MECHANISTIC STUDY OF THE ANTIBACTERIAL ACTIVITY OF FLAVONOIDS

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ABSTRACT OF THE DISSERTATION

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Foodborne pathogens are one of the major causes for food spoilage which happens in raw meat, vegetable, and dairy products. The emerging antibiotic resistance acquired by common pathogens is bringing urgency for food industry to explore more options for effective and safe antimicrobial agents. As one of the largest groups of plant metabolites, flavonoids were discovered to possess antimicrobial activity but much of the detailed mechanisms remain unclear.

In this study, we established a QSAR model using published datasets that can summarize and predict the redox behaviors of flavonoids with given structures. Then several flavonoids with similar backbone structures yet varied hyperactive groups were tested for antibacterial activity. We observed that flavonoid antibacterial activity has a positive correlation with its antioxidant potential, which was typically measured in acidic environments. Our ORAC analysis revealed that the most theoretically antioxidant flavonoids may exhibit prooxidant properties due to rapid degradation in weak alkaline conditions, a phenomenon directly associated with their antibacterial activities. We then focused our study on myricetin, the most potent one in the group that contains a C2=C3 double bond and B-ring pyrogallol group structure. We discovered that myricetin undergoes autoxidation to generate reactive oxygen species (ROS) at physiological conditions.

We then supplemented enzymes or cofactor metals that are essential for various cellular oxidative pathways to the growth medium of *E.coli* or *L. monocytogenes* with myricetin, and noticed significantly corresponding interference or improvement of the inhibitory effect. These data suggested that the antibacterial activities of myricetin are directly mediated by the ROS generated by its autoxidation. We also showed that the ROS generation in turn altered the physiological profile of *E.coli* cells that may lead to its demise. These alterations include the compromise of membrane integrity, increased leakage of cellular components, as well as transcriptional upregulations of multiple genes associated with oxidative damage prevention, DNA replication or DNA damage repair. Altogether, our findings highlighted the use of the previously underexplored natural flavonoid compound myricetin, among other flavonoids, as a potential and potent antibiotic with elucidated mechanisms of action that could be partially inferred from its structural features. The emergence of such broad-spectrum, strong and natural antibiotics will serve as one of the key solutions to the alarmingly rising problems of antibiotic resistance and will therefore be unmeasurably beneficiary to the food and drug industry.

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Chapter 1 Introduction

1.1 Food Preservation and Safety

Food preservation has a history as long as the history of food [1]. Before the modern invention of refrigerators, mankind has relied on freezing, drying, smoking, salting and fermenting to preserve raw meat or other products for an extended amount of time. Along with the development of technology and the in-depth understanding of food science, a growing numbers of preservation processes and techniques are applied in the food production pipeline [2]. Yet, the main purposes for these techniques, prevention of microbial growth and chemical composition changes, remain the core concerns in the food industry. Chemical changes are usually caused by hydrolytic or oxidative reactions with the external environment, and would result in overall compromise of the color, smell, taste and nutrition values of food. Microbial growth on the other hand, could have much more severe or even deadly consequences. As a matter of fact, foodborne diseases are becoming a serious challenge throughout the world: more and more new species of pathogens are being identified and isolated on a daily basis. Among those, some notoriously famous pathogens like *Escherichia coli* O157:H7, *Salmonella species*, *Listeria monocytogenes* have been recognized since as early as in the 1940s as major microorganisms responsible for foodborne illnesses. More recently, new emerging pathogens such as *Vibrio vulnificus* and *Cyclospora cayetanensis* have also come under the radar [3]. Even for those pathogens we are familiar with, they are constantly evolving rapidly to adapt environment, more often than not with increase of virulence. Most of the cases occurred with zoonotic pathogens and could be attributed to the massive use of antibiotics on animals. Some examples are methicillin-resistant *Staphylococcus aureus* (MRSA), species of *Salmonellae* and *Campylobacter*, all of which are known to be resistant to many antibiotics [4]. The emerging species of new pathogens and the limitation of effective medicine to fight old ones together pose a serious and luring threat to the public health.

Thus, food preservation has become one of the most challenging and pressing issues in the food industry.

In order to avoid spoilage in food, the widely used food preservatives normally possess either antimicrobial or antioxidant activity and have been extensively used in the manufacture process of food. Along with the exploding global population, the need for food products with longer shelf-life has also been sky rocketing. Indeed, food preservatives have a large margin of market and this market is expanding rapidly. By the year of 2021, the food preservatives market is about to reach 2.94 billion USD in terms of value. The increasing demand for effective and safe food preservatives has reflected the growing public awareness of food safety as now more than ever, consumers begin to pay more attention and concerns on the side-effects that may come along with food preservatives. Based on their sources, most of them can be categorized as natural preservatives (Class I) and artificial food preservatives (Class II). Class I preservatives include sugar, salt, syrup and other edible food ingredients and are generally considered less harmful to human health [5]. Class II artificial preservatives on the other hand, comprise of synthetic or chemical compounds and are usually the main reason that causes consumers' concern. Artificial food preservatives are advantageous in their expanded panel of derivatives to be applied widely and their capability to be manufactured on a large and cost-effective scale. They also have great potentials to be manipulated chemically for performance improvements. While impressive progress has been made with extended shelf-lives of food products achieved by the addition of artificial preservatives, sometimes consumers are exposed to the risk of carcinogenic chemicals or uncharacterized agents that can cause other diseases. Recently, many food manufacturers cut the use of artificial preservatives in the products and try to find an alternative approach as food products labeled with "natural preservatives" start to catch more eyeballs. Since natural preservatives usually come from native organisms or have already been proven as edible, consumers are much less worried about foodborne safety issues. This preference from consumers

and the true potential of safe natural food additives raise an exciting challenge for food scientists—searching for and characterizing natural antimicrobial agents for potential food grade preservatives.

1.2 Foodborne Pathogens

In the United States, about one in every six people can be exposed to the danger of consuming contaminated food. If we put aside the ones caused by chemical reagents, most of the food contaminations are due to microorganisms such as: bacteria, fungi, viruses, and some protozoa. The existence of specific microorganism may serve as the indicator of certain food product. Some of the main contributors to foodborne pathogens are detailed described here.

1.2.1 *Campylobacter jejuni* and *Campylobacter coli*

The *Campylobacter* species are curved or spiral-shaped Gram-negative bacilli. The illness caused by these bacteria is characterized by acute diarrhea, abdominal pain, and fever. Other serious conditions within the gastrointestinal tract have been associated with *Campylobacter spp.* infections, including intestinal bloody diarrhea, esophageal diseases, periodontitis, functional gastrointestinal disorders, celiac disease, cholecystitis, and colon cancer [7]. People acquire the infection of *Campylobacter* from having contaminated poultry, water or interacting with farm animals harboring these bacteria. Along with *Salmonella spp.*, *Campylobacter spp.* is one of the most frequently isolated foodborne pathogens.

1.2.2 *Salmonella typhimurium*

Salmonella typhimurium is found in human intestinal lumen as a pathogenic Gram-negative bacterium. It directly infects epithelial cells in the gut and triggers white blood cells infiltration, causing diarrhea in infected patients. *S. typhimurium* is extensively associated with foodborne

pathogen outbreaks in both developing and developed countries [8] by people consuming undercooked meat, dairy products and raw eggs [9].

1.2.3 *Escherichia coli* O157:H7

Escherichia coli is a Gram-negative, facultatively anaerobic, rod-shaped bacterium, which can be found in the lower intestine of human beings. Unlike most serotypes in the family, some *E. coli* strains may cause serious food poisoning. Large infection outbreaks of infamous *E. coli* O157:H7 have involved thousands of cases across the world. It was first identified as a foodborne pathogen in 1982 and categorized as enterohemorrhagic *E. coli* (EHEC) [10]. *E. coli* O157:H7 related diseases may result from contaminated meat, fruits, vegetables, and even person-to-person contact. Symptoms of *E. coli* O157:H7 include bloody diarrhea, non-bloody diarrhea, kidney disease, etc. Most of the studies believe these pathogens colonize in the large intestine by adhering to the cell membrane. Veron toxins or Shiga toxins (Stx) and cytotoxic factors, are produced by *E. coli* O157:H7 and other EHECs [10]. Even among pathogens that may cause severe diseases, *E. coli* O157:H7 is one of the most important EHEC serotype in North America.

1.2.4 *Listeria monocytogenes*

Listeria monocytogenes is a facultative anaerobic Gram-positive bacterium. It is widely distributed in nature, resistant to adverse environment, and able to survive in food for a long period of time. Listeriosis has become a major foodborne disease with high fatality rate over past decades. The outbreaks of *L. monocytogenes* were always related to milk products, meat and poultry products, and seafood. Consumption of contaminated food may lead to septicemia, meningitis, and meningoencephalitis, and abortion for pregnant women. A unique mechanism of pathogenicity of *L. monocytogenes* distinguishes it from other pathogens. It enters cells and transmits directly between cells. This mechanism reduces its exposure to antibiotics and makes it capable of penetrating into brain and placenta [11].

1.2.5 Foodborne fungi pathogens

Apart from bacteria, fungi also comprise of a major part of foodborne pathogens. While some fungi are central ingredients in the food industry (for example, *S. cerevisiae* is one of the most widely known agents in bakery and brewery), many pose threats to the human health and especially dangerous as sources of food spoilage. Two major ways of how fungi can be harmful are through the generation of toxic metabolites such as mycotoxins [12], or through active invasive fungal infections (IFIs). In both cases, the risks come from fungal contamination or spoilage of food and beverage that could lead to serious illnesses or outbreaks of infectious disease [13] . CDC estimates a few hundred species fungi out of a totality of millions that could cause human diseases, ranging from mild allergic reactions to deadly invasive infections. Among these, common foodborne fungal pathogens include *Alternaria*, *Aspergillus*, *Candida*, *Fusarium*, *mucormycetes* and *circinelloides* [13-16].

1.3 Antibiotics

Overview

Antibiotic is a large group of antimicrobial substances that act against bacteria. Some of them also possess antiprotozoal activity, yet most do not exhibit antiviral activities. Antibiotics can function by either killing the bacteria (bactericidal) or inhibiting the reproduction of the bacteria (bacteriostatic). Different classes of antibiotics have been described, primarily based on their chemical structure, the mode of actions, or the spectrum of activities. Antibiotics belonging to different classes, target various biosynthetic pathways. Most frequent targets of antibiotics include but are not limited to bacterial cell wall synthesis, nucleotide synthesis and protein synthesis [17]. These targeting mechanisms and effects on bacterial cells by commonly used

antibiotics, as well as indications in bacterial resistance to antibiotics will be discussed below in detail.

1.3.1 Antibiotics mode of actions

1.3.1.1 Antibiotics targeting cell wall

Bacterial cell walls are made of peptidoglycan, which consists of long sugar polymers. The base structure is formed by linear chains of two alternating amino sugars, N-acetylglucosamine (NAGA) and N-acetylmuramic acid (NAMA) [18]. The peptide chains extend from the sugars in the NAMA polymers and form cross links with the help of DD- transpeptidase. The D-alanyl-D-alanine of peptide chain is cross linked by glycine residues in the presence of penicillin binding proteins (PBPs). Cross-linking between amino acids in parallel amino sugar chains results in a three-dimensional structure, serving as a strong and rigid wall. The specific amino acid sequences and molecular structures vary with the bacterial species. Antibiotics belonging to the β-lactam family and the glycopeptide family inhibit cell wall synthesis [17].

β-lactam antibiotics, such as ampicillin and carbapenems, target primarily PBPs. It was proposed that the β-lactam ring mimics the D-alanyl-D-alanine structure in peptide chains that is normally bound by PBP. Once occupied by β-lactam ring, the PBP is then no longer available for the new peptidoglycan synthesis. This disruption of peptidoglycan layer synthesis eventually results in lysing of bacteria.

Glycopeptides, including vancomycin, are glycosylated non-ribosomal peptides. They target Gram-positive bacteria by binding to the D-alanyl-D-alanine terminus of the growing peptidoglycan, hence inhibiting cell wall synthesis [19].

1.3.1.2 Protein synthesis inhibitors

Bacterial protein biosynthesis is conducted by the 70S ribosome, consisted of two ribonucleoprotein subunits, namely the 30S and 50S subunits. Many groups of antimicrobials were reported to target either subunit of the ribosome, thus conveying inhibition to the bacterial protein biosynthesis.

Aminoglycosides, such as streptomycin and neomycin, are highly polar molecules, which cross the bacterial outer membrane via a self-promoted uptake process. However, the subsequent penetration into the inner membrane requires oxygen and is dependent upon electron transport [20]. Due to this reason, aminoglycosides work best in aerobic conditions and have poor antibacterial activities against anaerobes. Synergistic activities were observed for aminoglycosides in cocktail with antibiotics that inhibit cell wall synthesis (such as β-lactam and glycopeptides), which allows greater penetration of aminoglycosides at lower dosages. Once entered the cells, aminoglycosides bind with the 16S rRNA of the 30S subunit, causing perturbation of the translation elongation by impairing the proofreading process. The interference in turn leads to misreading and premature termination of translation [17].

Tetracycline antibiotics such as tetracycline, chlortetracycline and doxycycline are well known for their broad spectrum of activity, targeting both Gram-positive and Gram-negative bacteria, and also some protozoan organisms. Tetracycline penetrates into the outer membrane of Gram-negative bacterial cells via passive diffusion. The accumulation of tetracycline in the periplasm leads to their entering into the cytoplasm by ways including passive diffusion, proton motive force, and phosphate bond hydrolysis, thus being partially energy dependent. Once in the cells, tetracycline targets 16S rRNA of the 30S subunit for binding, arresting translation by sterically interfering with the tRNA docking to the A site during elongation[21].

Some other antibiotics target the 50S ribosomal subunit to inhibit protein synthesis process. These antibiotics include chloramphenicol, macrolides, and oxazolidinones. The detailed mode of actions varies despite the fact that all three families of antibiotics bind to the 23S rRNA of the 50S subunit. Specifically, chloramphenicol directly interferes with substrate binding in the ribosome, therefore inhibiting the peptidyl transferase activity [17]. As a comparison, macrolides sterically block the progression of the growing peptide, resulting in premature detachment of incomplete peptide chains. Oxazolidinones interfere with protein synthesis at multiple stages, including (1) inhibition of 70S formation and (2) blocking translocation of the peptide chain from A to P site once the 70S is already formed [22].

1.3.1.3 DNA replication Inhibitors

Fluoroquinolones target the bacterial DNA gyrase, which is an enzyme within the class of topoisomerase. The DNA gyrase activity is essential for unwinding the excessive positive supercoiling stranded DNA, allowing other enzymes to engage properly to the DNA strands for replication or transcription. Fluoroquinolones have high binding affinities to the bacterial gyrase, interfering its enzymatic functions, hence preventing DNA replication. This could result in double-strand break accumulation, replication fork stalling and eventually cell death. Mammalian topoisomerases have lower binding affinities for fluoroquinolones, therefore effectively avoiding high toxicities [17].

1.3.1.4 Folic acid metabolism inhibitors

Both prokaryotic and eukaryotic cells need folate for the biosynthesis of diverse types of cellular components. While mammalian cells possess ways to uptake and transport folate into the cytoplasm, bacterial cells must undergo *de novo* synthesis through the folate biosynthetic pathway. Due to the lack of conserved folate biosynthetic factors in the mammalian system, targeting the folate metabolism pathways by antibiotics has advantages in its specificity and

safety [23]. Sulfonamides and trimethoprim inhibit different steps in the same biosynthetic pathway during folic acid metabolism. Sulfonamides inhibit dihydropteroate synthase in a competitive manner, due to its higher affinity for the enzyme than its natural substrate. Trimethoprim inhibits dihydrofolate reductase at a later stage of the pathway. While used together, they show synergy and lead to a reduced mutation rate in bacteria for resistance [24].

1.3.2 Bactericidal and bacteriostatic

Antibiotics generally exhibit bacteria growth inhibition via bacteriostatic or bactericidal activities, with the major difference being if the cells were killed or merely suspended in growth while still alive [25]. Two terms are introduced, namely minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC), for determining an antibiotic to be bactericidal or bacteriostatic. MIC is defined as the lowest concentration of an antimicrobial drug that will inhibit the visible growth of a microorganism. MBC is defined as the lowest possible concentration of antimicrobial agent that kills the initial bacterial inoculum by ≥99.9%. While MIC represents the lowest dose of an antibiotic to inhibit bacteria growth, the MBC value describes the lowest dose of an antibiotic resulting in cell death, thereby being complementary to MIC. The determination of an antibacterial agent to be bactericidal is to calculate the MBC/MIC ratio (<4 for bactericidal agents) [26]. Apart from direct measurement of MBC and MIC, studies with more general antibiotics suggest that bacteriostatic and bactericidal drugs impact distinct pathways in the bacterial cells. Bacteriostatic drugs typically pause the protein synthesis pathways, with many being direct ribosome unit inhibitors [27, 28]. Meanwhile, the majority of bactericidal drugs target key players in DNA replication, DNA damage response, cell wall integrity [29]. Based on these definitions, one can expect that it is not mutually exclusive for an antibiotic to be bactericidal and/or bacteriostatic. In fact, it depends on multiple determinants such as the target strain susceptibility, *in vitro* or *in vivo* experiments, and whether a certain antibiotic is functioning alone or in combination with other antimicrobial reagents.

1.3.3 Side effects and antibiotic resistance

Currently, the development of applicable antibiotics includes screening for adverse effects before formal clinical use, thus are normally safe for human use. However, side effects were observed with some antibiotics, and can range from mild to severe symptoms depending on the specific antibiotic, the bacteria species as well as the specific environmental conditions. Some side effects may include hypersensitivity or allergic reactions, while others may lead to diarrhea, due to the disruption of normal protective gut flora (in the case of *Clostridioides difficile* -associated diarrhea). Antibiotics may also interact with other medicinal substances, such as the potential tendon damage from a quinolone antibiotic if co-administered with a systemic corticosteroid [30]. Sometimes, it may take years to reveal the true side effects of antibiotics. Oral administration of chloramphenicol was banned in the US back in 90s due to the increased number of chloramphenicol-associated cases of aplastic anaemia and its suspected carcinogenicity [31].

Antimicrobial resistance is recognized as one of the greatest threats to human health worldwide. This is largely attributable to the overuse of antibiotics that selects for bacteria or mutants that confer resistance. As described in the previous sections, antibiotics have their specific mode of actions with some of them sharing the same targets. Therefore, bacteria may either gain resistance to a single family of antibiotics or develop multi-drug resistance. For example, bacteria harboring β-lactamase is able to counteract β-lactam antibiotics and develop resistance. In other cases, some *Staphylococcus aureus* and *Escherichia coli* strains encode an enzyme that is able to modify the 23S rRNA of the 50S unit, allowing escape of the rRNA binding with multiple drugs including phenicols, lincosamides, oxazolidinones, etc [32]. Due to the increasing cases and types of emerging antibiotic resistant bacteria, scientists have devoted themselves for new antibiotics discovery as well as researching alternative ways to combat bacterial infections, including

developing vaccines and utilizing phage therapies [33]. Altogether, antibiotics developed in the past century have their advantages yet also underlying limitations and safety concerns that urgently require complementary approaches for mankind to gain full and safe protection from bacteria infections. In most recent years, more and more researchers have turned to natural products that harbor antibacterial capacities for antibiotic development.

1.4 Natural Antimicrobial Agents

As mentioned above, searching for natural antimicrobial agents is to relieve the increasing unease regarding the use of artificial preservatives. Other than that, the developing antibiotic resistance by pathogenic microorganisms is another reason why natural antimicrobial agents have received so much attention.

Natural antimicrobial agents can be obtained from a variety of sources including animals, plants and micro-organisms. Regarding antimicrobials of animal origin, chitosan [34, 35] and lysozyme [36] are among most understood ones. Antimicrobials originated from microorganisms on the other hand, include substances from bacteria, fungi and algae and also have great potential for practical use in food [34, 37-39]. In this dissertation, antimicrobials of plant origin will be discussed in detail.

Plant-derived antimicrobials are widely discovered in herbs, spices and plant by-products [6, 40-42]. Some compounds from fruits and vegetables are already proven to have considerable antimicrobial activity [43]. Surprisingly, many of those including peels, seeds and husks, which are normally considered waste, have showed even stronger antimicrobial activity than usable parts [44]. Considering the massive amount of waste from fruits and vegetables produced worldwide every year, this could be a major source of natural antimicrobial agents for economic reasons if utilized wisely.

In most cases, plant-derived antimicrobials are secondary metabolites, most of which have the major compound groups including phenolics, phenolic acids, flavonoids, tannins, saponins, terpenoids and alkaloids [45]. The differences in the antimicrobial activity may result from the differences in the chemical structure of these compounds [46]. (Figure 1.1) For example, phenolic compounds apply their antimicrobial activity through damaging the cell membrane of bacteria cells [47]. Certain function groups such as hydroxyl (-OH) group have been reported to play key roles in causing inhibition. Their effects include either depletion of ATP pool [48] or inactivation of enzymes [49] that may result in cell death. Other than the identities of function groups, the antimicrobial activity is also influenced by the position of these groups [50]. For example, difference in antimicrobial activity between carvacrol and thymol, two major compounds in essential oils, was observed despite their similar structure. Another example of the importance in hydroxyl group's position came from the report that flavanones and flavones showed activities against MRSA strains only when hydroxyl group appeared at 5-position [51]. In addition, phenolic structure has been proven to play a key role in compounds showing antimicrobial activity. This was revealed in the investigation of the relationship between major antimicrobial compounds in essential oils and their chemical structures [48, 50]. Thus, a better understanding of the relationship between structure and antimicrobial activity will pave the road for the discovery of new natural antimicrobials.

