method) Conc., µM</th>
<th>TEAC (ABTS Method) Conc., µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>&gt;2.0</td>
<td>887.89</td>
<td>2394.5</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>&gt;1.2</td>
<td>476.44</td>
<td>2394.25</td>
</tr>
<tr>
<td>Quercetin</td>
<td>&gt;1.5</td>
<td>83.11</td>
<td>2400</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>&gt;3.8</td>
<td>278.67</td>
<td>3.75</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>&gt;0.4</td>
<td>333.11</td>
<td>2336.25</td>
</tr>
</tbody>
</table>

Ascorbic acid used as a standard, though being lower than tannic acid in antimicrobial activity (MIC: 2.0), showed very good antioxidant capacity (2394.5 µM Trolox equiv. by ABTS) compared to tannic acid (2336.25 µM), which can play a major role in maintaining the proper functioning of the immune system by neutralizing...
potentially damaging free radicals. It is available in a variety of consumer products in both manufactured and natural products.

![Graph showing antimicrobial and antioxidant activities of polyphenols.](image)

**Figure 4.3 - Comparison of antioxidant capacities and antimicrobial activities of the polyphenols.**

Salicylic acid showed a weak antimicrobial effect and also a weak antioxidant capacity. Nevertheless, it is well known for its frequent usage as a commercial cosmetic preservative [Himejima et al. 1991].
CHAPTER 5

Recommendations for future research

This chapter provides a list of topics for future studies recommended based upon our work.

a) Polyphenols in our research exhibited very good antimicrobial and antioxidant activities against *S. mutans*, Gram-positive bacteria. The antimicrobial and antioxidant activities of polyphenols against Gram-negative bacteria and Gram-positive bacteria can be compared to see if they exhibit a same trend.

b) A more in depth study has to be done on quercetin as this polyphenol had color interference on the DMPD radical giving a totally contradicting result when compared with the ABTS method.

c) The DMPD and ABTS antioxidant activity results can be confirmed using electrochemical techniques like cyclic voltammetry and amperometry.
d) We did a HPLC analysis to check the enzyme activity of *Streptococcus mutans* in the presence of polyphenols. Dinitrosalicylic method can be used to measure the enzyme activity and the results can be compared with the HPLC results.

e) Further work can be done to check the glucosyltransferase enzyme activity in the presence of the different polyphenols by quantifying the exopolymers (glucans and fructans) production in the presence of different polyphenols.

f) In our work, the antibiofouling effects of polyphenols were verified before the biofilm formation. The antibiofouling effect of polyphenols has to be verified on a preformed biofilm because the bacteria in mature biofilms are resistant to antimicrobials and are harder to kill.

g) The antioxidant activity mechanism of polyphenols can be studied quantitatively based on the number and position of OH groups in their structures.

h) A relationship can be checked between the concentration of the polyphenols and their antioxidant potential. Several hypotheses may surround this type of experimentation given the balance between the antioxidant potential of the polyphenols and the possibility of creating prooxidants at higher concentrations in the solution.
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