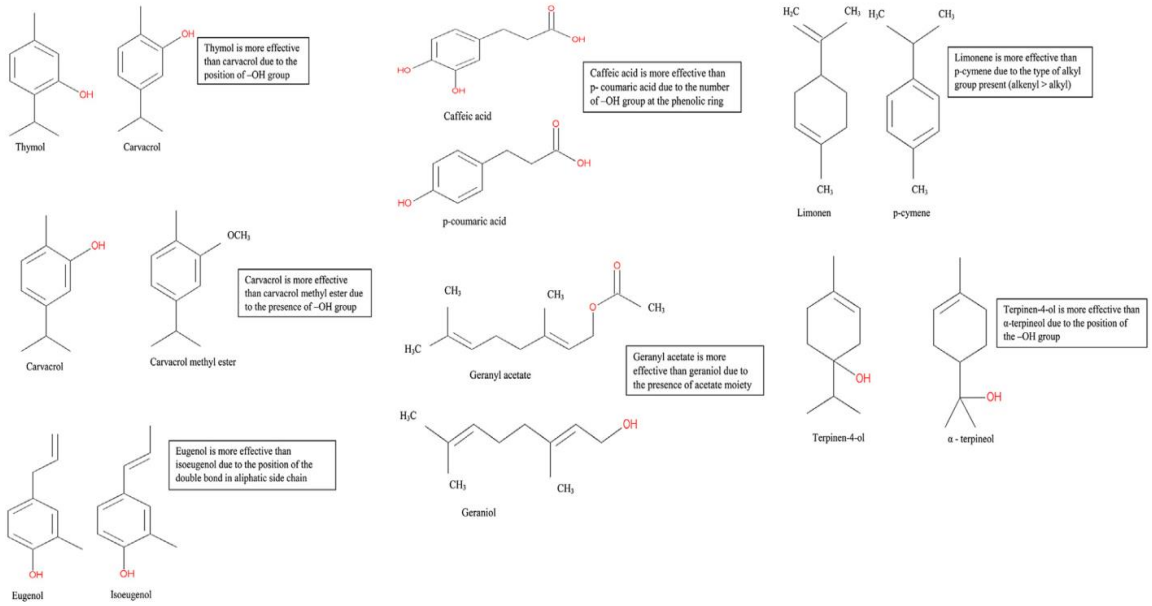

Figure 1. 1 Structural variation among functional groups in plant compounds alters their antimicrobial activity.

Adapted from Savoia et al., 2012 [46].

1.5 Flavonoids

The occurrence of flavonoids is widespread in the plant kingdom [52, 53]. Flavonoids are universally found in different parts of plants. Their distribution varies depending on the classes of plants, the types of organs and the surrounding environment [54]. Additionally, flavonoids are known as the secondary metabolic compounds derived from the low molecular weight plant polyphenols and those compounds usually play an essential role in plant organs. Initially, the function of flavonoids is considered to provide colors in flowers and aroma of fruits to attract plant pollinators [55]. In other reports, flavonoids are believed to protect plants from survival stresses like microbial infection, UV-B radiation [55], frost hardiness, drought, heat, and freezing [55-57]. Moreover, flavonoids are also involved in energy transfer, regulation of plant growth and photosynthesis [54].

Based on chemical structures, more than 4,000 flavonoid compounds have been isolated and identified [58]. The basic structure of flavonoids consists of two benzene rings (A and B) linked through a heterocyclic pyran ring (C) (Figure 1.2). According to different substitutions on the backbone structure, flavonoids are divided into several subgroups: flavone, flavonol, dihydroflavonol, catechin, flavanone, isoflavone, etc (Figure 1.3). The polyphenolic structure infers the antioxidant activity. In recent decades, other functions of flavonoids are revealed as anti-inflammatory [59], anticancer [60], antiviral [61], and antifungal properties [62]. Among all these functions, the antimicrobial activity of flavonoids draws more and more attentions due to its potential applications in the medical field and food industry. The flavonoid containing crude plant extracts that were widely used to fight infections mark the start of such research endeavors, which eventually led to the discovery of the knowledge base of antibacterial properties possessing flavonoids to this day. Representative flavonoid compounds belonging to different subgroups that have recorded activities against bacterial cell growth are introduced below for the current understanding of their antibacterial behaviors and underlying mechanisms [63].

1.5.1 Flavanone

Some flavonoids are classified based on the compound origins in biosynthesis. Flavanone is one of such classes known both as intermediates and end products in plant biosynthesis processes. The groundwork laying structure-activity relationships between these molecules and bacterial inhibition has been assessed using MRSA as a model system [64]. The analysis highlighted the importance of dihydroxylation at 2',4' or 2',6' on the B-ring as well as 5',7' on the A-ring. Other derivatives that promote the biofunction include long chain aliphatic groups substituted at C-6 or C-8, hydroxyl groups on C-5 and C-2' [51]. Other indicated that neither mono nor poly methoxylation derivatives of flavanones contributed to such functions [65].

Some of the most characterized flavanones are naringenin, nobiletin, tangeretin and liquiritigenin, and have been reported to be responsible for the bacterial protection from medicinal plants [66, 67]. The search for more representative and active flavanone is still showing promising progress marked by the recent discovery of lonchocarpol A, which robustly inhibits the micromolar synthesis in *Bacillus megaterium* [68]. Research groups working with *S. aureus* identified prenylation, geranylation, and lavandulylation as key boosters of flavanone activities. Three individual prenylated flavanones at C-6 or C-8 have been proven effective on the antibiotic resistance strain MRSA [69, 70]. In the case of geranyl and lavandulyl group substitutions, direct comparisons between the 6-lavandulyl containing flavanone sophoraflavanone G and leachianone G with 6-prenyl demonstrated that lavandulyl is a stronger enhancer than prenylation [64]. Naringenin who lacks the lavandulyl group on C-8 and hydroxyl group on C-2' corresponding exhibit weaker activities against bacteria. Similar to the prenyl group, both geranyl and lavandulyl tend to occur on C-6 and C-8, suggesting that these sites are hotspots for antibacterial promoting substitutions.

1.5.2 Flavone

Flavone is structurally distinct from flavanone mainly via the unsaturated C3-C4 bond, making its chemical skeleton planar. The flavone family have long been popular candidates in flavonoid antibacterial activities. Like the case with flavanones, Pioneer works have identified prenylation to be a critical impacting factor in flavone antibacterial activities. For example, a di-prenylated flavone named kuwanon C showed activities against both Gram positive (*S. epidermis* and *S. aureus*) and Gram negative strains (*E.coli* and *S. typhi*) [71]. On the other hand, Corylifol C, a flavone with only a mono prenyl group isolated from *Psoralei corylifolia*, exhibited negligible growth inhibition of *S. aureus* or *S. epidermis* [72]. Morusin, also a di-prenylated flavone, has limited antigrowth capacities only on Gram positive bacteria. This reduction of antibacterial properties can be explained on the structural level as evidence revealed that the C-3 prenyl group on morusin likely reacts with the other prenyl group on C-8 internally, forming a ring-like substituent with the C-7 OH group, losing both active prenyl groups. The collective published evidence thus further strengthens the importance of the prenylation of flavones in their antibacterial capabilities.

Another important chemical impactor is suggested to be acylation on flavones. Multiple groups have observed enhanced activities against bacteria when O-acyl substituents are present. Moreover, investigations on a variety of Oroxylin A or chrysin derivatives have revealed strong positive correlations between their antibacterial activities and presence of nitrogen, phenyl groups, long alkyl chains, or long distances from the alkyl amines to the core ring structure, realized by engineered spacer insertions [73-75]. Altogether, acylation is likely an impactful additive on the flavone structure for its functional enhancement.

1.5.3 Flavonol

Flavonol is structurally close to the family of flavones where a hydroxyl group is present at the C-3 position instead of hydrogen. While many flavonols are found to be active agents against both Gram-positive and Gram-negative bacteria, other evidence also seem to suggest that their activities can be largely affected by the substituent groups instead of in the backbone structural components. For example, some flavonol with only either a methoxyl or a hydroxyl moiety typically exhibit only modest inhibitions on the bacterial growth especially when compared with some commonly used antibiotics, limiting their potentials to be utilized as novel antibiotic agents. On the other hand, having other active moieties present may greatly enhances their potency. Some commonly active substituent groups include prenyl groups (papyriflavonol A against multiple strains), hydrocarbonyl (kenusanone C against various bacteria) and lavandulyl (kushenol X against *S. aureus* and *B. subtilis*) [71, 76]. Despite the emerging novel derivatives of flavonols, some of the most characterized flavonols were chosen in this study to further examine their modes of action in fighting against bacterial growth.

Quercetin, a dietary flavonoid, widely exists in our daily food such as onion, tomato and lettuce. Instead of existing as its original structure, it usually is bound with sugars, ethers or phenolic acids. Quercetin and its derivatives have received increasing attention for their bioactive activities in many aspects including antioxidant, anti-obesity, anti-cancer, anti-inflammatory and antimicrobial activities [77-80]. Galangin is one of the most important and naturally active flavonoids, which is extracted primarily from the roots of various herbal medicines such as *Alpinia officinarum Hance, Alnus pendula Matsum, Plantago major L,* and *Scutellaria galericulata L*. Like some other flavonoids, galangin was reported to possess many biological activities, including anti-inflammatory, antimicrobial, anti-obesogenic, and antioxidant effects. In addition, many studies have indicated an anticancer effect of galangin against various malignancies, including breast cancer, lung cancer, colorectal cancer, melanoma, hepatocellular carcinoma and etc [81].

Myricetin is very common in berries, vegetables, teas and wines produced from various plants. It exists in both free and glycosidically-bound forms. Myricetin is recognized for its iron-chelating, antioxidant, anti-inflammatory and anti-cancer properties [82]. Additional studies also demonstrated that myricetin possesses antihypertensive activity, immunomodulatory activity, as well as activity against neurological disorders, rendering it a high valuable therapeutic use [82]. The antimicrobial activities of myricetin against various bacteria and viruses by itself or in combination with other antibiotics were also described, with various mechanisms case by case. Most importantly, among intense examinations on *in vitro* and *in vivo* models, very few of these studies have raised concerns with adverse effects of myricetin, making it a safe food additive option.

Kaempferol is a flavonoid found in many edible plants including tea, broccoli, cabbage, kale, tomato, strawberries, grapes and plant-based traditional medicine. Epidemiological studies have revealed that kaempferol-containing food consumption seemed to be linked with reduced risks of various cardiovascular diseases and cancer development. Other studies have demonstrated that kaempferol and some of its glycosides derivatives possess a wide spectrum of pharmacological functions, including but not limited to antioxidant, anti-inflammatory, antimicrobial, anticancer, anti-osteoporotic, analgesic and antiallergic activities [83].

1.5.4 Flavonon-3-ol

Flavonon-3-ol is a family of flavonoids that are also known as dihydroflavonols. Similar as flavanones, flavonon-3-ols classes are also both biosynthesis intermediate and end products in plant metabolism. Many flavonon-3-ols showed even more robust and broad activities than traditional antibiotics and the MRSA strains were found sensitive to most dihydroflanovols [84]. Due to their high potentials in being used as novel antibiotics, two promising agents were also

chosen to undergo a series of experimentations to profile their potency and possible mechanism of action when significant.

Ampelopsin, also known as dihydromyricetin, is a flavanonol. It is widely found in *Ampelopsis* species which are used as herb medicine in East Asia. For example, *Ampelopsis grossedentata*, a wild plant in South China, has been reported to possess lots of pharmacological properties, such as antioxidant, analgesic, antimicrobial, relieving cough and reducing blood pressure, etc [85]. As a good source of flavonoid, *A. grossedentata* has been confirmed to present ampelopsin as its major flavonoid, taking up more than 20% (w/w) [86]. The successful cultivation of *A. grossedentata* makes large-scale production possible for dihydromyricetin, enabling its massive use in functional food and medicine. However, the poor solubility restricts its functions. Thus, increasing its solubility or dispersibility in liquid system will be a great improvement for its application in food and pharmaceutical industry.

Taxifolin, or dihydroquercetin, is a flavonoid commonly found in onion, milk thistle, French maritime pine bark and Douglas fir bark. Taxifolin has promising pharmacological activities in the management of inflammation, microbial infections, oxidative stress, cardiovascular, and liver disorders. Additionally, taxifolin demonstrates prominent anticancer activities, evaluated by multiple *in vitro* and *in vivo* experiments, and is suggested to be further developed as a new drug for human use [87].

1.5.5 Anthocyanidin and Proanthocyanidin

Unlike flavonones and flavonon-3-ols, anthocyanidins and proanthocyanidins are found in plant tissues only as accumulative end products of biosynthesis. Proanthocyanidin are oligomers of flavan Separate studies unveiled three individual proanthocyanidins to exhibit antivirus functions

against coxsackie B virus and HSV [88, 89]. The proanthocyanidin family were reported to be effective on both drug sensitive and resistance bacterial strains [90, 91]. Some examples of characterized ones include EPA, PB3 and PEC that were recently isolated and all have inhibitory effects on Gram positive and Gram negative bacteria. A popular theory is that the oligomeric status has a direct positive correlation to their antibacterial behaviors, backed up by the comparisons between some common flavanol monomer, dimer, trimer and oligomers [92, 93]. The molecular mechanisms for many of such oligomers remain unclear and will require extensive investigations before being converted to medicinal or antibacterial applications.

1.5.6 Aurone, isoflavonoid and Chalcone

Aurone, isoflavonoids and chalcones represent flavonoid families in relative minority yet with notable potentials in being fully utilized for their strong antibacterial capabilities. Aurone naturally exist as common pigments for plant color displays. Besides, some extracted aurones also possess antimicrobial, anticancer and antidiabetic indications [94, 95]. An isolated heptasubstituted aurone glucoside was able to fight against 5 out of 22 different bacterial species [96]. The double bond in aurones grants it with either the Z or E configuration and interestingly, only the Z configuration adopting aurone isomers are thought to be directly active in bacteria growth, underscoring the potential role of geometric configurations in flavonoid functions.

Isoflavonoids represent flavonoids with a 3-phenylchromen-4-one backbone and with the B ring attached to the C ring at the C-3 position. They can be further classified into subgroups just as regular flavonoids into isoflavones and isoflavanones accordingly and could have distinct potencies at targeting bacteria that is currently at a premature stage to be thoroughly understood solely based on their structural components. Lupalbigenin is found to be strong against *S. aureus* and MRSA SK1 with an MIC at 4 μg/mL [97]. Gancaonin G was derived from genestein with substituted prenylation at C-6 position and methylation on 7-OH and shows only moderate

activities against the oral pathogen *S. mutans* [98]. Glabridin on the other hand, showed robust inhibitions against Gram positive bacteria with MIC values typically below 10 µg/mL, yet fairly poor with Gram negative ones with MIC scores usually greater than 100 µg/mL [99].

Chalcones are the family mostly found in edible plant tissues and structurally has an open linkage made of three carbons between its two ring components. Systematic studies have inferred that chalcone may possess even stronger bacterial targeting properties than flavanones or flavone against MRSA and streptococci. A closer investigation on the chemical moieties have identified the C-2' hydroxyl group to be specifically important in conveying the activities [64, 100]. In contrast, fluorination or chlorination moieties on the C-4 position of the B ring were dispensable for bacterial inhibition [51]. Recent discoveries also added to the story regarding the importance of hydroxyl groups at the C-4' position, as exemplified by the 2',4'-dihydroxychalcone showing activities against both MSSA and MRSA, but that the 2' hydroxyl group alone in the A ring is not necessary [101]. On the other hand, methoxylation seems to completely abolish these activities of chalcones [102]. In the case of dihydrochalcones, their antibacterial behaviors are considerably vulnerable, when reduction of the double bond, C-benzylation or the dihydrochalcone dimers all render the compound inactive against pathogens such as *M. tuberculosis* [103].

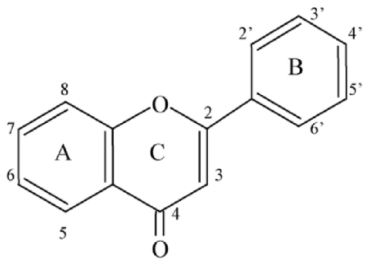

Figure 1. 2 The skeleton structure of flavones.

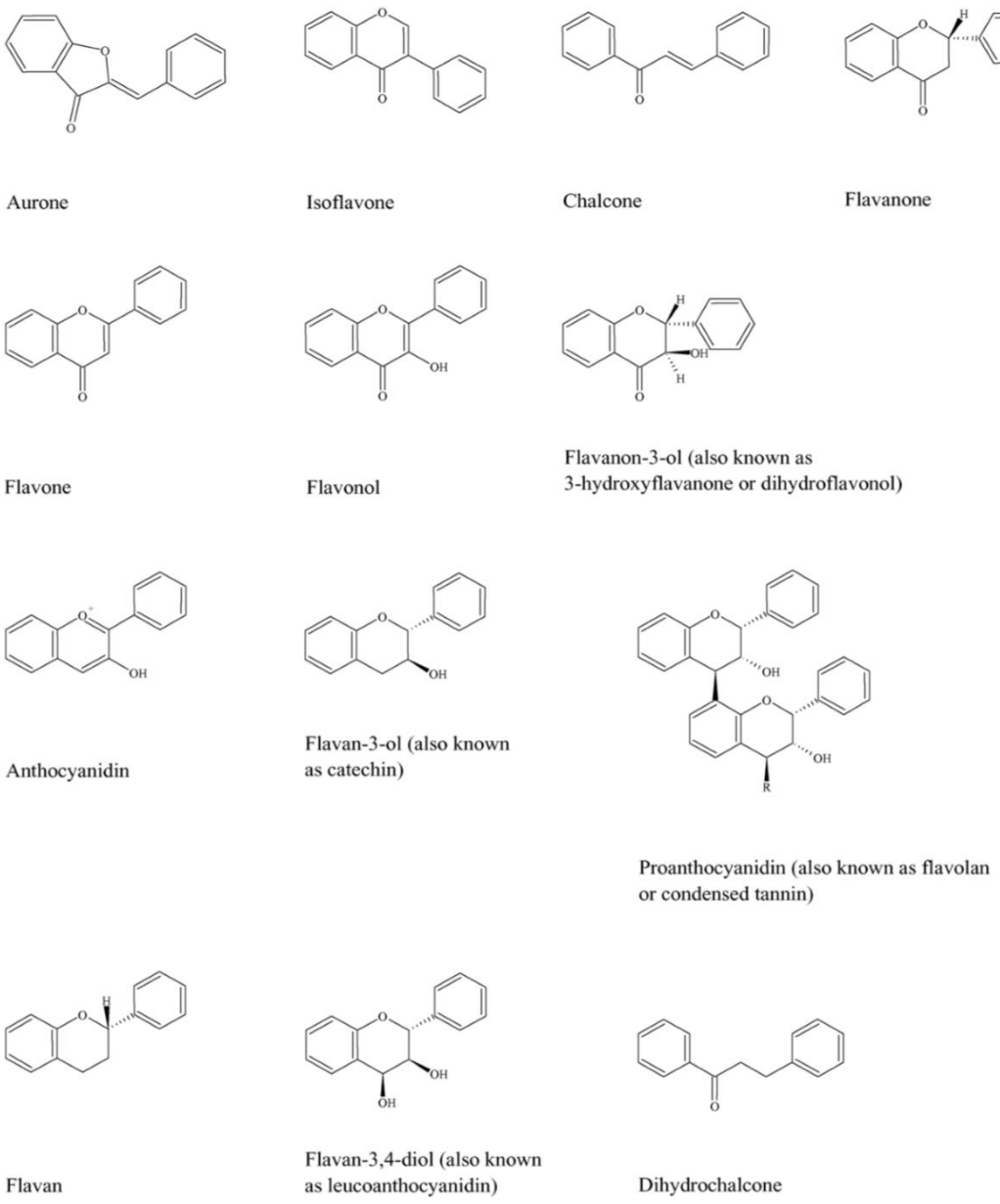

Figure 1. 3 The skeleton structures of the main classes of flavonoids.
Adapted from Cushnie et al., 2005 [104].

1.5.7 Antioxidant Activity of Flavonoids

1.5.7.1 Flavonoids as Antioxidants

Flavonoids are well-known as effective antioxidants [105]. Owing to the phenolic hydroxyl groups at certain positions of benzene rings [106], flavonoids demonstrate their antioxidant

activities by scavenging free radicals, donating protons, quenching singlet oxygen, and chelating metal ions. The beneficial effects of flavonoids to human body are highly related to their antioxidant activities as well. For example, free radicals, e.g. superoxide ($O_2^{\bullet -}$), alkyl peroxyl (ROO•), alkoxyl (RO•), and hydroxyl (HO•), are believed to participated in several life events in cells such as energy production, intercellular signal transmission, and cell growth regulation. However, severe cell damage may occur when excessive free radicals are generated, leading to breakage of DNA strands, lipid peroxidation, and inactivation of enzymes, etc. Multiple defensive mechanisms are applied by human body to avoid such outcomes which potentially result in aging, cardiovascular diseases, and even cancer [60]. Therefore, antioxidants such as flavonoids may strengthen the body defense by scavenging extra free radicals.

1.5.7.2 Structural basis for the antioxidant action of flavonoids

The Structure-Activity Relationship (SAR) of flavonoids have been systematically studied for their high structural diversity [107]. Although the mechanisms through which flavonoids act as health-promoting agents are still not well explained [108], their antioxidant activities are known highly dependent on the presence of function group substitutions at different positions of carbon backbone structure. Naturally occurred flavonoids are mostly glycosides, but sugar moieties are not the major groups responsible for the biological activities. Rather, common substitution groups such as hydroxyl and methoxyl groups alter the activity of flavonoids differently according to their number and position. Electronic properties, hydrophobicity, topology, and steric effects are several influencing factors that eventually lead to activity alterations [109].

The structural diversity of flavonoids attracts enormous interest and makes them excellent subject for QSAR studies [110, 111]. SAR and QSAR studies provide insightful information for model construction and help to elucidate the mechanism of action. Prediction of the activity of target

compounds and synthesis of potent analogues can both be realized by analyzing the activities of a variety of flavonoids [112-116].

The relationship of flavonoid structures and their antioxidant activity has been discussed and some general conclusions have been drawn [117, 118]. In general, the antioxidant activity of flavonoids is highly linked to hydroxyl groups: more potent antioxidant activity can be achieved when more hydroxyl groups are present especially on A and B rings, while the extent of conjugation between B and C rings also plays a role [112, 119-126]. More specifically, Bors et. al., raised three criteria as general structural requirements for flavonoids with antioxidative potential [127]:

1) The catechol structure on the B ring, which renders phenoxyl radicals of flavonoids high stability by hydrogen bonding and expanded electron delocalization;

2) The C2=C3 double bond, which brings coplanarity to the backbone structure and stabilizes phenoxyl radicals by electron delocalization;

3) 3-OH and 5-OH groups, which leads to the enhancement of the radical scavenging capacity and the radical absorption ability of flavonoids.

Other statements have been claimed in the studies from other groups: catechol moiety on the A ring contributes to antioxidant activity in the absence of the same structure on the B ring [128, 129]. The importance of flavonoid backbone structure was demonstrated only when *o*-dihydroxy structure is absent [130]. Additionally, 3-OH group was proven to be critical to the antioxidant activity. Either glycosylation at C-3 position or removal of 3-OH decreased their antioxidant activity dramatically [131]. Future criteria should be addressed in the manner that the contribution of each single structure is discussed under different circumstances.

1.5.7.3 Mechanisms of the antioxidant action of flavonoids

1) Scavenging of reactive free radicals.

As one of the major mechanisms of the antioxidant activity of flavonoids, scavenging reactive free radicals is realized through two possible pathways: H-transfer (i) and Electron-transfer (ii). The reaction equations are shown as below:

(i) $FOH + ROH\blacksquare \rightarrow FO\blacksquare + ROH$

(ii) $FOH + ROH\blacksquare \rightarrow FOH^{+}\blacksquare + ROH^{-} \rightarrow FO\blacksquare + ROH$

H-transfer is controlled by the bond dissociation enthalpy between hydrogen and oxygen atom, while electron transfer is related to the ionization potential and the reactivity of the $FOH^{+}\blacksquare$ cation [132]. Radical $FO\blacksquare$ is the thermodynamically favorable form in both mechanisms proposed, which explains its reactivity as a radical scavenger. For H-transfer, the structural requirements are similar to the ones for the antioxidant activity of flavonoid [127]: catechol structure in the B ring, C2=C3 double bond, and C4 carbonyl group. Flavonoid phenoxyl radical is formed through deprotonation and may further donate hydrogen to a second radical to become a stable quinone.

Electron-transfer may dominate the scavenging action when no hydrogen atom is available for donation. For example, 3-OH and 5-OH monohydroflavones have very limited ability of deprotonation or H-transfer radical scavenging due to the strong hydrogen bond between the hydroxyl group and the C4 carbonyl group.

2) Chelating of trace metal ions.

Transition metal ions, such as Fe^{2+} and Cu^{+}, have been proved to be important in oxygen metabolism and free radical formation [105]. Through Fenton reaction, for example, highly reactive oxygen species are generated in the presence of iron (II) or copper (I):

$H_2O_2 + Fe^{2+} (Cu^{+}) \rightarrow HO\blacksquare + OH^{-} + Fe^{3+} (Cu^{2+})$

Flavonoids limit the prooxidant actions of transition metal ions by chelating them. Potential binding site is 3',4'-OH groups on the B ring, and this partially explains why catechol structure is required for demonstrating antioxidant activity.

3) Inhibition of enzymes involved in free radical production.

Prooxidant enzymes, such as NADPH oxidases, xanthine oxidase, endothelial nitric oxide synthase and mitochondrial oxidases, play important roles in generating reactive oxygen species (ROS) and oxidative stress regulation *in vivo* [105, 133]. The *in vivo* antioxidant activity of flavonoids may partially result from inhibiting the prooxidant enzyme activity. Therefore, several flavonoids are of great interest for designing new compounds to be potentially used for disease treatment.

1.5.8 Prooxidant Activity of Flavonoids

Although flavonoids are generally regarded as antioxidants, these polyphenolic compounds can also act as prooxidants [134-136]. The prooxidant activity of flavonoids is related to their hydroxyl group substituent pattern. Catechol moiety on the B ring, one of the key factors of the antioxidant activity of flavonoids, may also contribute to the prooxidant activity. It makes it possible for some flavonoids to go through autooxidation or enzymatic electron oxidation with the formation of semiquinone and quinone. As electrophiles, these metabolites bind to macromolecules in the cells by forming covalent adducts. Meanwhile, they participate in redox cycling, resulting in the production of ROS and oxidative stress. The transformation of flavonoids may be accelerated in the presence of transition metal ions and are believed to be the basis of the prooxidant effect [105].

Some flavonoids which show toxic effect may apply the mechanism mentioned above. Quercetin has been found to form alkylating quinone-type metabolites and its mutagenicity was identified [137, 138]. DNA damage was observed for several flavonoids with multiple hydroxyl groups in their A or B ring [139, 140]. Copper (II) ion plays a key role in the ROS generation and DNA binding.

To sum up, flavonoids can demonstrate both antioxidant and prooxidant activity within the same compound. There is a possibility that the most active antioxidant happens to be one potent prooxidant since hydroxyl groups are responsible for both two activities [141]. Thus, cytotoxicity should be taken into consideration when designing certain functional compound based on SAR of flavonoids

1.6 Oxidative stress in bacteria

The existence of molecular oxygen (O_2) does not have a history as long as the existence of life. The appearance of photosynthetic organisms not only set up a milestone for more advanced life forms but also created a crisis for most of the living organisms three billion years ago – oxygenation of atmosphere. The small size and non-polarity of oxygen render it the ability to rapidly diffuse across biological membranes. Other than participating in aerobic respiration to provide energy, oxygen reactively accepts electrons from environment to generate superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO▪). These Reactive Oxygen Species (ROS) proactively oxidize biomolecules such as nucleic acids, lipids, and amino acids. The rate of cell respiration is not fast enough to lower the intracellular O_2 concentration to a level that exposes no threat to cell growth. Microorganisms have thus developed a variety of self defense mechanisms of escaping oxidative stress: obligate anaerobes and microaerophiles reside in anaerobic microhabitats; aerobes have developed a series of strategies to defend themselves against the hostile environments.

1.6.1 Formation of O_2^- and H_2O_2 in aerobic cells

For a long time, O_2^- and $H_2O_2$are considered generated constantly inside microbe cells when they are exposed to oxygen-containing environments. Other researchers suggest otherwise by mutating SOD in *E. coli*, *S. cerevisiae*, and other organisms [142-144]. So far there is no enzyme that has

been reported to generate O_2^- or H_2O_2 as a stoichiometric product in *E. coli*. In Contrast, O_2^- or H_2O_2 are formed when redox enzymes accidently transfer electrons to oxygen molecules instead of their designated substrates.

The respiratory chain includes a series of redox enzymes which serve as electron donors to oxygen. Both O_2^- and H_2O_2 have been detected when respiratory vesicles are incubated with reductive substrates *in vitro* in the presence of oxygen [145-148]. Membrane vesicles derived from glucose-grown cells consume oxygen and release as O_2^- at approximately 0.2% while the figure is doubled with H_2O_2. The electron transfer between oxygen and respiratory enzymes can happen at a higher rate in cells initially cultured under anaerobic conditions. Thus, the release of O_2^- and H_2O_2 is elevated correspondingly.

In the normal process, respiratory dehydrogenases use reductive substrates as electron donors, passing the electrons on to secondary redox moieties (quinones or iron-sulfur clusters) from flavin cofactors where the electrons made their first stop. O_2^- and H_2O_2 can be generated when $FADH_2$ from reduced flavin transferred the electrons incorrectly to oxygen [149]. The electron transfer from flavins is univalent and nonspecific.

1.6.2 Biomolecules that O_2^- and H_2O_2 damage

The toxicity of O_2^- and H_2O_2 was studied by constructing bacterial SOD and catalase/peroxidase mutants [142, 150, 151]. These mutants demonstrate metabolic defects resulting from the dysfunction of a family of dehydratases [152, 153], which contain a $[4Fe\text{-}4S]^{2+}$ cluster structure. Both O_2^- and H_2O_2 can bind the critical iron atom at the active site of enzymes to release iron and $[3Fe\text{-}4S]^+$ species, which are unstable and inactive. The released unincorporated ferrous iron ions are further oxidized by H_2O_2, rendering hydroxyl radicals which cause DNA damage. Thus, O_2^- and H_2O_2 are both mutagenic agents to cells [154, 155].

1.6.3 Defensive strategies against oxidative stress

Superoxide undergoes spontaneous dismutation but still requires scavenging systems to be maintained at a low intracellular level. Gram-negative bacteria usually adopt two systems, cytoplasmic and periplasmic, since O_2^- cannot penetrate membranes at neutral pH [156, 157]. *E. coli* deploys two cytoplasmic SOD isozymes (MnSOD and FeSOD) and one periplasmic enzyme (CuZnSOD). The steady-state concentration of O_2^- is maintained at approximately 0.1 nM while the intracellular formation rate is about 5 μM/s [147]. The synthesis of SODs is triggered by the activation of SoxRS system.

Hydrogen peroxide in *E. coli* is scavenged by peroxidases (primary) and catalases (secondary). NADH peroxidase AhpCF is a two-component protein. AhpC is firstly oxidized by H_2O_2 to form a Cys46-Cys165 disulfide bond, which can be reduced by other Ahp cysteinyl residues to form a second disulfide. Then NADH-reducible flavoprotein AhpF reversibly binds to this bond for reduction [158]. This mechanism renders Ahp high activity for intracellular H_2O_2 controlling. Hydrogen peroxide exceeding 20 μM saturates Ahp and activates OxyR regulon, inducing *katG*-encoded catalase [147]. Ahp and catalase are both with high turnover rate to maintain an H_2O_2 concentration difference across membranes.

1.7 Rationale and Research Objectives

Due to the increasing attention paid to food safety, the use of artificial food preservative has raised concerns from consumers. The requirement for natural food preservative is challenging for not only food manufacturers, but also food scientists. One of the good sources for natural antimicrobials is plant by-products. These by-products are massively produced in food manufacturing process and generally regarded as waste. Plant by-products, such as peels, seeds

and husks, normally serve as a natural defense to prevent fruits from spoilage. Flavonoids are a group of phenolic compounds commonly extracted from plants. Flavonoids have been reported to have anti-inflammatory, anti-cancer, anti-proliferative and antimicrobial activities. However, the mechanisms behind the antimicrobial activities still remain unclear. On the other hand,poor bioavailability has restricted the application of flavonoids in food industry. This study focuses on the antimicrobial activities of flavonoids against foodborne pathogens, including their structure-activity relationship, antimicrobial molecular mechanism and approaches of how flavonoids can be incorporated into food products more efficiently.

Research Objectives

1.7.1 Establish a QSAR model to probe the relationship between flavonoid structures with antibacterial activities.

A mathematical equation was built using QSAR analysis to model the redox behaviors of flavonoids based on their structural features in chapter 2. Next, antimicrobial activities of selected flavonoids against common foodborne pathogens are investigated, including Gram-positive and Gram-negative bacteria. The flavonoid redox profiles obtained from the QSAR model accounted for the differences in antimicrobial activities from the variety of flavonoids. Furthermore, the QSAR model provided further explanations for how the flavonoid redox potentials may dictate their antibacterial activities in chapter 2 and chapter 4.

1.7.2 Study on the antibacterial mechanism of myricetin and ampelopsin.

According to literature, flavonoids may apply their antimicrobial activity by triggering cell leakage resulting from membrane damage. The mechanism study in this dissertation is focused on cell membrane integrity, oxidative stress response and gene transcription in chapter 3. Detailed discussions on the compound metabolism and possible defense mechanisms from bacteria cells are covered in chapter 4. Extended studies on the full dissection of bacteria biological response,

expansion on other antimicrobial potentials, compound impact on gut microbiome and biosafety, as well as directions for improvement by formulation are proposed as immediate future applications in chapter 5. These would further add to the understandings of these compounds on the basis of this study.

Chapter 2 QSAR of redox behavior and bacterial inhibition efficiency of flavonoids

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2.1 Abstract

As one of the largest groups of plant metabolites, flavonoids have been discovered to possess antimicrobial activity but some of the mechanisms are still unclear. In this study, several flavonoids with similar backbone structures were studied for their antibacterial activity. Utilizing both computational prediction as well as experimental approaches, we observed a correlation between predicted redox behavior and antibacterial function of flavonoids. In fact, flavonoids with low anodic potential render prooxidant environments in aqueous solution under physiological pH, likely due to their rapid degradation, that inhibit bacterial growth. Among all flavonoids tested, myricetin, which contains B-ring pyrogallol group structure and C2=C3 double bond, demonstrates a strong prooxidant activity and was found to be the most potent one in antibacterial function. Our study establishes a correlation between the redox behavior and antibacterial activities of flavonoids, while laying a foundation for prediction on antibacterial activity of a given flavonoid structure.

Keywords: Flavonoids, antibacterial activity, anodic potential, prooxidant activity, quantitative structure activity relationship (QSAR)

2.2 Introduction

Flavonoids, as common plant metabolites, are universally found in different plant organs. Their distribution can vary depending on the classes of plants, the types of organs and the environment [54]. The functions of flavonoids are originally thought to provide colors in flowers and aroma of fruits to attract plant pollinators [55]. Other studies also claimed that flavonoids protect plants from survival stresses, including ultraviolet B (UVB) radiation [55], injury recovery, signal transfer [56, 159, 160], frost hardiness, drought, heat, and freezing [55, 56, 161]. Moreover, flavonoids are also involved in energy transfer, regulation of plant growth and photosynthesis [54]. More than 4,000 flavonoid compounds have been isolated and identified, based on their chemical structures [58]. Flavonoids share a common chemical structure of two benzene rings (A ring and B ring) linked through a heterocyclic pyran ring (C ring). The polyphenolic structure of flavonoids infers the antioxidant activity, which has been largely associated with multiple beneficial activities, including anti-inflammatory [59], anticancer [60], antiviral [61], and antifungal properties [62]. Especially, the antimicrobial activity of flavonoids draws more attention due to potential applications of these compounds in as antibiotics and food preservatives.

The antibacterial activity of flavonoids has been increasingly documented in recent years. Crude extracts from plants with a history of use in folk medicine have been screened *in vitro* for antibacterial activities [162]. Other research groups also isolated and identified the structure of flavonoids that possess antibacterial activity, which include galangin, quercetin and various quercetin glycosides and kaempferol together with its derivatives [162]. The mechanisms of some of these antibacterial actions were also described and may vary for each flavonoid molecule. These mechanisms include membrane disruption [7], biofilm formation interference [163], DNA synthesis inhibition [164], as well as cell membrane synthesis inhibition [165]. For example, quercetin was shown to reduce the thickness of the membrane bilayer in a reconstituted model system [166]. Myricetin has also been found to perturb bilayers leading to thermal property

alterations, interpreted from NMR analyses [167]. Some flavonoids were also reported to block DNA replication pathways either by direct intercalation into dsDNA, shown in *Proteus vulgaris* and *Staphylococcus aureus (S. aureus)* [168], or interacting with essential enzymes such as DNA gyrase or replicative helicases in *Escherichia coli (E. coli)* [169]. Other discoveries have also suggested that different subsets of flavonoids could inhibit ATP synthases and reduce the ATP bioavailability in *E. coli* and *S. aureus* [170, 171]. However, very limited evidence has been shown to support that the redox status of flavonoids attributes directly to their antibacterial functions.

We aimed to establish the connection between the antibacterial activity of flavonoids and their redox status. Firstly, we established a quantitative structure activity relationship (QSAR) model for predicting the anodic potential (E_{ap}), as a measurement of redox activity, of flavonoids based on their observed chemical structure. Next, we evaluated six flavonoids compounds and their antibacterial activity on both *E. coli* (Gram-negative) and *Listeria monocytogenes* (Gram-positive). We observed a correlation between a lower E_{ap} and a higher antibacterial activity. In particular, flavonoids with low E_{ap} render prooxidant environments in aqueous solution under physiological pH, likely due to the rapid degradation, resulting in an unfavorable condition for bacteria to grow in. Our study is the first, to our best knowledge, to establish a direct correlation between the redox status and antibacterial activities of flavonoids, while also providing underlying molecular rationales for such correlations. In addition, our study laid a foundation for future investigations on predicting antibacterial activity of flavonoids with given structures.

2.3 Methods and Materials

Bacterial Strains

Frozen stocks of *Escherichia coli* O157:H7 (ATCC 43895) and *Listeria monocytogenes* (*L. monocytogenes*) Li 20 (ATCC 19111) were stored at −80 °C in Tryptic Soy Broth (TSB) (Sigma-Aldrich) containing 15% glycerol (v/v). All cultures for use were prepared by transferring a loop of culture from frozen stock onto Tryptic Soy Agar (TSA) plates (Sigma-Aldrich) for isolation and incubating at 37ºC for 24 h. One single isolated colony was transferred into a 50 mL tube with sufficient volume of fresh liquid media and incubated at 37 °C with agitation. For all the MIC assays in the study, overnight culture was diluted 1:10,000 to achieve approximately 10^5 colony forming units (CFU)/mL and continually grown at 37 °C.

Minimum Inhibitory Concentration (MIC) Assay

Stock solutions of flavonoids were prepared freshly in DMSO. For MIC determination, 100 µl of broth containing twice the needed maximum concentration of flavonoids were added to the first column of a polystyrene non-treated 96-well plate (Falcon), and 1:1 serially diluted with 5% DMSO. The compound solutions were subsequently inoculated with 100 µl of bacteria cells to achieve approximately 10^5 CFU per ml. All wells were mixed by micropipetting and gentle shaking after the addition of bacterial cells. The plate was then covered with sterile sealing film to prevent the vaporization of the medium. The plate was inserted into a temperature controlled 96-well plate reader (Biotek) and incubated at 37 °C for 24 h, while taking continuous measurements of the turbidity of sample at wavelength 595 nm, with time intervals of 1 hr. OD_{595} readings were recorded and graphed as a function of time. The MIC was defined as the lowest concentration of compound that inhibits the growth of microorganisms, which is the concentration that showed no OD increase. The flavonoids chosen for this assay were: galangin, kaempferol, quercetin, myricetin, taxifolin, and ampelopsin. Ampicillin was selected as control group.

Oxygen radical absorbance capacity (ORAC) assay

A 96-well black plate (VWR) was used for this assay. 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) (Sigma-Aldrich) and fluorescein sodium (Sigma-Aldrich) solution were freshly prepared with 0.1M PBS buffer before use. Each well contained 240 µL reaction mixture of fluorescein sodium (0.8µM), AAPH (10mM) and flavonoids (1.6µM). The plate reader was set at 37 °C with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The experiment ran for 2.5 h with a time interval of 5 min.

High Performance Liquid Chromatography (HPLC) analyses of flavonoids degradation

All flavonoids were dissolved in 0.1M PBS buffer with 5% DMSO and incubated at 37 °C. HPLC was conducted on Hewlett-Packard Agilent 1100 series HPLC-MS. Analysis was performed with a Hypersil ODS C18 column (Thermo Scientific), 5µm, 250 × 4.6 mm at ambient temperature with 1.0 ml/min flow rate. The mobile phases consisted of solvent A (0.1% phosphoric acid in water (v/v)) and solvent B (acetonitrile). For myricetin and quercetin, the gradient elution was 10-50% B in 10-15 min, 50% B in 15-20 min and the wavelength was 370 nm. For ampelopsin and taxifolin, the isocratic elution was 25% B in 0-20 min and the wavelength was 290 nm.

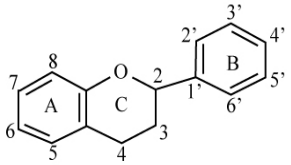
Regression modeling and statistical analysis

E_{ap} value training set was acquired from Firuzi et. al., 2005 [172]. For univariate analysis, one-way ANOVA/student T-test was used for all 9 dependent variables. For multivariate analysis, a bi-directional stepwise linear regression was performed from the full model. The best model is defined as $E_{ap} \sim R3 + R5 + R4' + C2{=}C3 + A\_pattern + B\_pattern$.

2.4 Results

Quantitative Structure Activity Relationship (QSAR) analysis of the redox status of flavonoids.

To thoroughly understand the prooxidant and antioxidant activities of various flavonoid compounds, we sought to explore the correlation between their redox status and chemical structures. While a previous QSAR analysis has touched briefly upon such correlation and predictability assessments, the model was trained with a broad spectrum of flavonoid families and thus lacks the resolution to distinguish among highly similar ones that share the polyphenolic structure with only a few differentiating functional groups [173]. Therefore, we aim for establishing a model that better suits quantitative comparisons among most closely related flavonoids. Specifically, we analyzed 13 flavonoids with distinct functional groups on the A and B rings in an attempt to build a mathematical model by QSAR analysis. The key features, including chemical moiety compositions at positions R3, R5, R7, R8, R2', R3', R4', R5', the presence of a double bond between C2 and C3, as well as the electroactive group patterns on A and B rings, were taken into consideration and laid out in detail in Table 2.1. The corresponding anodic potential (E_{ap}) values of all chosen flavonoids but ampelopsin that directly reflects their redox activities were extracted from published results [172].



	R3	R5	R7	R8	R2'	R3'	R4'	R5'	C2=C3	A*	B*	E_{ap}**	Predict E_{ap}***
myricetin#	OH	OH	OH	H	H	OH	OH	OH	+	R	G	0.30	0.30
quercetin#	OH	OH	OH	H	H	OH	OH	H	+	R	C	0.39	0.39
rutin	Ogl	OH	OH	H	H	OH	OH	H	+	P	C	0.46	0.46
fisetin	OH	H	OH	H	H	OH	OH	H	+	P	C	0.39	0.39
kaempferol#	OH	OH	OH	H	H	H	OH	H	+	R	P	0.45	0.45
taxifolin#	OH	OH	OH	H	H	OH	OH	H	-	R	C	0.46	0.46
naringenin	H	OH	OH	H	H	H	OH	H	-	R	P	0.89	0.89
catechin	OH	OH	OH	H	H	OH	OH	H	-	R	C	0.45	0.46
apigenin	H	OH	OH	H	H	OH	H	H	+	R	P	0.90	0.90
5-OH flavone	H	OH	H	H	H	H	H	H	+	P	B	1.20	1.20
galangin#	OH	OH	OH	H	H	H	H	H	+	R	B	0.59	0.59
3-OH flavone	OH	H	H	H	H	H	H	H	+	B	B	0.68	0.68
hesperetin	H	OH	OH	H	H	OH	Ome	H	-	R	P	0.71	0.71
ampelopsin#	OH	OH	OH	H	H	OH	OH	OH	-	R	G	N/A	0.37

Table 2. 1 Substitution patterns and E_{ap} of the series of flavonoids.

* Electroactive Groups for A ring and B ring: Gallic (G), Catechol (C), Resorcinol (R), Phenol (P), Benzene (B)

** Reference: Firuzi *et. al*., 2005

*** Predicted in this study.

Flavonoid compounds whose antibacterial activities were investigated in this study.

We first performed univariate analysis to assess the impacts from each of the 9 structural features on the final E_{ap} score by one-way ANOVA (Figure 2.1). Among the 6 individual structural groups (R3, R3', R4', R5, R5', R7) under investigation, 3 brought statistically significant impacts on the E_{ap} values: R3 (P=0.0016), R4' (P=0.0461) and R7 (P=0.0420). At all 3 positions, having an OH group instead of H-atom decreased the E_{ap} score of the compound. For non-OH or H groups, Ogl group on R3 has a similar decreasing effect as OH, whereas the OMe group on R4' did not show a deviation from the ones with hydrogen. On a different perspective, having a double bond between C2 and C3 on the B ring did not significantly alter the flavonoid redox status in the selected group. Notably, given previous reports that suggested the importance of C2=C3 in flavonoid functions [174], the discrepancy could be due to the sample size limitation in the current study and requires more thorough examinations. The overall electroactive groups on A and B rings were also categorized into Gallic (G), Catechol (C), Resorcinol (R), Phenol (P) and Benzene (B) and subjected to similar one-way ANOVA analysis. While the different group patterns on the A ring did not significantly influence the readout, the B ring patterns caused substantial alterations to the final E_{ap} scores, ranking in the following order: G < C < P ~ B (P=0.0495). Altogether, the statistical analysis suggested that the OH groups at some specific positions more than others, as well as the moiety patterns on the B ring, were strongly responsible for the E_{ap} values of flavonoids.

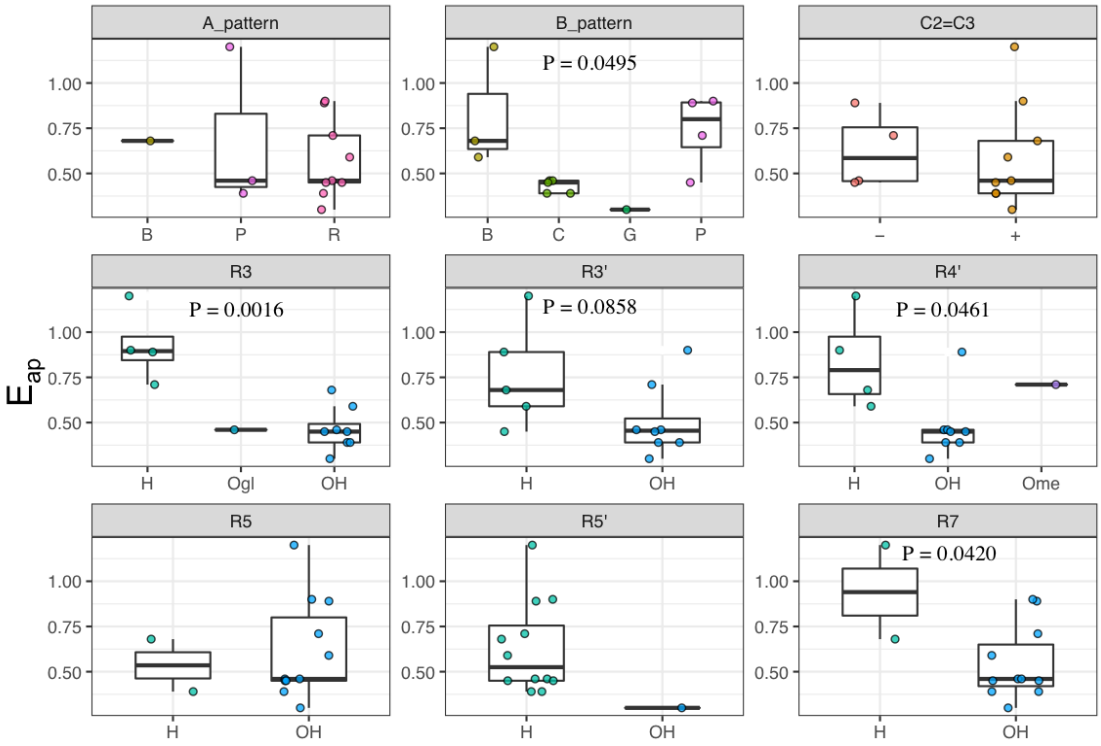


Figure 2. 1 Variance of E_{ap} explained by structural groups of flavonoids.

E_{ap} were plotted with each structural groups or patterns by positions. P values were indicated in plots with significant differences, analyzed by one-way ANOVA.

Next, we sought to build a mathematical model to connect the important key groups with the E_{ap} values that would enable predictability for other uncharacterized flavonoids in the same subfamily by multivariate linear regression analysis. The same 13 model flavonoids were used as the training dataset with up to 12 categorical independent variables and their E_{ap} values as the responding variable. We obtained Equation (1) as the best fitting model following various attempts:

$$\begin{aligned} E_{ap} = {} & 1.12\ (\pm 0.01225) - 0.54(\pm 0.01414) I_{3Ogl} - 0.375(\pm 0.01323) I_{3OH} + \\ & 0.235(\pm 0.01658) I_{5OH} - 0.075(\pm 0.01323) I_{4'OH} - 0.255(\pm 0.01323) I_{4'Ome} - \\ & 0.065(\pm 0.00866) I_{C2\ =\ C3} - 0.09(\pm 0.01414) I_{AP} - 0.325(\pm 0.02179) I_{AR} - \\ & 0.125(\pm 0.01323) I_{BC} - 0.215(\pm 0.01658) I_{BG} - 0.065(\pm 0.01658)\ I_{BP} \end{aligned} \quad (1)$$

$$n = 13 \quad r^2 = 0.9993 \quad F = 1481$$

In this equation, all *I*'s represent indicator variables with the value ascribed as 1 in the presence of corresponding structural entities, and $I = 0$ where absent. n is of the number compounds, r2 represents the adjusted correlation coefficient and F represents the ratio of explained over unexplained variances. Our equation is highly reliable in predicting the flavonoid E_{ap} with given structural features, confirmed when plotting the predicted E_{ap} vs the experimental value using the existing dataset (Figure 2.2). Overall, we have established a working mathematical model that well summarizes the SAR of flavonoids and could potentially be used to infer the redox activities of an uncharacterized flavonoid compound.

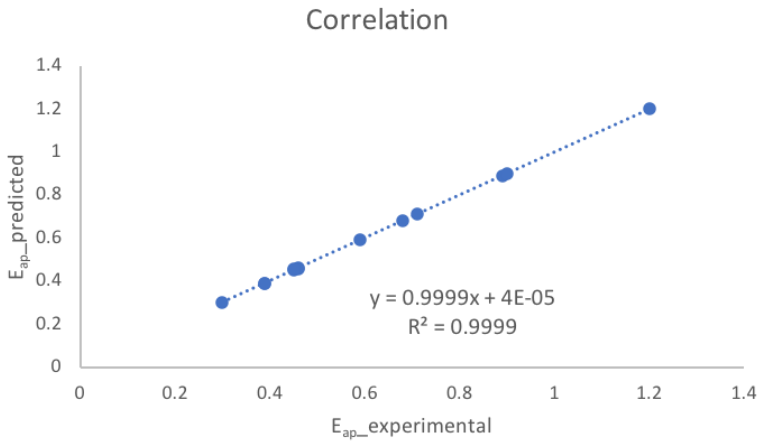


Figure 2. 2 Predicted E_{ap} correlates with experimental value.

Predicted E_{ap} was plotted against reported experimental E_{ap}.

Six flavonoids with similar structures were evaluated for their antibacterial activities.

To examine whether our QSAR model is physiological relevant, we pursued six flavonoids that had been reported to exert antibacterial functions, including galangin, kaempferol, quercetin, myricetin, taxifolin and ampelopsin [82, 162, 175]. Since the E_{ap} of ampelopsin was lacking from literature, we calculated its theoretical E_{ap} value as 0.37 based on Equation (1) (Table 2.1). Even though these six flavonoids contain similar structures, they display a wide spectrum of E_{ap} ranging from 0.30 – 0.59, allowing us to further dissect the functional groups that may contribute to the redox status of flavonoids and to infer their impacts on antibacterial activities. Specifically, all six flavonoids display a common 3-ring structure yet differ in either the B ring side chains, or the C2-C3 bond on the C ring (Figure 2.3). Profoundly, the numbers of hydroxyl groups on B ring differ for most of the selected flavonoids. Myricetin and ampelopsin both contain three hydroxyl groups on position R3', R4' and R5', presenting as a typical pyrogallol group structure. Quercetin and taxifolin both have two hydroxyl groups, but with R3' and R4' hydroxyl groups on quercetin and R4' and R5' on taxifolin. In contrast, kaempferol only has one R4' hydroxyl group, while galangin has none on its B ring. Another noted difference lies in the nature of the C2-C3 bond in each flavonoid. Taxifolin and ampelopsin have a C2-C3 single bond, while the other four all have a C2=C3 double bond, rendering conplane B and C rings. Based on our QSAR model, the E_{ap} values of these six flavonoids are in the following order: myricetin (0.3) < ampelopsin (0.37) < quercetin (0.39) < kaempferol (0.45) < taxifolin (0.46) < galangin (0.59). We then would like to examine how the antibacterial activities of these six flavonoids would correlate with the order of E_{ap}.

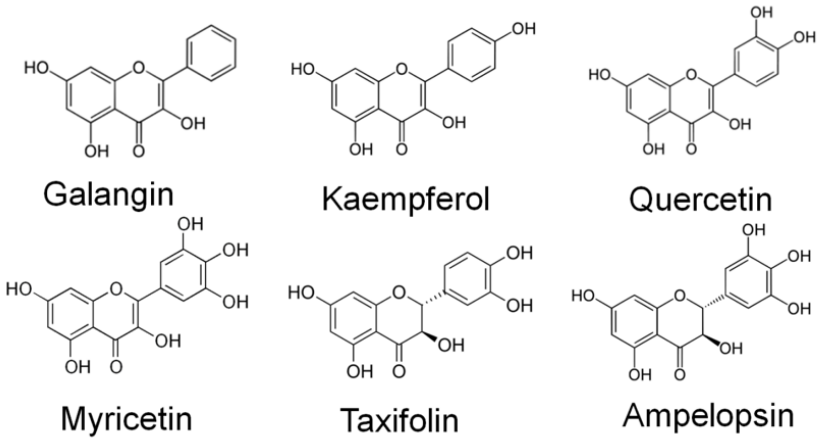


Figure 2. 3 Structures of the six flavonoids selected in the study.

To study the antibacterial activities of these flavonoids, growth inhibition assay was conducted with two bacterial strains to monitor their susceptibility to various flavonoids. *E. coli* O157:H7 and *L. monocytogenes* Li 20, belonging to Gram-negative and Gram-positive bacterium respectively, are the two common representative foodborne pathogens. Flavonoids were added to the growth media at log phase with each to the concentration of 0.5 μmol/ml, the highest concentration at which the least soluble compound can stay in the aqueous solution with 5% DMSO vehicle. No bacterial growth was observed in the ampicillin treated group as a positive antibiotic-treated control. Bacteria can grow in media supplemented with 0.5 μmol/ml flavonoids, but at a lower rate compared to the compound-free media control, suggesting all flavonoids under investigation has antibacterial activity against *E. coli* (Figure 2.4A) and *L. monocytogenes* (Figure 2.4B) to certain extents. For both *E. coli* and *L. monocytogenes*, myricetin has the most potent antibacterial effect at a similar level as ampicillin, followed by kaempferol. Bacterial cells treated with galangin, quercetin, taxifolin and ampelopsin have comparable growth rates. To further probe the maximum growth inhibition capacity of these flavonoids, a higher dose of 2 μmol/ml flavonoids were supplemented to the media. Unfortunately, galangin and quercetin have very poor solubility at 2 μmol/ml and thus were dropped from this dataset. At the increased concentration, ampelopsin abolished the bacterial growth to a comparable extent as myricetin and ampicillin. Taxifolin also inhibits bacterial growth at 2 μmol/ml yet with less impact (Figure 2.4C-D). To summarize, the antibacterial capacity of these flavonoids can be ordered as: myricetin > kaempferol > ampelopsin > taxifolin (galangin and quercetin were not eligible for fair comparison due to their poor solubility in this assay). With the exception of kaempferol, which likely possesses additional antibacterial mechanisms and will be further discussed later, the order of antibacterial activities for the other three flavonoids is the in reverse to that of their predicted E_{ap} values based on our model. Our next focus is to address the mechanisms of this negative correlation.

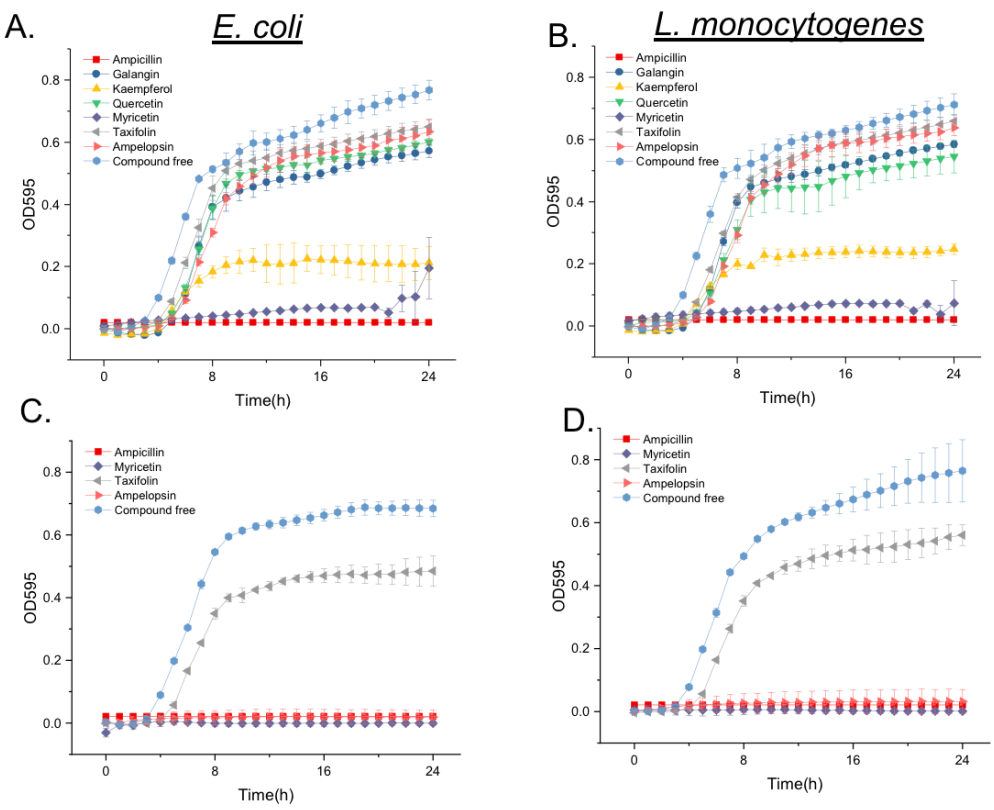


Figure 2. 4 Selected flavonoids inhibit the growth of both Gram positive and negative bacteria.

E. coli and *L. monocytogenes* were grown in TSB media for overnight and cells were re-inoculated into fresh media to a concentration of 10^5 CFU/ml. Growth curve of *E. coli* (A, C) and *L. monocytogenes* (B, D) under the treatment of different flavonoids at 0.5 µmol/ml (A, B) and 2 µmol/ml (C, D) or ampicillin at 100 µg/ml was monitored over the course of 24 hours.

Flavonoids demonstrate various redox activities when supplemented in bacterial media.

To further explore our observation on the correlation between E_{ap} values of flavonoids and their capacity of bacterial inhibition, we measured the free radicals scavenging activity of these six flavonoids using an *in vitro* biochemical ORAC (Oxygen Radical Absorbance Capacity) assay. 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) is known to produce peroxyl radicals upon thermal upshift, resulting in the decay of fluorescence and serving as an internal control in this experiment [176]. We supplemented the ORAC reaction with one of the six flavonoids and measured the fluorescence decay for up to 150 minutes. Myricetin, with the lowest E_{ap} value among flavonoids tested, caused the most rapid decay of the fluorescence intensity while added to the reaction, suggesting that it had induced a strong prooxidant environment (Figure 2.5A). This is followed by ampelopsin with the second lowest E_{ap}, which similarly induced a prooxidant outcome. In contrast, kaempferol, taxifolin, quercetin or galangin slowed down the fluorescence decay, suggesting that these flavonoids display antioxidant properties that protected the fluorescence from the attack of peroxyl radicals released by AAPH (Figure 2.5A). This prooxidant properties of myricetin and ampelopsin to a less extent is likely the underlying mechanism for their bacterial growth inhibition capabilities.

One possible explanation for the induction of prooxidant environments by myricetin and ampelopsin is their intrinsic instabilities. We found that myricetin underwent rapid degradation within 4 hours in aqueous solution under physiological pH conditions followed by ampelopsin, while taxifolin remained mostly stable after 24 hours (Figure 2.5B). This led us to propose a hypothesis that myricetin and ampelopsin, upon degradation in aqueous solution under physiological pH, may generate byproducts that in turn induce a prooxidant environment. To conclusively test this hypothesis, further studies on the degradation profiles of these flavonoids are required.

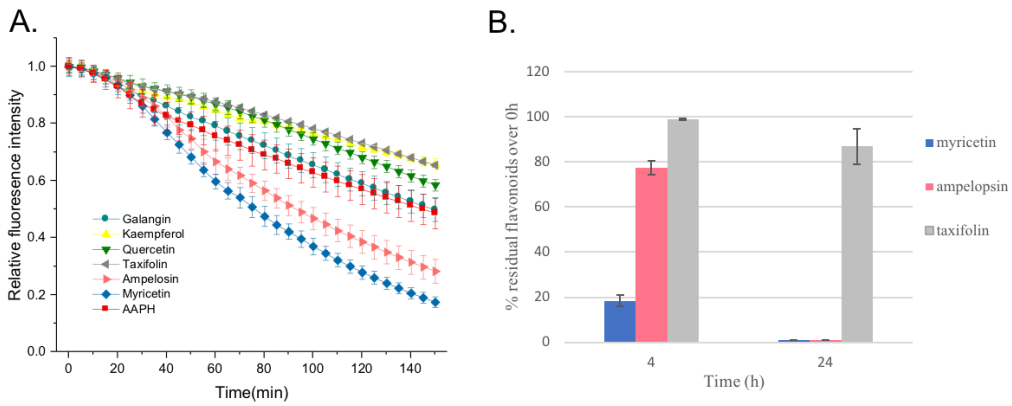


Figure 2. 5 Flavonoids demonstrate various anti- or pro-oxidant activities.

(A) Different flavonoids were incubated with fluorescein sodium and 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) for ORAC assay. AAPH with no flavonoids serves as internal control. (B) Degradation of indicated flavonoids measured by HPLC (pH = 7.4).

2.5 Discussions

Flavonoids, as a large group of natural plant metabolites, have been known for their antioxidant properties. These antioxidant activities are associated with multiple beneficial effects to human, including anti-inflammation, anti-aging, anti-cancer, anti-cardiovascular disease activities [177-179]. Some flavonoids also process antibacterial activities, such as galangin, kaempferol, quercetin, myricetin, taxifolin and ampelopsin, yet it is not known whether or how the redox activities of the flavonoids contribute to their antibacterial functions. In this study, we developed a QSAR model for predicting the redox activity based on the chemical structures of the highly resembling flavonoids. We then examined the antibacterial activities of the six flavonoids mentioned above on both *E. coli* and *L. monocytogenes* and found that the antibacterial capacity is correlated with the anodic potential (E_{ap}) of these flavonoids. Further investigation suggests that myricetin and ampelopsin, with relatively low E_{ap}, undergo rapid degradation that leads to a prooxidant effect, which is likely the mechanism for inhibiting bacterial growth.

The electrochemical profile of flavonoids is dictated by their structures and directly reflects their redox behavior, which is potentially the foundation of most if not all of their biological functions. The mechanism of action of flavonoids as antioxidants involves the ability to scavenge radicals by an H-atom or electron transfer process [180]. Model establishment provides a possibility for predicting the redox behavior of certain flavonoid with a given structure. We developed a QSAR model for predicting the E_{ap} value, as a measurement to evaluate the redox activity of different flavonoids based on their chemical structures. Our equation successfully and accurately recapitulates the experimental E_{ap} of the model flavonoids and allows us to calculate an E_{ap} for ampelopsin as 0.37. From our modeling results, five variables have been selected and considered to have significant impact on E_{ap} value, which are: position 3, 4', 5, and hydroxyl group pattern on A and B ring. Normally, a hydroxyl substitution on these positions results in lower E_{ap}. Although the training dataset we chose accomplished the job by differentiating the flavonoids in

the study, more data should be included in the future for prediction of flavonoids with more complex substitutions, such as methoxylation and glycosylation. Noteworthily, collecting E_{ap} values from different flavonoids under identical conditions is critical for accurate model training. It would therefore be ultimately beneficial to establish a universal experimental criterion for E_{ap} value acquisition, especially when it is not accessible to include a large number of flavonoids in electroanalytical assays.

Redox environments can be sensed and responded to by bacteria, thus impacting their physiological features and growth [181]. By measuring the oxidative environment through ORAC, of six investigated compounds four showed antioxidant activity under the experimental conditions, whereas the other two (myricetin and ampelopsin) were prooxidants. Bacteria experience hostile environment when the surrounding oxidative stress is raised by prooxidants. Our further discovery of a correlation between the flavonoid degradation and prooxidant activity suggests phenolic autoxidation of myricetin and ampelopsin, the initial step of which generates semiquinone and superoxide radicals. These radicals can damage major macromolecules (DNA and proteins) [182] and this could be the main reason why the two compounds showed potent antibacterial activities. Moreover, it is reasonable to speculate that there is a cutoff E_{ap} value below which compounds are prone to degradation, inducing prooxidant state in the presence of oxygen. From our results and prediction values for the investigated compounds, this cutoff value should be very close to the E_{ap} range of ampelopsin (0.37) and quercetin (0.39). It is worth noting that E_{ap} is pH dependent and can serve as an indicator for predicting actual redox environments with various flavonoids. Therefore, flavonoid redox behaviors should always be specifically evaluated under actual conditions taking consideration of possible switches in relation to the cutoff E_{ap} value.

The fact that all flavonoids selected showed certain amount of antibacterial activity in spite of their different redox states suggests that flavonoids apply their antibacterial activity through a combined mechanism of action. It has been found that flavonoids can also inhibit the activity of certain redox active enzymes, such as NADPH oxidase, lipoxygenase and cyclooxygenase [183, 184], which may serve as an alternative mechanism of the antibacterial activity of flavonoids. Kaempferol, a flavonoid with relatively weak prooxidant property in ORAC assay yet provides a strong bacterial inhibition outcome, sets an example that the redox behavior of flavonoids may not be the sole explanation of their antibacterial activities. Some reports mentioned that kaempferol inhibits sortase A, which plays a key role in the adhesion to and invasion of hosts by Gram-positive bacteria [185]. Alternatively but not exclusively, kaempferol C3 has been proposed to intercalate into bacterial cell membrane and such disruption may contribute to its antibacterial activities [186].

As we examined the antibacterial activities of flavonoids, we found that myricetin demonstrated the strongest capacity to inhibit bacterial growth, with an MIC of 0.5-2 μmol/ml. Compared to ampelopsin, myricetin has an additional C2=C3 double bond and exhibits low E_{ap} values as well as better bacterial inhibitory effects, suggesting that C2=C3 double bond contributes to the potential autoxidation. Although detailed mechanisms were not clear, a reasonable speculation is that the semiquinone and superoxide radicals generated upon myricetin degradation are likely the causes for its antibacterial functions. In addition, myricetin demonstrated similar inhibitory impacts on both Gram-positive *L. monocytogenes* and Gram-negative *E.coli*, making it a good candidate for broad-spectrum antibiotic use. Further studies may focus on understanding the cellular mechanisms myricetin utilizes to inhibit bacterial growth as well as to develop formulation solutions of myricetin to improve its usage as a potential antibiotic or food preservative.

Overall, our study described a QSAR model to assess the relationship between the structure and E_{ap} property of flavonoids. We also evaluated the refined correlation between the E_{ap} value and the antibacterial activity of flavonoids, opening up the possibility to predict an antibacterial capacity of a flavonoid with given chemical structure. This analysis also provides insights on differential functional groups and their contributions to the antibacterial activity, which will aid a better design on flavonoid derivatives for drug and antibiotic screening. Additionally, we identified myricetin as a promising candidate as an antibiotic or food preservative. These findings will boost the understanding of the structure activity relationship of flavonoids and their antibacterial properties. Nevertheless, further investigations should focus on evaluating and expanding the universality of the described QSAR model and elucidating the detailed mechanisms flavonoids apply to inhibit bacterial growth upon autoxidation.

Competing Interests

The Authors declare that do not have competing interests.

Chapter 3 Myricetin and ampelopsin inhibit bacterial growth by generating ROS and regulating gene expression

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3.1 Abstract

Foodborne pathogen is one of the causes for food spoilage which happens in raw meat, vegetable, and dairy products. The emerging antibiotic resistance is bringing urgency for food industry to explore more options for effective and safe antimicrobial agents. As one of the largest groups of plant metabolites, flavonoids have been discovered to possess antimicrobial activity but some of the mechanisms are still unclear. In this study, two representative flavonoids, myricetin and ampelopsin, were investigated for their bacteria growth inhibition activities. Underlying antibacterial mechanisms for the more potent one, myricetin, was thoroughly elucidated. This study has discovered that myricetin undergoes autoxidation to generate reactive oxygen species (ROS) for bacterial inhibition. As a consequence, membrane integrity of *E. coli* was found compromised and cellular components were subject to severe leakage. Moreover, the transcription of multiple genes associated with oxidative damage prevention were drastically regulated, suggesting that the compound induces profound physiological alterations to bacterial cells. Our findings have rationalized potential antibiotic use of myricetin and provided further possibilities for improvement.

Keywords: Myricetin, Ampelopsin, Reactive oxygen species, Antibacterial activity

3.2 Introduction

Foodborne pathogens including bacteria, viruses and parasites cause more than 250 mild to severe diseases, posing serious threats to the public health. Among the commonly recognized pathogens, bacteria such as *Escherichia coli* O157:H7 (*E. coli*), *Salmonella* species, *Listeria monocytogenes* (*L. monocytogenes*) are major responsible microorganisms for foodborne illnesses that researchers have identified in the past decades. Humans can be exposed to pathogens through various sources including direct contact with infected animal/plant products or indirect consumption of contaminated food [187]. The first characterized antibiotic, penicillin, was first discovered by Alexander Fleming in 1928 [188]. Since then, more antibiotics have been discovered and thus revolutionized the treatment of infectious diseases caused by most major bacterial and fungal pathogens. However, while the pace of new antibiotics discovery started to slow down in the 1970s, the massive use of antibiotics in medicine, veterinary and agriculture gave rise to the appearance of antibiotic-resistant bacteria strains [189, 190]. Up to 2020, CDC estimates that approximately 48 million people suffer from foodborne diseases annually while new pathogen species are being identified on a regular basis. Therefore, there is an urging need for more candidates in the drug discovery pipeline with more variations in their classifications, mechanisms and antimicrobial spectrums to fight foodborne bacterial infections.

Flavonoids are one of the largest groups of metabolite compounds in plants widely found in live tissues as well as dietary products that originate from plants, such as fruit, vegetables and tea. Many flavonoids from plant extracts were found to contribute to the defense system against microbial pathogens. The antibacterial mechanisms of flavonoids could include membrane disruption [7], biofilm formation [163], DNA synthesis inhibition [164], and cell envelope synthesis inhibition [165]. In this study, myricetin and ampelopsin are two model flavonoids that were under investigation on their antibiotic activities. Myricetin and ampelopsin (also named dihydromyricetin, or DMY) are the two major flavonoids in *Ampelopsis grossedentata*, a

flavonoid-rich plant whose extracts have been long and infamously exploited to use in traditional Chinese herbal medicine to treat pyretic fever or cough [191, 192]. Both flavonoids were reported to demonstrate either antioxidant or prooxidant activity under different circumstances [193]. Moreover, both compounds have been implicated to exhibit potential antibacterial activities [104, 194]. However, the detailed mechanisms on the cellular level are largely unclear. Therefore, we aim to evaluate their impacts on foodborne pathogenic bacteria and elucidate the possible mechanisms to find a better candidate as a food preservative. We showed that the prooxidant properties of myricetin and ampelopsin are directly associated with their antibacterial activities, and that the bacteria undergo a series of physiological alterations and transcriptional regulation upon myricetin treatment. These bacterial stress response activities triggered by the drug expand the understanding of antibiotic resistance mechanisms and could shed light upon other medicinal targets for bacterial infections. Moreover, this study has provided substantial evidence that myricetin and ampelopsin could be utilized as potential antibiotics for effective food preservation.

3.3 Methods and Materials

Bacterial Strains

Frozen stocks of *Escherichia coli* O157:H7 (ATCC 43895) and *Listeria monocytogenes* Li 20 (ATCC 19111) were stored at −80 °C in Tryptic Soy Broth (TSB) (Sigma-Aldrich) containing 15% glycerol (v/v). All cultures for use were prepared by transferring a loop of culture from frozen stock onto Tryptic Soy Agar (TSA) plates (Sigma-Aldrich) for isolation and incubating at 37°C for 24 h. One single isolated colony was transferred into a 50 ml tube with sufficient volume of fresh liquid media and incubated at 37°C with agitation. For all the MIC assays in the study, overnight culture was 1:10000 diluted to achieve approximately 10^5 colony forming units (CFU)/ml before use. For the experiments of flow cytometry, RT-qPCR, SDS-PAGE and glutathione measurement, overnight culture was 1:100 sub-cultured to fresh media and continually grown at 37°C. Bacteria cells were harvested at 0.4 of OD_{595} by centrifuge at 5000 × g and then resuspended with same volume of PBS buffer for myricetin treatment.

Minimum Inhibitory Concentration (MIC) Assay

Stock solution of myricetin or ampelopsin was prepared freshly in DMSO. For MIC determination, 100 µl of broth containing twice the needed maximum concentration of flavonoids were added to the first column of a polystyrene non-treated 96-well plate (Falcon), and 1:1 serially diluted with 5% DMSO. The compound solutions were subsequently inoculated with 100 µl of bacteria cells to achieve approximately 10^5 CFU per ml. All wells were mixed by micropipetting and gentle shaking after the addition of bacterial cells. The plate was then covered with sterile sealing film to prevent the vaporization of medium. The plate was inserted into a temperature controlled 96-well plate reader (Biotek) and incubated at 37°C for 24 h, while taking continuous measurements of the turbidity of sample at wavelength 595 nm, with time intervals of 1 hr. OD_{595} readings were recorded and graphed as a function of time. The MIC was defined as

the lowest concentration of compound that inhibits the growth of microorganisms, which is the concentration that showed no OD increase.

Assay for antibacterial activity of flavonoids under different pH

Following the method described in the MIC assay, TSB medium with two different pH (6.0 and 7.4) was used. The pH of acidic medium was adjusted to 6.0 with 0.1N HCl. The growth curves were recorded during a total test time of 24h.

Assay for antibacterial activity of flavonoids in the presence/absence of Cu^{2+}

Following the method described in the MIC assay, peptone yeast glucose (PYG) broth (1% peptone, 0.125% yeast extract and 1% glucose) was used in this assay. The concentration of $CuCl_2$ added was 100 μM. Bacteria growing in the PYG broth without Cu^{2+} was used as control group. The growth curves were recorded during a total test time of 24h.

Assay for antibacterial activity of flavonoids in the presence/absence of Superoxide Dismutase (SOD)

The procedure followed the method described in the MIC assay. The final concentration of SOD (Sigma-Aldrich) was 125 IU/ml. Bacteria grown in TSB broth was used as control group. The growth curves were recorded during a total test time of 24h.

Assay for antibacterial activity of flavonoids in the presence/absence of catalase

The procedure followed the method described in the MIC assay. The final concentration of catalase (Sigma-Aldrich) was 100 IU/ml. Bacteria grown in TSB broth was used as control group. The growth curves were recorded during a total test time of 24h.

Glutathione (GSH) Measurement

The 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB)-glutathione reductase recycling assay [161, 195] was used to measure cellular GSH levels. In specific, 25 ml of a grown cell culture was first pelleted by centrifugation at OD_{595} 0.4. The cell pellet was then gently resuspended in 200 µL of the stock buffer (143 mM sodium phosphate/6.3 mM Na_4EDTA, pH 7.4) by micropipetting and added with 100 µL of 10% 5-sulfosalicylic acid solution (Sigma-Aldrich). Any protein precipitation was removed by centrifugation at 13,000 x *g* for 5 minutes. 10 µl of lysed cell supernatant was used for cellular GSH measurement.

Real-time PCR

Cells were cultured in TSB as described above. Total RNA was extracted using Trizol (Invitrogen), and was reversely transcribed into cDNA with the High-Capacity cDNA reverse Transcription Kit (Applied Biosystems), and assayed using SYBR Green PCR Master Mix (Applied Biosystems) with Mx3000P real-time PCR system (Agilent Biosystems). The primers (Integrated DNA Technologies) used to analyze expression of 12 genes including a housekeeping gene *gapA* were listed in Table 3.1 [196-198]:

Gene name	Primer sequences
ahpC [198]	forward, GACCCGTAACTTCGACAACA; reverse, ATGCCTTCAGCGGTAACTTC
katG [198]	forward, TACTGGGTGCCAACTTCGAT; reverse, GGTCGCTTTCCACTCGTAAC
yaaA [198]	forward, GCTGTTAGACAATTCCCAGCAG; reverse, AAGCCGGTGTAGACATCACCTT
oxyR [197]	forward, CGCGATCAGGCAATGG; reverse, CAGCGCTGGCAGTAAAGTGAT
dps [197]	forward, CAAAACCCCGCTGAAAAGTTAC; reverse, GATATCTGCGGTGTCGTCATCT
sodA [197]	forward, CCGATTATGGGCCTGGAT; reverse, CAAAACGTGCCGCTGCT
trxA [197]	forward, TGCGGTCCGTGCAAAATGAT; reverse, AGCAGCAGAGTCGGGATACCA
polB [196]	forward, GCATTAACGATAGTGCCATTG; reverse, CCGCAAGGGACAGAAGTCTC
dinB [196]	forward, CATTGAACCGTTGTCACTGG; reverse, GTAATCACAAACTGGCCGTTG
umuD [196]	forward, GTGTGGCTTTCCTTCACCG; reverse, CTTCACTACTGATGGTAATG
recA [196]	forward, GCGTCACAGATTTCCAGTGC; reverse, GGTAAAGGCTCCATCATGC
sbmC [196]	forward, GCAAAATCATCACCGACTACAC; reverse, CGAGATTAAGCAGGAAGAG
gapA [197]	forward, GTCGCTGAAGCAACTGGTCT; reverse, AAGTTAGCGCCTTTAACGAACAT

Table 3. 1 Primers for RT-qPCR.

HPLC-MS analysis of flavonoid degradation

Flavonoids were dissolved in 0.1M PBS buffer with 5% DMSO and incubated at 37ºC. HPLC-MS was conducted on Agilent 1100 series LC/MSD instrument (Waldbronn, Germany). The HPLC-MS interface used an electrospray ionization source (ESI) and the MS featured an ion trap analyzer. HPLC was performed with a Hypersil ODS C18 column (Thermo Scientific), 5µm, 250 × 4.6 mm at ambient temperature with 1.0 ml/min flow rate. The mobile phases were consisted of solvent A (0.1% phosphoric acid in water (v/v)) and solvent B (acetonitrile). For myricetin, the gradient elution was 10-50% B in 10-15 min, 50% B in 15-20 min and the wavelength was 370 nm. For ampelopsin, the isocratic elution was 25% B in 0-20 min and the wavelength was 290 nm. For mass spectrometry analysis, ampelopsin was dissolved in solution for less than 30 minutes before sample application.

SDS-PAGE

E. coli cells were grown to log phase and harvested at 5000 × g for 5 min at room temperature. The cells were then washed twice and resuspended in PBS buffer (pH 7.4). Myricetin (1 µmol/ml) and PMSF (1mM) was added to the cell suspensions and the samples were incubated at 37 ◦C for 2, 4 and 6 hours. The suspension was centrifuged and the supernatant was collected. Proteins in the supernatant were precipitated by adding Trichloroacetic acid (TCA) (0.28g/ml) and centrifugation. The precipitate was dissolved with urea solution (6M). 50ul of sample solution was then mixed with 25ul of sample buffer (1M Tris–HCl (pH 6.8), 50% glycerol, 10% SDS, 10% beta-mercaptoethanol, 1% Bromophenol blue) and heated at 100◦C for 10 min. Proteins were separated in 10% SDS-PAGE gel, washed with wash buffer (10% acetic acid, 20% methanol) and stained with Coomassie blue. Control samples were prepared similarly without myricetin treatment.

Flow cytometry

E. coli cells were grown to log phase and harvested at 5000 × *g* for 5 min at room temperature. The cells were then washed twice and resuspended in PBS buffer (pH 7.4). Myricetin (1μmol/ml) was added to the cell suspensions and the samples were incubated at 37 ◦C for 2, 4, 6 and 24 hours. The staining process was performed with a LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen). 2 ml of each *E. coli* cells sample (1×10^6 bacteria/ml) was stained with 6 ul of combined reagent (SYTO 9/Propidium iodide = 1:1) and incubated at room temperature in the dark for 15 minutes before being subjected to a BD LSRFortessa Flow Cytometer and analyzed using FlowJo 10.6.2 (BD Biosciences) .

3.4 Results

Myricetin and ampelopsin exhibit bacterial growth inhibition.

To study the potential dose dependent antibacterial activities of myricetin and ampelopsin, a quantitative approach was applied with liquid bacteria cultures to determine their minimum inhibition concentrations (MIC). Both Gram-negative (*E. coli*) and Gram-positive (*L. monocytogenes*) bacteria were examined to evaluate the functional spectrum of the compounds. Myricetin or ampelopsin was added to the growth media for both *E. coli* and *L. monocytogenes* to the final concentration 0-2 μmol/ml. Both myricetin and ampelopsin showed decent growth inhibition on both bacterial cultures. Specifically, myricetin delayed bacterial growth at the concentration of 0.5 μmol/ml and fully abolished the growth at 1 μmol/ml. Thus, the MIC of myricetin is 1 μmol/ml for both *E. coli* (Figure 3.1A) and *L. monocytogenes* (Figure 3.1B). We also looked at the efficacy of ampelopsin against *E. coli* and *L. monocytogenes*, which had shown reduced antibacterial activity than myricetin across the dilution series of the compounds. We report a 2 μmol/ml MIC of ampelopsin on both *E. coli* and *L. monocytogenes*, which is 2-fold higher than myricetin (Figure 3.1C-D). Therefore, both flavonoid compounds efficiently blocked bacterial growth in log phase liquid cultures, with myricetin exhibiting a slightly better potency.

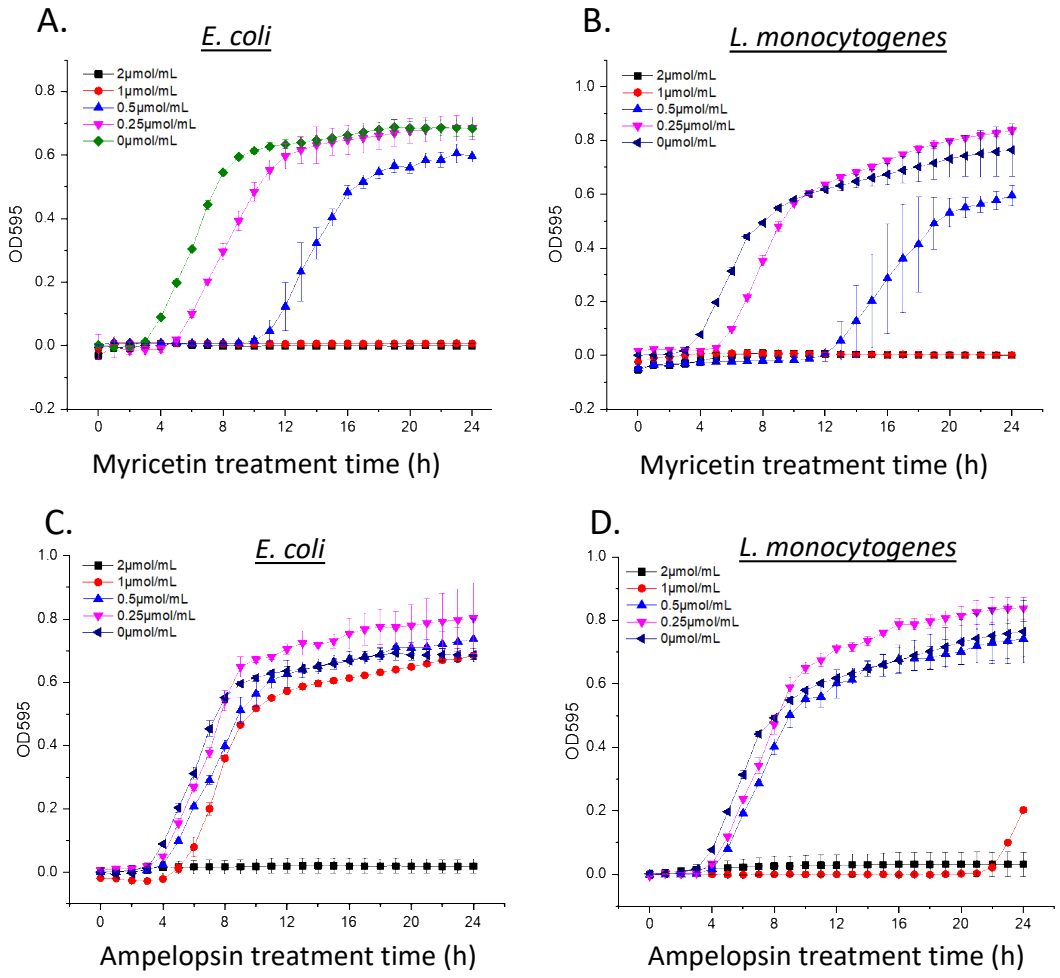

Figure 3. 1 Myricetin and ampelopsin inhibits *E. coli* and *L. monocytogenes* at an MIC of 1 μmol/ml.

E. coli and *L. monocytogenes* were grown in TSB media for overnight and cells were re-inoculated into fresh media to a concentration of 10^5 CFU/ml. Growth curves of *E. coli* (A) and *L. monocytogenes* (B) under the treatment of myricetin at various concentrations was monitored over the course of 24 hours by OD_{595}. Similar graphs of *E. coli* (C) and *L. monocytogenes* (D) growth in response to ampelopsin were also plotted.

The antibacterial activity of myricetin is associated with its degradation under alkaline condition.

At physiological pH 7.4, myricetin went through rapid degradation in aqueous phase, which gave the solution a dark brown color within 30 minutes (Figure 3.2A left). This led us to hypothesize that myricetin is oxidized to the quinone form, the polymerization of which causes browning effects [199]. Similar phenomenon happened to ampelopsin at a slower rate with semiquinone and quinone formation confirmed by LC-MS analysis (Figure 3.3), but not observed with quercetin and taxifolin (data not shown). Next, HPLC was utilized to study the rate of degradation of myricetin in aqueous solution. At pH 7.4, ~50% of myricetin was degraded after 50 min, and only 20% remained after 4 hours (Figure 3.2B). Almost complete degradation was observed by 24 hours (Figure 3.2C). In contrast, myricetin degradation rate was largely reduced at pH 6, with 80% still remaining by 4 hours and 20% remaining by 24 hours (Figure 3.2C). To study whether the antibacterial activity of myricetin is consequential to its pH-dependent degradation, the inhibition of bacterial growth of myricetin was directly compared in media at pH 6 or pH 7.4. Consistent with previous observations, for both *E. coli* and *L. monocytogenes*, myricetin has a MIC of 1 μmol/ml at pH 7.4 (Figure 3.2D-E). In dramatic contrast, bacteria growth was largely rescued in media with up to 2 μmol/ml of myricetin at pH 6, suggesting that the weak alkaline condition is needed for myricetin antibacterial activity (Figure 3.2D-E). We did not test beyond 2 μmol/ml myricetin to examine its MIC at the low pH due to solubility limitations. Altogether, our data demonstrated that myricetin undergoes rapid degradation at a weak alkaline condition, which is closely associated with its potency in antibacterial activities. Likewise, ampelopsin degrades rapidly at pH 7.4 (Figure 3.4), at which condition it confers better antibacterial activities (Figure 3.5). Our results suggested that the pH sensitivities of both myricetin and ampelopsin in their degradation have drastic impacts on bacterial growth.

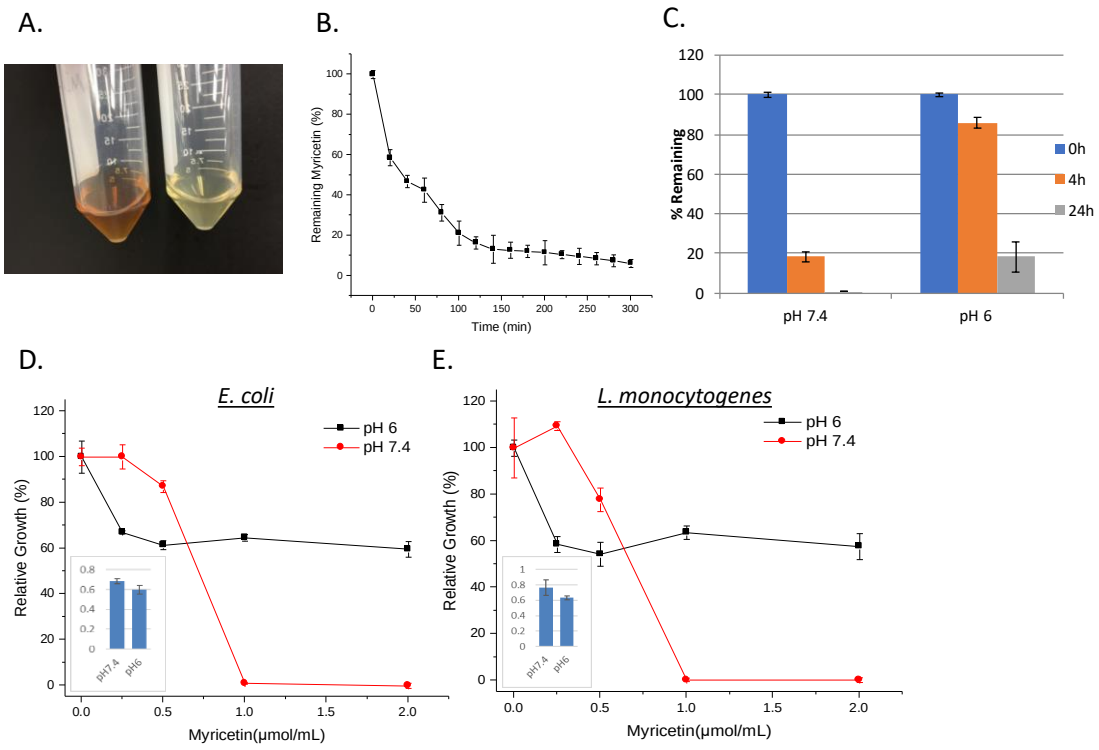

Figure 3. 2 Myricetin inhibition of bacterial growth is pH dependent.

(A) Myricetin (1 μmol/ml) in TSB media for 30 min (left) and less than 1 min (right). HPLC measurement of myricetin degradation in aqueous solution (pH adjusted by HCl-NaOH) at pH 7.4 for 5h (B), pH 7.4 and pH 6 for 24 hours (C). % Remaining myricetin was normalized to its starting concentration (1 μmol/ml). *E. coli* and *L. monocytogenes* were grown in TSB media for overnight and cells were re-inoculated into fresh media with pH 6 or pH 7.4 to a concentration of 10^5 CFU/ml. Relative growth of *E. coli* (D) and *L. monocytogenes* (E) in media supplemented with different concentrations of myricetin was normalized to the growth of bacteria at control media at 24 h (indicated in the inner figures). Growth were measured by OD_{595} at 24 h.

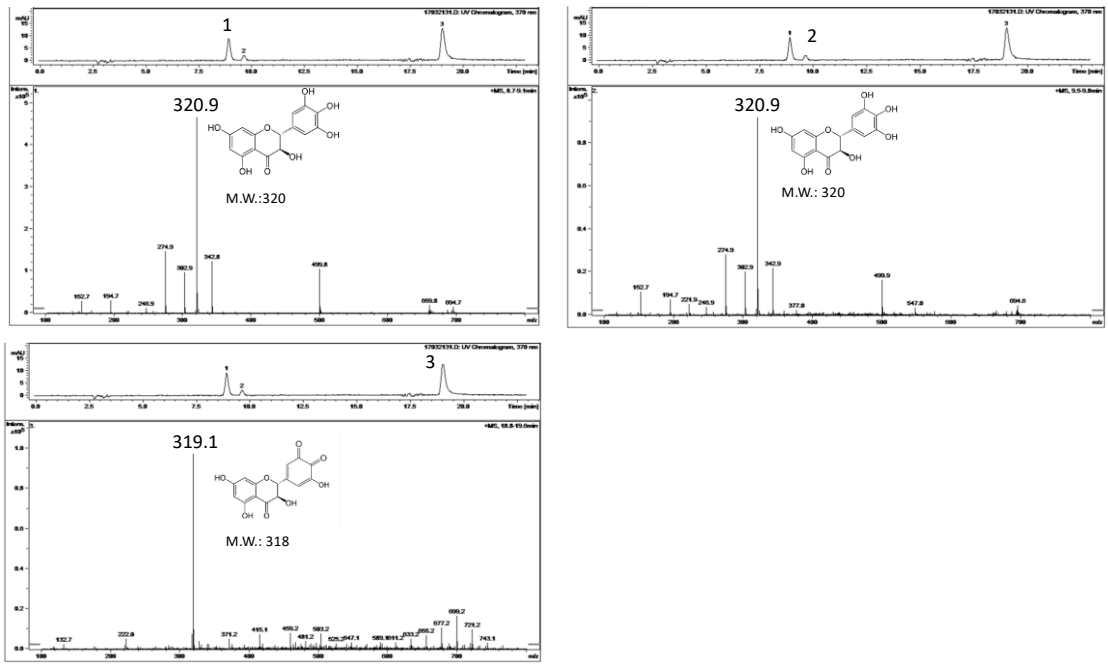

Figure 3. 3 Ampelopsin degradation produces quinone.

LC/MS analysis of ampelopsin (1 µmol/ml) degradation in aqueous solution at pH 7.4. LC and MS spectra of (iso)ampelopsin (M.W. 320) and ampelopsin quinone (M.W. 318).

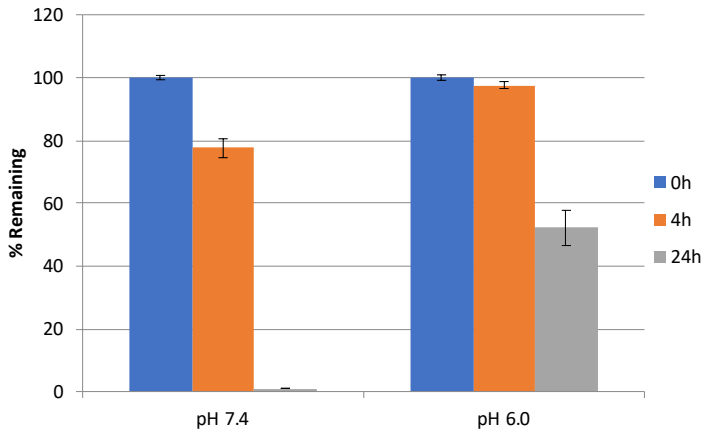

Figure 3. 4 Ampelopsin degrades at weak alkaline condition.

HPLC measurement of ampelopsin degradation in aqueous solution at pH 7.4 and pH 6 for 24 hours (pH adjusted by HCl-NaOH). % Remaining ampelopsin was normalized to starting concentration (1 μmol/ml).

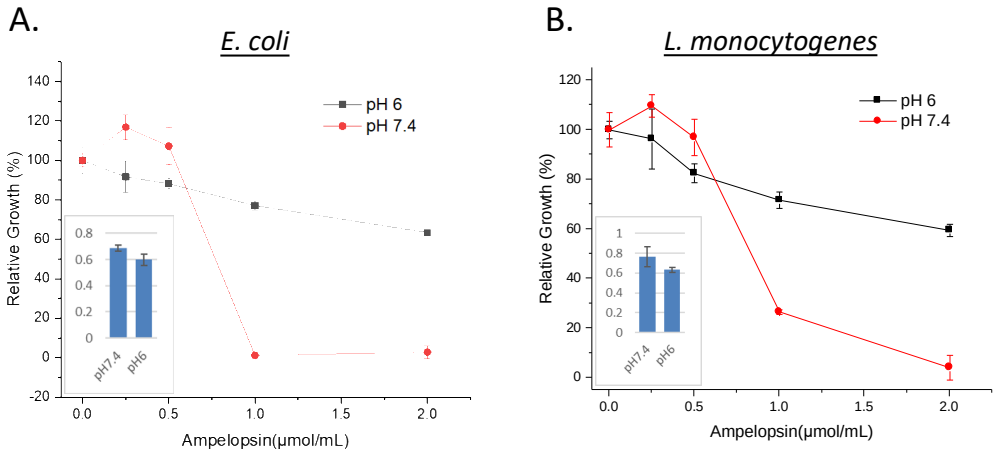

Figure 3. 5 Ampelopsin inhibition of bacterial growth is pH dependent.

E. coli and *L. monocytogenes* were grown in TSB media for overnight and cells were re-inoculated into fresh media with pH 6 or pH 7.4 to a concentration of 10^5 CFU/ml. Relative growth of *E. coli* (A) and *L. monocytogenes* (B) in media supplemented with different concentrations of ampelopsin was normalized to the growth of bacteria at control media at 24 h (indicated in the inner figures). Growth were measured by OD_{595} at 24 h.

Myricetin induces oxidative stress within bacterial cells by generating ROS.

The potential quinone formation upon myricetin degradation could generate reactive oxygen species (ROS) [200]. To study whether myricetin induces oxidative stress to exposed bacteria, *E. coli* cells were incubated with various concentrations of myricetin and levels of intracellular glutathione (GSH) were measured. In normal bacterial cells, GSH is present mainly in its reduced form but will be converted to the oxidized form (GSSG) if oxidative stress is introduced. Thus, cellular GSH level can be used as a redox environment indicator. We observed a dose dependent loss of GSH in concurrence of an increased concentration of myricetin (Figure 3.6A), suggesting that GSH are oxidized by ROS associated with increased level of myricetin.

To further test if myricetin inhibits bacterial growth via generating ROS, we examined the add-on effects of transition metal ions, considering its ability to catalyze the oxidation process of myricetin. We hypothesize that if ROS plays an active role in the antibacterial properties of myricetin, this growth inhibition would be correlated with the level of metal ions in the medium. Peptone Yeast Extract Glucose broth (PYG) was used in this assay for its low cation basal level. *E. coli* and *L. monocytogenes* were grown in PYG media with or without copper ions. Higher MIC of myricetin was observed for both *E. coli* and *L. monocytogenes* in PYG media compared to TSB, which is correlated with the lower level of cation (Figure 3.6B-C). In contrast, when supplemented with copper, myricetin in PYG displayed a more robust antibacterial activity, with an MIC of 2 μmol/ml for *E. coli* (Figure 3.6B) and 1 μmol/ml for *L. monocytogenes* (Figure 3.6C). Similar observations were obtained when using ampelopsin (Figure 3.7A-B). Our data support the hypothesis that with the assistance of metal ions, myricetin and ampelopsin go through a faster oxidation process to realize the antibacterial functions.

We then sought to further dissect the oxidation pathway undertaken by myricetin to inhibit bacteria growth. Superoxide dismutase (SOD), a common antioxidant defense mechanism in

living cells, is known to catalyze the dismutation of the superoxide radical (O_2^-) into hydrogen peroxide:

$$\cdot O_2^- + \cdot O_2^- \xrightarrow[SOD]{2H^+} H_2O_2 + O_2$$

Flavonoid oxidation generates superoxide radicals which are considered the source of the antibacterial activity of many flavonoids [201]. To investigate if myricetin degradation generates superoxide radicals and if so, whether such superoxide radicals are required for its antibacterial activity, *E. coli* and *L. monocytogenes* were grown in media with or without SOD supplementation. The antibacterial activity of myricetin demonstrated major reduction when SOD was added (Figure 3.6D-E), which had a MIC at 1 µmol/ml in control media compared to 2 µmol/ml in SOD supplemented media. Similar observations were made with ampelopsin (Figure 3.7C-D). These results indicate that the formation of superoxide radicals contributes to the antibacterial process.

Another common ROS defensive enzyme found in living organisms, catalase, is known to specifically utilize hydrogen peroxide as the substrate to generate water and oxygen:

$2\ H_2O_2 \rightarrow 2\ H_2O + O2.$

Similar to SOD, catalase plays a critical role in protecting cells from oxidative damage and it is one of the enzymes with the highest turnover rates, implicating superior potential for its oxidative damage protection effects [202]. To assess if catalase protects bacteria from myricetin toxicity through ROS, *E. coli* and *L. monocytogenes* were grown in media with or without catalase supplement. Indeed, the addition of catalase significantly impaired the antibacterial activity of myricetin (Figure 3.6F-G). Similar observations were also obtained when using ampelopsin (Figure 3.7E-F), suggesting that hydrogen peroxide is a major influencer for inhibiting bacterial growth by myricetin and ampelopsin. Overall, our data supports a mechanism of antibacterial activity for myricetin and ampelopsin: these flavonoids rapidly degrade in aqueous solution and

generates ROS in the process, including superoxide radicals and hydrogen peroxide, which induces oxidative stress to exposed bacteria, leading to their demise.

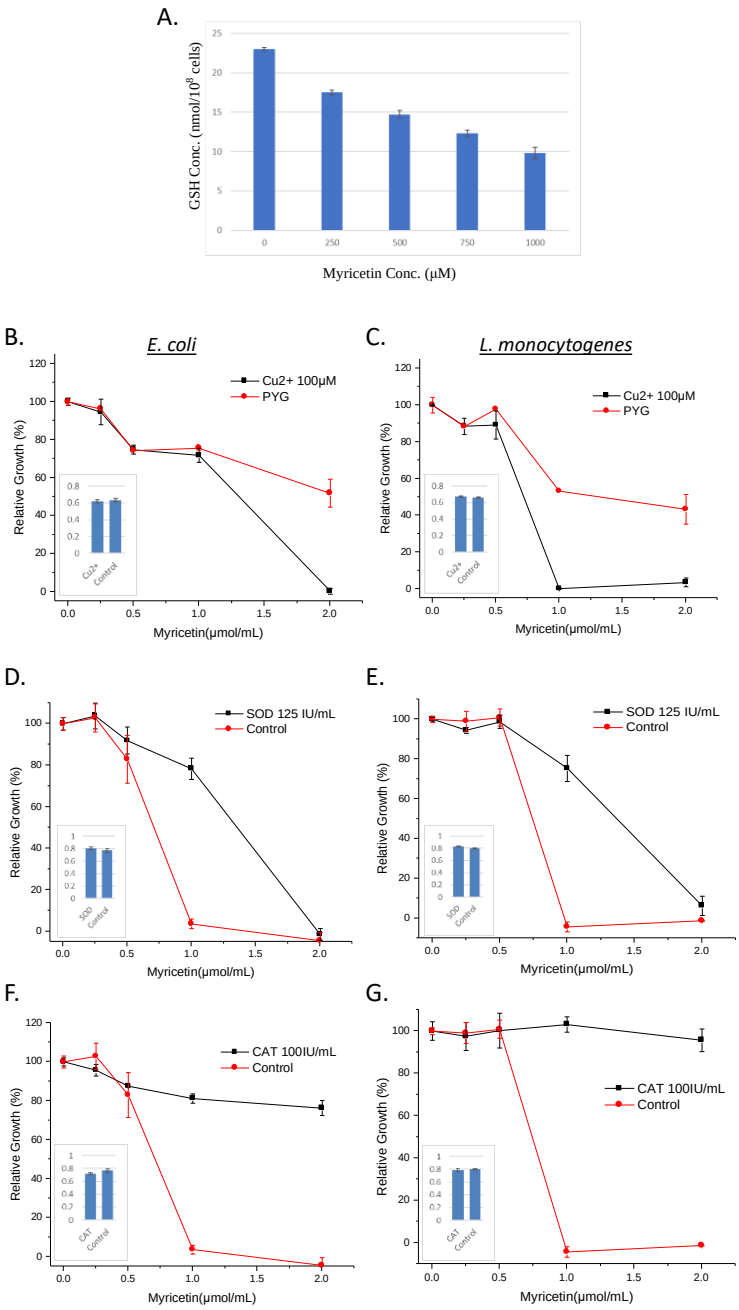

Figure 3. 6 Myricetin inhibits bacterial growth by inducing oxidative stress.

(A) *E. coli* cells were grown overnight in TSB and cells were re-inoculated (v/v 1:100) into fresh media to OD_{595} 0.4. Cells were then treated with various concentrations of myricetin in PBS for 24 hours. Lysed cellular glutathione levels were measured. *E. coli* and *L. monocytogenes* were grown in TSB media for overnight and cells were re-inoculated into indicated fresh media to a concentration of 10^5 CFU/ml. Relative growth of *E. coli* (B, D, F) and *L. monocytogenes* (C, E, G) was normalized to the growth of bacteria at control media at 24 h (indicated in the inner figures). Growth were measured by OD_{595} at 24 h.

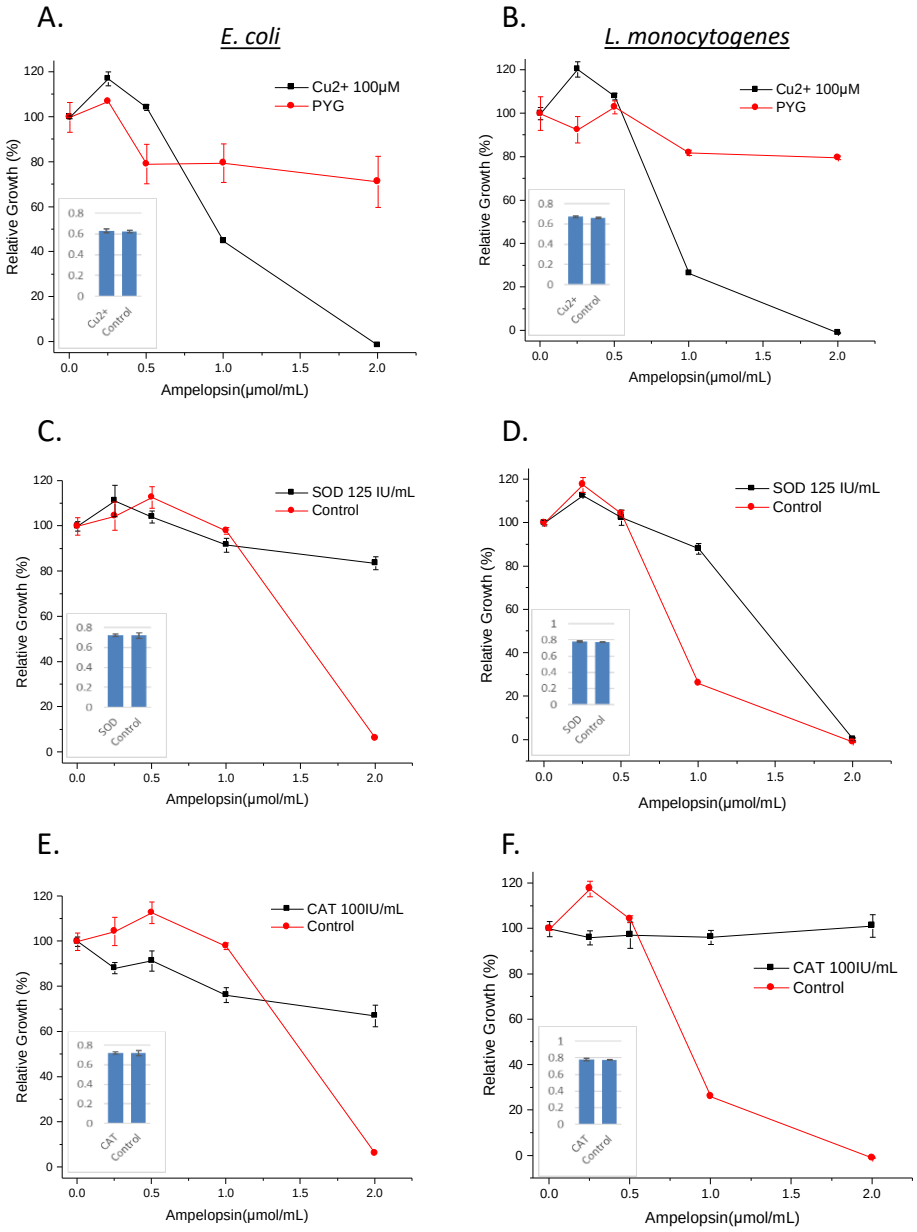

Figure 3. 7 Ampelopsin inhibits bacterial growth by inducing oxidative stress.

E. coli and *L. monocytogenes* were grown in TSB media for overnight and cells were re-inoculated into indicated fresh media with various ampelopsin doses to a concentration of 10^5 CFU/ml. Relative growth of *E. coli* (A, C, E) and *L. monocytogenes* (B, D, F) was normalized to the growth of bacteria at control media at 24 h (indicated in the inner figures). Growth were measured by OD_{595} at 24 h.

Myricetin compromises cell membrane integrity and causes cell leakage.

To study the potential damage on bacteria caused by myricetin, we examined the bacterial cell membrane integrity as this is one of the most common mechanisms by which flavonoids could impair bacteria growth. *E. coli* cells were treated with 1 µmol/ml myricetin for 2, 4 or 6 hours before being pelleted by centrifugation. The supernatant was harvested and subjected to SDS-PAGE analysis. Increased protein abundance was observed in the media supernatant from the samples treated with myricetin for 4 and 6 hours compared to the control groups (Figure 3.8), despite a slower growth rate of *E. coli* in the presence of myricetin (data not shown). In addition, we applied flow cytometry analysis to assess bacterial cell membrane permeability upon myricetin treatment. *E. coli* cells were treated with 1 µmol/ml myricetin for a time course up to 24 hours. *E. coli* cells harvested at 2, 4, 6 and 24 hours of myricetin treatment were stained with propidium iodide (PI) and SYTO 9. PI binds to DNA after travelling across damaged cell membrane while SYTO 9 has a higher affinity to live cells (Figure 3.9A). The cell population which emits strong fluorescence for both PI and SYTO 9 are the live ones with compromised membrane integrity. While the live cell (SYTO 9-positive) numbers were on the same level with or without myricetin treatment, more *E. coli* cells became permeable to PI after treatment with myricetin (Figure 3.9B-C). This suggests that myricetin permeabilizes the cell membrane even though sparing the viability of these bacterial cells at 1x MIC. Together with the observation of more protein being released to the extracellular environment, we propose that one of the antibacterial approaches of myricetin is by compromising normal cell membrane function. The membrane disruption grants cellular proteins free diffusion out of the cells, which inhibits bacterial growth and may kill bacterial cells especially when some of the proteins are responsible for critical metabolic functions.

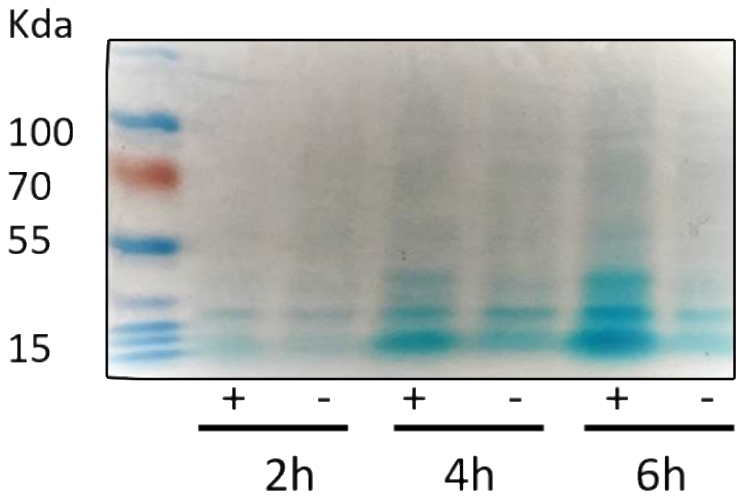

Figure 3. 8 Myricetin induces bacterial cellular leakage.

E. coli cells were grown overnight in TSB and cells were re-inoculated (v/v 1:100) into fresh media to OD_{595} 0.4. Equivalent numbers of cells were treated with 1 µmol/ml myricetin in PBS for the indicated hours. An equal volume of supernatant was subjected to SDS-PAGE and stained with Coomassie Blue.

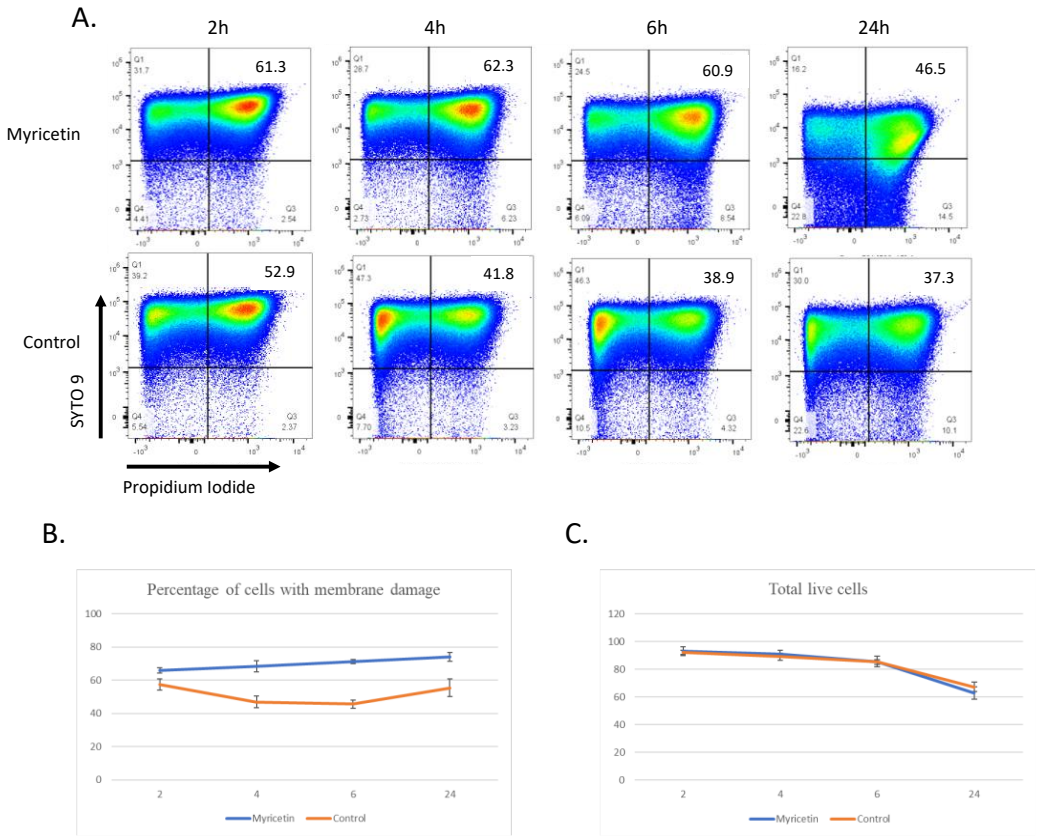

Figure 3. 9 Myricetin induces bacterial membrane leakage.

E. coli cells were grown overnight in TSB and cells were re-inoculated (v/v 1:100) into fresh media to OD_{595} 0.4. Cells were treated with 1 µmol/ml myricetin in PBS for the indicated hours. Cells were then stained with SYTO 9 and PI for viability and membrane permeability respectively (A). Percentage of cells with compromised membrane (B, SYTO 9^+ PI^-) and total live cells (C, SYTO 9^+) were quantitated.

The change of cellular redox environment alters gene transcription profiles.

The transcription of twelve genes related to the responses of *E. coli* to oxidative stress was examined by RT-qPCR (Figure 3.10). Detailed gene functions are listed in the table (Table 3.2) and *gapA* gene, which codes for GAPDH, was used as reference. Targeted genes were chosen based on their functions associated with ROS degradation, DNA repair or free-iron control. The expression level of multiple genes involved in ROS regulation was largely upregulated compared with GAPDH control upon myricetin treatment. They include coding genes of some enzymes essential to ROS degradation, such as *katG* (~5-fold induction, encodes a catalase-peroxidase), *sodA* (~5-fold induction, encodes superoxide dismutase) and *ahpC* (~3-fold induction, encodes alkyl hydroperoxide reductase). Genes involves in the responses to ROS, including *oxyR* and *yaaA* are also upregulated 11-fold and 4-fold, respectively. In addition, genes encoding DNA repair proteins including *polB*, *dinB* and *recA*, are induced from 1 to 10-fold upon myricetin exposure. Interestingly, *dps* gene encoding the DNA protection during starvation protein, which was demonstrated to protect DNA from oxidative damage by sequestering intracellular Fe^{2+}, was downregulated upon myricetin treatment. Additional experiments examining the intracellular ferrous/ferric levels are needed to further interpret this observation. Overall in agreement with our other findings, the transcription profile analysis suggests that myricetin creates an oxidative stressful environment for bacteria, triggering its physiological responses on the transcription level.

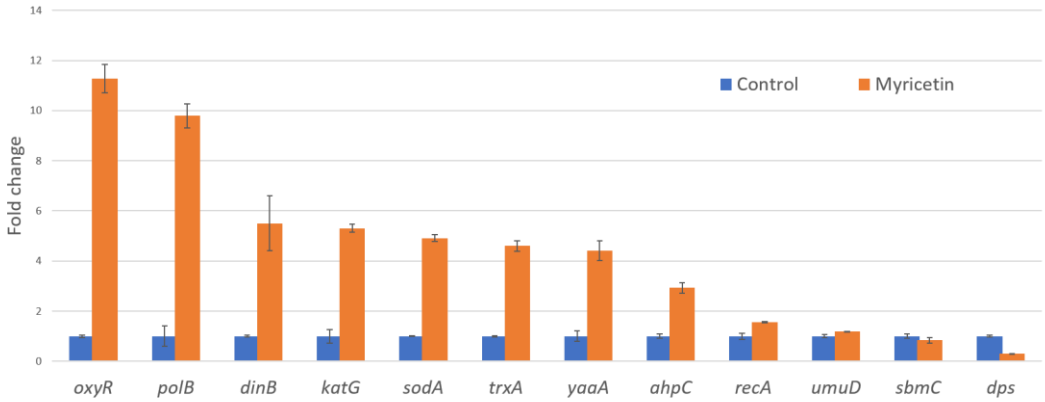

Figure 3. 10 Myricetin alters gene transcriptional profile of exposed bacteria.

E. coli cells were grown overnight in TSB and cells were re-inoculated (v/v 1:100) into fresh media to OD_{595} 0.4. Cells were treated with 1 µmol/ml myricetin in PBS for 24 hours. RT-qPCR was conducted for targeted genes. Fold changes were calculated by ΔΔCt to gapA in control samples.

Gene	Protein	Function
oxyR	Hydrogen peroxide sensor	activates the expression of a regulon of hydrogen peroxide-inducible genes
polB	DNA polymerase II	DNA repair and mutagenesis
dinB	DNA polymerase IV	DNA translesion repair and untargeted mutagenesis
katG	Catalase-peroxidase	catalase and peroxidase
sodA	Superoxide dismutase	superoxide anion radicals degradation
trxA	Thioredoxin 1	catalyzes dithiol-disulfide exchange reactions
yaaA	Peroxide stress resistance protein	cellular response to hydrogen peroxide stress by diminishing unincorporated iron
ahpC	Alkyl hydroperoxide reductase C	catalyzes the reduction of hydrogen peroxide to water and alcohols
recA	Protein RecA	homologous recombination and the bypass of mutagenic DNA lesions by SOS response.
umuD	DNA polymerase	translesion repair and UV protection.
sbmC	DNA gyrase inhibitor	inhibits the supercoiling activity of DNA gyrase.
dps	DNA protection during starvation protein	protects DNA from oxidative damage by sequestering intracellular Fe^{2+}

Table 3. 2 Functions of selected genes responsive to myricetin.

3.5 Discussion

Microbial hazard in food has been threating the public health and life quality. Better strategies to prevent bacterial contamination in food products as well as to preserve food quality are needed due to frequently emerging foodborne pathogen outbreaks and other food-related illnesses. Our study focused on the antibacterial activities of two model flavonoids, myricetin and ampelopsin, on *E. coli* and *L. monocytogenes*, representing Gram-negative and Gram-positive bacteria, respectively. We found that myricetin has the more potent antibacterial efficacy against these two common foodborne pathogens, with an MIC of 1 μmol/ml for both, while ampelopsin also has the capability to inhibit both bacteria with a slightly lower sensitivity. Additionally, rapid degradation of myricetin was observed in aqueous solutions, a phenomenon closely associated with the production of reactive oxygen species (ROS), including superoxide radicals as well as hydrogen peroxide. Upon supplementing the enzymes for catalyzing these ROS reactions, the antibacterial activity of myricetin was impaired, suggesting that the ROS released by myricetin degradation is critical for inhibiting bacterial growth. In addition, alterations of intracellular redox environment, including a reduced level of GSH and upregulation of ROS-responsive genes and DNA repair genes, were demonstrated with *E. coli* under myricetin treatment. Most of the representative phenotypes were also observed for ampelopsin. The collective data suggest that upon sensing the environmental oxidative stress, a series of defense mechanisms are triggered in the bacteria to maintain the cellular redox balance. Furthermore, we also found that myricetin causes membrane disruption of *E. coli* cells and cellular content leakage. To summarize, our study characterized the antibacterial activity of myricetin from multiple fronts, and elucidated a multi-functional antibacterial mechanism by myricetin: it compromises the bacterial cell membrane causing cellular leakage, and alters the cellular redox environment, the latter likely mediated by ROS production. Given that ampelopsin phenocopies many characteristic impacts as myricetin on the cells, it is highly possible that these two compounds exert similar ROS mediated antibacterial mechanisms.

Generation of superoxide and hydrogen peroxide during myricetin and ampelopsin autoxidation has been specifically indicated in this study. Flavonoid autoxidation is a complex non-enzymatic process which plays a role in defending exogenous microorganisms [203]. Catechins containing pyrogallol groups autoxidize with an initial step of generation of semiquinone and superoxide. O_2^- in turn oxidizes reduced catechin to form H_2O_2 [204]. Semiquinone serves as a better electron donor to O_2 than its reduced form [205] and is converted to quinone [206]. Quinones are powerful antibiotics which alkylate proteins [207] and undergo browning process to form quinone polymers [206]. Based on our data, we propose that myricetin goes through the similar autoxidation pathway in the weak alkaline physiological condition (Figure 3.11). Although direct interruption to normal cellular metabolic activities was also observed, superoxide and hydrogen peroxide exert their major toxic effects through elevating cellular hydroxyl radical level. H_2O_2 easily transforms into HO· by reacting with the cellular pool of unincorporated iron while O_2^- induces the synthesis of iron importers resulting in intensification of Fenton reaction [208]. As the most potent one out of these three ROS, hydroxyl radical can damage DNA and other cell components directly, including lipids, Cys/Met residues, mononuclear iron enzymes, etc [209]. We believe the presence of myricetin greatly changes the redox environment in the prooxidant direction with the observation that cellular glutathione (GSH) levels are reduced. GSH is an antioxidant which is widely distributed in living cells. GSH is present mainly in its reduced form and will be converted into the oxidized form GSSG when oxidative stress is introduced [161]. It plays a key role by in this process, thus preventing cellular components from ROS damage. Therefore, cellular GSH level is typically used as a redox environment indicator. The decrease of GSH level we observed supports the hypothesis that the cells encounter a more prooxidant environment upon exposure to myricetin.

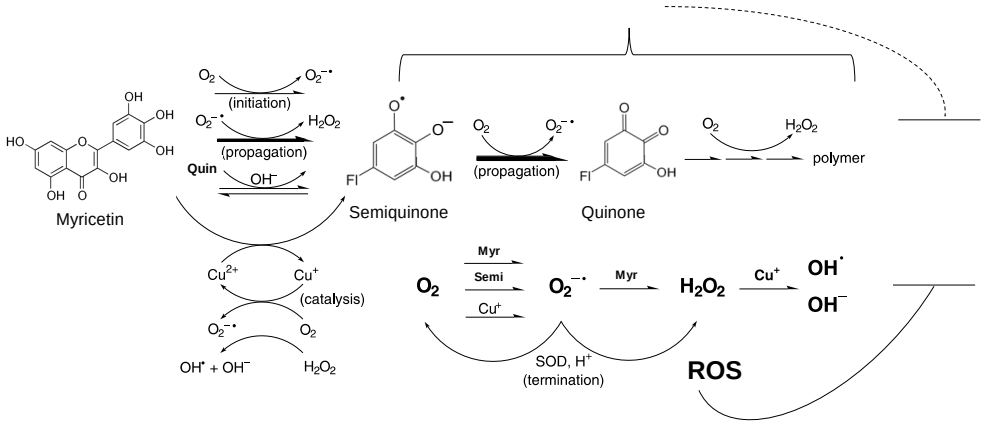

Figure 3. 11 Proposed mechanisms for myricetin autoxidation at physiological conditions. Adopted from Mochizuki, M. *et al.* 2002 [204].

Further supporting the hypothesis that myricetin triggered ROS generation thereby disrupting the bacteria cellular function, we observed transcriptional upregulation of a group of ROS-responsive genes upon myricetin exposure. *E. coli* possesses two major regulatory systems in response to oxidative stress, OxyR and SoxRS, which are triggered by peroxides and superoxide/nitric oxides, respectively [208]. Together with SOS response, these three systems directly contribute to the survival of *E. coli* against exogenous ROS attack [208]. All the genes selected in this study are categorized as following: OxyR (*oxyR, katG, ahpC, dps, yaaA*), SoxRS (*sodA*), SOS response (*recA, polB, dinB, umuD*), and *trxA* & *sbmC* (not related to the three systems). *SodA*, which is the gene encoding Mn-SOD, was upregulated, and is associated with the generation pathway of superoxide. In the SOS response gene cluster, the activation of *recA* leads to a drastic transcription upregulation of *polB* (pol II) and *dinB* (pol IV), but not much for *umuD* (pol V). This is likely because pol II and pol IV are both involved in the early stage of DNA repair during replication, while pol V only comes later for translesion synthesis when template is not accessible. Two individual genes, *trxA* (redox reactions catalyzation) and *sbmC* (DNA gyrase inhibition), are not regulated by those three systems yet theoretically could be indirectly involved in oxidation stress response [196, 197]. The thioredoxin protein encoded by *trxA* likely facilitates the reduction of other proteins under oxidative stress. A slight decrease of *sbmC* transcription suggests that it was not used as the DNA damage defense approach in this scenario. The most interesting finding comes from OxyR regulon, which upon drug treatment is oxidized by H_2O_2 and induces the upregulation of *katG* (hydroperoxidase I), *ahpC* (alkyl hydroperoxide reductase), *yaaA* (peroxide stress resistance protein), yet reduces the transcription of *dps*. Dps, an iron-storage protein, uses H_2O_2 as electron acceptor for free iron scavenging [210]. The transcription decease may be due to the exhaustion of unincorporated iron in the wake of H_2O_2 consumption through Fenton reaction. Finally, we observed a permeabilized cell membrane and leakage of cellular components into the media upon myricetin treatment. Cell membrane comprises of lipid and proteins that are likely the targets of ROS. Previous study demonstrated an alternative

mechanism that certain flavonoids may intercalate into membrane by their hydrophobic groups [186]. However, this observation came from a liposomal model but not on live cell membranes. Future experiments are needed to examine direct intercalation of flavonoids with bacterial cell membrane. Besides the previous proposals, our data provide an alternative explanation for the membrane compromise that may be induced by ROS damage.

E. coli O157:H7 and *L. monocytogenes* Li 20 are two strains selected in this study as respective representatives of Gram-negative and Gram-positive bacteria. Both myricetin and ampelopsin apply a very similar inhibition effect on these two strains. This result can be well explained by the broad-spectrum toxic effect of ROS on cells from different organisms. However, considering some bacteria strains may respond differently even to the same external stimuli, it is worth examining the responses from more additional bacteria strains, including both Gram-negative and Gram-positive, as well as pathogenic and probiotic ones to gain a thorough knowledge. To better characterize and evaluate the potential applications of myricetin as food supplements, additional studies are needed to evaluate any potential impacts or toxicity on mammalian cells and plant cells. Moreover, with *in vivo* animal models, how myricetin metabolizes in the circulation system and a full evaluation of its dosage safety will be highly valuable. Taking into consideration of the emerging importance of microbiota diversity in healthy individuals, it is also critical to explore the change myricetin may bring to the microbiota profile from the more complicated redox environment under anaerobic conditions.

In summary, we have examined the antibacterial activities and further characterized the potential mode of actions of two flavonoids in detail, myricetin and ampelopsin. We discovered that myricetin and ampelopsin undergo rapid oxidation in aqueous solutions and release multiple types of ROS as well as quinones that possibly inhibit the optimal growth of bacterial cells in the environment (Figure 3.11). We then showed these ROS reduce bacteria fitness likely by altering

the bacterial redox balance and impairing the cell membrane integrity. More evidence was also provided regarding how myricetin alters the *E. coli* transcriptional profiles of key ROS responding genes. Detailed key molecular rationales of how they impede bacterial growth and metabolism were also investigated. The ROS response mediated actions strongly indicate that they attack the pathogens via multiple biological pathways simultaneously, a feature further supported by their almost unbiased effect on different bacteria subtypes. This broad-spectrum of antimicrobial capability makes myricetin a superior candidate antibiotic, as it is less likely to be impacted by common mechanisms by which bacteria gains antibiotic resistance (i.e. gain-of-function or loss-of-function genetic mutations). This study altogether highlighted the potential use of myricetin as a novel antibiotic against bacterial pathogens in food products. Moreover, the flavonoids themselves are generally considered to carry minimal toxicity towards higher organisms as they are one of the most common natural extracts from live plants. Nevertheless, future research should focus on examining the use of myricetin on expanded target bacteria repertoire and evaluating the efficacy and safety in usage as a food preservative.

Competing Interests

The Authors declare that do not have competing interests.

Chapter 4 Discussions

Microbial hazard in food has been threating the human health and their life quality. Better strategies to prevent bacterial contamination in food as well as to preserve food quality are urgently needed. In the current study, the antibacterial activity of several flavonoids with common backbone structures were examined on *E. coli* and *L. monocytogenes*. A highly predicative mathematical QSAR model was established by linear regression analyses that connects flavonoid chemical structural features with their redox behaviors. We then discovered that the most theoretically antioxidant flavonoids may exhibit prooxidant properties in weak alkaline environments through degradation, a mechanism that could eventually accounts for their strong antibacterial capabilities. In a focused study, we found that myricetin has the most potent antibacterial efficacy against these two common foodborne pathogens, with an MIC of 1 μmol/ml for both. Additionally, rapid degradation of myricetin in solution is associated with the production of reactive oxygen species (ROS), including superoxide radicals as well as hydrogen peroxide. We have demonstrated that upon myricetin treatment, the intracellular GSH level of *E. coli* has reduced, with transcriptional upregulation of ROS-responsive genes and DNA repair genes. In addition, myricetin also causes membrane leakage of *E. coli* cells. Here, we described a potential mode of action, where the emerging ROS due to myricetin oxidation inhibits bacterial growth via altering bacterial cell redox balance and membrane integrity. Our study contributes to the better understanding of flavonoids antimicrobial mechanisms, laying the foundations for application development of flavonoids as promising food preservatives. The antimicrobial features and application of flavonoids including myricetin is an exciting field, yet it is still underdeveloped field and there are numerous additional questions and concerns that need further investigations. Some aspects of these will be covered in this chapter and immediate future directions will also be discussed.

4.1 Flavonoids, including myricetin, harbor antibacterial capabilities via a wide variety of molecular mechanisms.

Plant extracts have been used as a practical antimicrobial solution in early medicine long before the discovery of penicillin, the first fully characterized antibiotic in 1928 [54]. Modern pharmacological research has identified flavonoids as one of the most abundant and effective species of small molecules in plants. Previous studies have highlighted multiple molecular mechanisms for how flavonoids induce toxicity against bacteria, most of which are closely related to the functional structural groups on the B and C rings of flavonoids [162, 183, 211, 212]. The most common approach of these is through compromising the integrity of bacteria cell membrane. A previous fluorescent polarization assay has examined 11 flavonoid, polymethoxyflavone or isoflavonoid compounds, and revealed a strong positive correlation between the flavonoid anti-*E.coli* activities and the reduction of model double lipid layer membrane fluidity [213]. A similar yet more physiological cell membrane depolarization effect was also seen with 3 different flavonone and chalcone molecules on the methicillin-resistant *Staphylococcus aureus* (MRSA) strain [214]. An earlier study also focused on *S. aureus* using galangin, which belongs to the flavone family. The authors observed cell membrane damage mediated potassium loss into the medium, possibly as a downstream effector of bacteria cell metabolism blockage [215]. Recently, 3 flavonols (quercetin, rutin and tiliroside) were shown to reduce the thickness of the membrane bilayer in a reconstituted model system [166]. Myricetin itself has also been found to perturb bilayers leading to thermal property alterations, interpreted from NMR analyses. However, this model had required supporting evidence from physiological data, as it was using an artificial DPPC membrane [167]. In this study, myricetin induced membrane disruption and cellular content leakage have been observed in live *E.coli* cells, confirming the critical role of this mechanism *in vivo*. Notably, the membrane bilayer damage in bacteria, caused by a subgroup named catechins, were shown to be a direct consequence of an oxidative burst by ROS [216]. Considering the strong alterations also observed in this study, it is reasonable to hypothesize that

myricetin targets membranes via a similar mechanism. Interestingly, this phenotype could also be independent on ROS generation given that myricetin was also demonstrated to act against the anaerobic oral pathogens in periodontal region where ROS is not effectively generated[217]. Thus, myricetin might be able to kill bacterium via ROS independent mechanism as well.

Associated with the membrane perturbation function of flavonoids is inhibition of biofilm formation, either through induction of bacteria aggregation, blockage of cell envelope synthesis or direct targeting of biofilm signaling pathways. This has been shown in multiple bacteria strains including several flavones on *E.coli* [218], several flavonols on *S. aureus* [219], as well as a flavanone on *E. faecalis, S. aureus, E. coli* and *P. aeruginosa,* etc [220]. Flavonoids are also reported to block DNA replication pathways either by direct intercalation into dsDNA, shown in *P. vulgaris* and *S. aureus* [221], or interacting with essential enzymes such as DNA gyrase or replicative helicases in *E. coli* [169]. This mechanism is also echoed by results from this study that showed a significant transcriptional upregulation of DNA polymerases induced by myricetin. Other discoveries have also suggested that different subsets of flavonoids could inhibit ATP synthases and reduce the bioavailability in *E. coli* and *S. aureus* [170, 171]. It is also believed that flavonoids could interact with metal ion pools in the cells and manifest its activities [222], which is also confirmed in this study by the alteration of transcription profiles of metal scavenging genes upon myricetin treatment. Notably, all the aforementioned mechanisms are not independent of one another, but rather are deeply interconnected. For example, the ATPase inhibition could contribute to the biofilm formation pathway blockage or cause an inability to maintain the membrane potential. This further showcased the potential of myricetin to serve as a complex antibiotic that attacks the bacteria on multiple perspectives.

4.2 Myricetin can be either an antioxidant or a prooxidant

Myricetin plays dual roles as both an antioxidant and a prooxidant. Like many other flavonols family members (Quercetin, Kaempferol, Isorhamnetin, etc.), myricetin shows strong scavenging activity for free radicals and ions. Using high pressure liquid chromatography coupled with an electrochemical detector, S. Rafat Husain et al. found myricetin showed high activity to remove free hydroxyl radicals derived through UV photolysis of H_2O_2 and the scavenging activity is associated with the hydroxyl groups substituted in the aromatic B-ring [223]. Accordingly, myricetin was shown to alleviate H_2O_2 induced cell death through inhibition of ROS generation and activation of antioxidant enzymes [224]. In addition, myricetin was able to prevent lipid peroxidation, which leads to the use of myricetin as a food preservative to extend the shelf life of fats and oil containing products [225]. In contrast to its protective role of ROS production, myricetin was also proved capable of generating ROS. In the presence of Cu(II), myricetin generated active oxygen species which caused DNA strand scission in vitro [226]. Additionally, auto-oxidation of myricetin was reported to inhibit mitochondrial succinoxidase and generate O_2^- and H_2O_2, resulting in mitochondrial respiratory bursts [227]. In line with this, here we showed that myricetin displayed antimicrobial activity via autoxidation to generate ROS.

How myricetin switches between these two opposite functions remained enigmatic. Several studies tried to reveal some of the control factors. Miranda J. Laughton et al. showed concentration is a key to determine the function of myricetin. At low concentrations (IC50<1.5 uM), myricetin exhibited antioxidant effects, inhibiting lipid peroxidation. Interestingly, at a higher concentration (100 uM), it enhanced hydroxyl radical generation by up to eight-fold. Thus, they concluded myricetin acted as an anti-oxidant at low concentration and as a prooxidant at high concentration [228]. However, in our study, we found that even at low concentrations (0.25 uM and 0.5 uM), myricetin still showed antimicrobial activity and decent ROS generation. This suggests that absolute concentration is not the only factor to control the effects of myricetin,

although it may affect functions of myricetin in specific contexts. Another study has explored the contribution of reducing agent (ascorbic acid) and Fe ion to the effects of myricetin. They found that myricetin showed antioxidant effects in the presence of ascorbic acid, while pro-oxidative effects prevailed in ascorbic acid free systems [229]. This indicates different food may need "customized" preservatives, for example, Vitamin C/E-rich foods are likely to compromise the antibacterial effects of myricetin and call for other candidates.

The transition metals are essential for the antibacterial effects of myricetin, which mediate the transfer of electrons during ROS generation. In our results, copper deficient medium remarkedly mitigated bacteria inhibition effects. In the presence of oxygen and transition metals, myricetin undergoes auto-oxidation and produces a semiquinone-type radical at certain pH conditions. The semiquinone-type myricetin then transfers electrons to transition metals and turns into quinone-type myricetin. This is followed by the transfer of electrons from the reduced metals to molecular oxygen, leading to the generation of superoxide anion radicals and other ROS [229, 230]. The degradation and auto-oxidation of myricetin invokes the pro-oxidant reaction chain, thereby generating ROS and exhibiting antibacterial effects. It has been reported that the degradation of myricetin is tightly control by pH. Stephen J. Franklin etc. found the most stable condition for myricetin was pH 3 buffer (T50=1155h) while the least stable was pH 8 buffer (T50=0.1h) [231]. Agreeing with this finding, our data showed that myricetin underwent rapid degradation and displayed more efficient antibacterial effects at pH7.4 compared to other pH conditions.

4.3 Myricetin-derived ROS may interrupt bacterial electron respiratory chain.

In this study, myricetin demonstrated prooxidant property rather than antioxidant activity. One of the most important avenues for the ROS induced anti-bacteria effect is through the disruption of the electron transport chain (ETC) [232]. As the main powerhouse of the organism, ETC provides

energy by pumping protons across the membrane, establishing a proton motive force to allow the synthesis of ATP [233]. In bacteria, there are redundant electron transport systems (ETS) so that the bacteria can adapt to different conditions. For example, E. coli can synthesize both a higher-redox-potential respiratory quinone, ubiquinone (UQ), and a more ancient respiratory quinone, naphthoquinone (NQ) as electron carriers [232]. It has been shown that UQ and NQ are respectively associated with aerobic and anaerobic conditions [232]. One hypothesis implicated that when switching gears under oxidative stress, the resource allocated to ROS defense system may prevent the bacteria from growing [232]. Interestingly, since semiquinone and quinones are also generated upon myricetin oxidation, it is highly possible that myricetin can also disrupt ETC via imbalanced quinone production.

Throughout evolution, variants of some respiratory components emerged while some of the core components in ETC remain conserved from bacteria to mammals. The major eukaryotic energy production machinery, mitochondria, synthesizes the energy unit ATP during oxidative phosphorylation. By coupling pyruvate and fatty acid oxidation with ETC, they give out massive amounts of ROS and regulate cellular redox reactions, highly resembling bacteria actions [234]. Notably, while bacterial cell walls mainly contain the unique structures of rigid peptidoglycan layers, it was also reported that the membranes could form microdomains consisting of lipid species, namely “lipid ordering” [235]. The strikingly similar lipid raft structures are present in eukaryotic mitochondria membranes that compartmentalize the cytoplasm. Related studies have demonstrated that accumulation of ROS such as H_2O_2 could destabilize mitochondria membrane that in turn trigger DNA damage responses and/or reduces mitochondria ETC protein functions [236, 237]. Major enzyme components that impact such ROS signaling pathways in human mitochondria include superoxide dismutase (SOD1 or SOD2), catalase and glutathione peroxidase, all of which have functionally conserved counterparts in the prokaryotic organisms [238, 239].

Altogether, these reports tightly connected bacteria ETC and mammalian mitochondria respiratory pathways, providing substantial support to a well perceived theory that mitochondria is transformed from bacteria endosymbiosis in eukaryotes through ~1.5 billion years of evolution [240]. This proposal also flags the possibility that the antibacterial flavonoids under investigation in this study may possess similar targeting capabilities towards mitochondria through conserved mechanisms. In fact, some human mitochondrial diseases exhibit antibiotic sensitivity and believed to be correlated with its bacterial origin [241]. On this note, it is of particular significance to understand the impacts of flavonoids on mitochondrial functions for safety measurements as food additives.

4.4 Flavonoids induce apoptosis through mitochondrial pathway in mammalian cells.

There are two major apoptotic pathways in mammalian cells, intrinsic mitochondrial pathway and extrinsic death receptor pathway. Tea catechins have been studied for their anti-carcinogenic and anti-tumor effects in both *in vitro* cell culture and *in vivo* animal cancer models [242]. Among all the polyphenols in green tea, EGCG is the most effective in inducing selective cytotoxicity to cancerous cells through the two pathways. EGCG triggers intrinsic apoptotic signaling via activation of Caspase-9 in PC3 prostate cancer and MCF-7 breast cancer cells [243, 244]. Similar findings were confirmed by the induction of Cytochrome C, Caspase-3, and Caspase-9 [245]. The mitochondrial pathway can be induced by oxidative stress [246]. It has been reported that EGCG induces human mesothelioma cell death by inducing reactive oxygen species [247]. Given the similar pyrogallol group structure in myricetin and EGCG, the mechanism behind myricetin induced bacteria killing and EGCG induced apoptosis may be very similar. Therefore, the metabolism of bacteria cells may be interrupted both via extracellular ROS membrane attack and electron respiratory chain disturbing through a combined mechanism by myricetin.

4.5 Bacteriostatic vs bactericidal potentials of myricetin

The wide variety of antimicrobial compounds from plant extracts possess either bacteriostatic vs bactericidal activity, depending on their structure and the bacteria strain they encounter [222, 248]. The typical assay that researchers conduct to distinguish the two models is called the time-kill kinetic assay [249]. In short, the bacteria in study would be inoculated to early log phase, supplemented with the antibiotic to a final concentration of 4x MIC, then plated at different time points after drug treatment. The number of CFU per mL would be recorded as a readout for the cell viability as a drug response. Several studies using this approach have suggested that flavonoids across multiple sub-families exhibit bactericidal activities, although questions have been raised arguing that an effect of cell aggregation may also reduce the number of CFU and be mis-interpreted as bactericidal [27, 162, 212, 214]. While there is a lack of ideal methods to directly address the aggregation effect in time-kill assays, the question of whether flavonoids kill bacteria cells may be tackled from another angel, through common mechanisms of bacteria cell deaths. Bacteriostatic drugs typically pause the protein synthesis pathways, and perhaps more importantly here, do not generate hydroxyl radicals [27, 28]. Meanwhile, the majority of bactericidal drugs target key players in DNA replication, DNA damage response, cell wall integrity, and work on both Gram-positive and Gram-negative cells [29], although not all bactericidal antibiotics necessarily trigger ROS responses [198]. These collective findings were made based on multiple primary types of antibiotics, beyond those that induce bacteria aggregation, and therefore are highly likely to be applied more universally including in flavonoids.

The results from this study with myricetin highly match the features of bactericidal antibiotic activities (ROS response, DNA replication alterations, membrane permeabilization, broad spectrum bacteria targeting, etc.) The bactericidal effect is most likely coming from hydroxyl radicals generated from Fenton reaction, as superoxide and hydrogen peroxide have been

recognized as bacteriostatic agents [250]. The.only exception observed was that the flow cytometry data showed the presence of live cells with compromised membrane. This may be explained by the definition of bactericidal agents. Typically, the determination if an antibacterial agent is bactericidal is based on MBC/MIC value (<4 for bactericidal agents) [26]. However, myricetin was only applied at a mild final concentration of 1x MIC in the flow cytometry experiment, an effort to observe intermediate cellular properties of the bacteria, and it is highly likely that the drug concentration is not high enough to evaluate its full killing capability. Altogether, our data strongly suggest that myricetin possesses bactericidal properties as a potent candidate antibiotic. While direct evidence would ideally be needed to make a final conclusion either by flow cytometry or microscopy at a higher myricetin concentration, this is unfortunately not feasible by our current formulation method due to myricetin solubility limitations. On another note, while most antibiotics tend to target cellular pathways that massively consumes energy, bacteriostatic and bactericidal antibiotics could interfere with the respiration pathways in bacteria via distinct mechanisms. A recent report suggested that bacteriostatic antibiotics reduce cellular respiration, but bactericidal antibiotics instead accelerate respiration, supported by their noticeable activity reductions resulted from attenuating respiration [251]. To further understand the mechanism of how myricetin inhibit bacteria growth and conclude its role as either bacteriostatic or bactericidal reagent, it will be intriguing to explore the respiration rate upon myricetin treatment.

4.6 Pyrogallol group-containing compounds and their antimicrobial activities.

Our data demonstrated that myricetin and ampelopsin exhibited effective antibacterial activities. Both compounds contain a pyrogallol group in ring B. It has been reported that the pyrogallol group is used to generate superoxide anions during stable free radical formation [252]. Therefore, compounds with pyrogallol group are prone to be pro-oxidant and have high antibacterial activities. Among the flavonoids, tannins are popularly used polyphenols in a wide range of

products, such as green tea, red wine, beer and fruits. Many tannins including myricetin, gallic acid, baicalein, tannic acid, gallagic acid, epigallocatechin gallate (EGCG) and Theaflavin-3-gallate [253] contain pyrogallol group. Similar to myricetin, the other compounds also displayed decent antibacterial activities. Gallagic acid was able to inhibit both Gram-negative and Gram-positive bacteria [254]. Baicalein was considered as a potential antibacterial agent for foodborne pathogen Shiga toxin-producing enterohaemorrhagic *E. coli* as it inhibited both Shiga toxins 1 and 2 [255]. Tannic acid was widely used as antiviral and antibacterial agent, as it has been showed to antagonize viral pathogens such as Influeneza A virus, Papilloma viruses, noroviruses, Herpes simplex virus type 1 and 2, and human immunodeficiency virus (HIV). It also has decent inhibitory effects on both Gram-positive and Gram-negative bacteria strains as *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, and *Listeria innocua* [256]. Although there is no direct evidence on whether gallagic acid has antibacterial or antiviral activity, gallagic acid containing Caribbean did show anti-*Helicobacter pylori* and antiulcer action in rodent models [257]. Recent studies found that EGCG served as a natural antibacterial agent to regulate the growth and toxin production of *Staphylococcus aureus*, and aztreonam-EGCG combination inhibited multidrug-resistant *Pseudomonas aeruginosa* [258, 259]. Theaflavin-3-gallate from black tea displayed antimicrobial activities at nanomolar levels against *Bacillus cereus* [260]. Taken together, our work and all these findings suggest pyrogallol group within flavonoid compounds is a key function unit associated with antibacterial activities.

4.7 Structural stability analysis of myricetin.

Antioxidant/radical scavenging activity has been claimed to be one of the most relevant chemical properties of flavonoids. It has been proposed that there are three structural features majorly contributing to this property [127]:

1. 3’, 4’-hydroxyl groups on the B ring

2. 2, 3-double bond in conjugation with 4-keto group

3. 3-hydroxyl groups on the C ring

The result from our ORAC assay suggested high radical scavenging capacity of quercetin, which satisfies all the requirements above. It has also been widely reported that hydroxy substitutions in the B ring exert great impact on the electron donor properties of flavonoids [261, 262]. The antioxidant activity can be categorized in their redox behavior, which is characterized by anodic peak potential (E_{ap}): the lower the potential, the more antioxidant flavonoid is. Some of the flavonoids selected in our study were included in a previous review and sorted by anodic peak potential in an order of: myricetin < quercetin < taxifolin < kaempferol [180].

Phenol structure is often oxidized by losing one electron and one proton to the thermodynamically unstable phenoxy radical, which exists in three isomeric forms. The following hydrolysis results in *ortho*- and *para*-positions, while the *meta*-position is not favored [263, 264]. These positions lead to the irreversible oxidation of mono-phenol structure, which explains the relatively high E_{ap} of kaempferol. These actions above may provide an explanation how kaempferol demonstrates antibacterial activity in spite of its prooxidant activity. More evidence of such can be found in the lipid peroxidation pathway by monohydroxybenzoic acids [182].

The presence of C2=C3 double bond in quercetin stabilizes the generated phenoxyl radical by enhancing the electron delocalization, resulting in lower E_{ap} than taxifolin [182, 262, 265]. Therefore, it is reasonable to presume that the E_{ap} of ampelopsin is higher than myricetin. The additional hydroxyl group on the B ring of myricetin and ampelopsin even increases the electron donor ability on top of catechol, resulting in superior antioxidant activity for autoxidation taking place. The initial step of autoxidation generates semiquinone and superoxide radical, which accelerate autoxidation and lead to H_2O_2 formation [204, 266]. The presence of transition metals

enhances the prooxidant activities of myricetin and ampelopsin and produces hydroxyl radicals through Fenton reaction. Therefore, the overall redox behavior of flavonoid should be considered in the context of concentration, pH and the presence of transition metal ions [267, 268].

4.8 Myricetin, ROS and mammalian cells.

ROS commonly include superoxide (O2−), hydrogen peroxide (H2O2), and hydroxyl radical (OH·), which are by-products of aerobic metabolism [269]. Cellular ROS concentration can be presented at either physiological or pathological levels. The physiological level of ROS is maintained low for normal cellular functions. At a pathological level on the other hand, the drastically elevated ROS will trigger oxidative stress response that may be eventually detrimental to the cells [269]. Under physiological conditions, low levels of ROS can activate signaling pathways so that the organisms can adapt to it. For example, SOD1 and SOD2, namely superoxide dismutases 1 and 2, located in the cytosol/mitochondrial intermembrane space and mitochondrial matrix respectively, are responsible for preventing superoxide accumulation by converting them into H2O2 [269]. In contrast, oxidative stress induced by high levels of ROS would cause damage to DNA, protein or lipids [269]. ROS aggregation in liver could lead to severe hepatic injury through lipid per-oxidation and pro-inflammatory cytokine induction [270]. More importantly, abnormal ROS accumulation would make the cells become tumorigenic by promoting proliferation, growth and survival signaling pathways [269].

Myricetin as a plant-derived flavonoid widely used in in various foods and beverages, has been reported to show both anti- and pro-oxidant effects [82, 271]. Depending on various conditions, myricetin may either produce ROS or prevent ROS generation, and both ways could be beneficial to the vertebrate hosts. On one hand, consistent with our findings that myricetin can inhibit bacteria by generating ROS, in vertebrate system, ROS can also kill pathogens by causing direct

oxidative damage to DNA, protein or lipids of the pathogens [272]. Moreover, *in vivo* ROS are capable to eliminate the pathogens by activating immune response, such as pattern recognition receptors signaling and T cell activation [272]. On the other hand, the antioxidant activity provided by myricetin can also be beneficial to the vertebrates. Previous study demonstrated that ROS can be inhibited by myricetin by up to 34.5% based on the fatty liver cell model using HepG2 [270]. To note, the intracellular ROS decreased with myricetin in a dose-dependent manner in mammalian cells, suggesting their antioxidant capacity in inhibiting oleic acid-induced triglyceride over-accumulation and steatosis [270]. The cytotoxicity of myricetin with ROS production on mammalian cells and in animal models are still not clear. Further investigations are thereby needed on this matter considering the deleterious outcome potentially induced by ROS.

4.9 Broad spectrum antimicrobial activities potentiate myricetin as a promising food preservative.

Bacteria antibiotic resistance strains are threatening current food preservations. Some well-known strains such as asmethicillin-resistant *Staphylococcus aureus* and antibiotic-resistant *Campylobacter spp.* have been reported as sources of food contamination [273, 274]. Since these strains showed resistance to most common antibiotics while some effective antibiotics carry cytotoxicity, seeking new non-toxic antibiotics is an urgent mission in food industry.

In this study, we found myricetin had antibacterial effects on both Gram-positive and Gram-negative bacterium at aerobic condition mainly through ROS generation. Myricetin also displays antimicrobial effects against several other organisms. In combination with ampicillin/sulbactam, amoxicillin/clavulanate and cefoxitin, myricetin showed a strong activity against *Klebsiella pneumonia* [275]. Myricetin was bactericidal to *B. cepacian*. through protein synthesis inhibition [276]. It also inhibited the growth of *M. lysodiecticus, B. subtilis, K. pneumonia, C. diphtheria, C. diphtheriticum, P. aeruginosa, S. aureus, S. sonneie, S. epidermidis, S. saprophyticus, E. faecalis,*

S. pneumonia, S. pyogenes, E. coli, E. faecium, P. mirabilis, S. typhi, S. paratyphi, S. dysenteriae and *S. flexneriae* [277]. In addition, myricetin showed activity against *Mycobacterium tuberculosis* [278].

Myricetin was able to inhibit the activities of Rauscher murine leukemia virus (RLV) and human immunodeficiency virus (HIV) reverse transcriptase and thus could act as a strong antagonist of RLV and HIV [279]. A drug screen assay for Ebola virus inhibitor identified *Limonium morisianum* as the most potent inhibitor extract with myricetin as the main component. Mechanism-wise, myricetin could inhibit the interaction between VP35 with the viral dsRNA [280]. For the Severe Acute Respiratory Syndrome (SARS) coronavirus, myricetin inhibited the coronavirus helicase protein through affecting the ATPase activity thus inhibiting the SARS-coronavirus [281, 282].

The broad-spectrum antimicrobial activities of myricetin make it a strong candidate as a food preservative against bacteria, fungi and viruses. It was reported that daily consumption of myricetin in traditional Greek diet is 0.9 to 1.9 mg along with various other flavonoids [283], indicating that oral myricetin intake may not have severe negative effects on human health and can be use as food additives. However, systematic future research should be done to examine the safety level of myricetin as a food additive.

Chapter 5 Future Directions

5.1 Investigation of the role of ROS generation in bacteria physiological alterations.

Several immediate experiments could be pursued to strengthen and expand the current study. Firstly, we have shown that the ROS generation upon myricetin oxidation is associated with altered GSH level in the cells. More direct evidence could be established by measuring the intracellular level of GSH upon myricetin treatment in the presence of SOD and/or catalase. The reversion of GSH level upon adding in SOD and/or catalase would indicate that the impact on GSH is mediated by ROS released by myricetin oxidation. More approaches could be applied to measure the protein release in the SDS-PAGE experiments and PI staining in the flow cytometry experiment, with/without SOD and catalase. If showed similar results, these data will strongly support our hypothesis that ROS is responsible for mediating the damages. On the contrary, it is also possible that the additional SOD and catalase, which reduces the antibacterial activity of myricetin shown in the growth assay, may not affect the physiological alterations of the bacterial cells. This possibility, though in contrast to what we proposed, should lead to two alternative hypotheses: First, the myricetin itself may interact with cell components. Kaempferol C3 has been proposed to intercalate into bacterial cell membrane and such disruption may explain its antibacterial activities [186]. Myricetin has a same C3 conformation and can potentially function in the same way. Second, though the complete oxidation product profile is yet to be determined, semiquinones and quinones are known to be generated [193]. Quinones have been studied for years and are well-known for their bactericidal effects through targeting protein synthesis or interruption of cell wall proteins. One way or another, this collective approach would directly establish the relationships between ROS generation and bacterial growth response.

5.2 Assessment of myricetin as a potential broad-spectrum antifungal antibiotic.

As invasive fungal infections are bringing more and more nonnegligible problems in the food industry, the need for novel and various types of antifungal drugs has been raised. Flavonoids has been shown in the new century to exhibit great potentials to target and inhibit fungal growth [183]. However, the details for their mode of actions within fungal cells are largely missing, hindering their potentials to be widely used as antifungal drugs. More importantly, among all the studies that associated flavonoids with fungus killing potentials, far from enough were focused on foodborne fungal pathogens. A few recent publications have highlighted the use of particular nanoparticles with antimicrobial properties that could target foodborne fungi pathogens with more mechanistic details [284, 285]. Interestingly, some of these proposed modes of actions for fungi killing include the use of metal ions to inhibit fungal biofilm formation, cell toxicity from ROS generations and disruption of cell membranes. Studies with PMF (polymethoxylated flavones) have also shown that they inhibit the growth of a foodborne fungal pathogen *Aspergillus niger* by triggering Na^+ and K^+ leakage and altering the chitin contents in the plasma membrane [286, 287]. These MOAs highly resemble the ones flavonoids, especially myricetin, undertake to target other pathogens. Therefore, it is worth exploring if and how myricetin may contribute as a fungal antibiotic.

Myricetin has been implied in the growth inhibition of *C. glabrata* at an MIC of 3.9 - 64 μg/ml, and was speculated to act through targeting DNA synthesis [288, 289]. On top of this, it would be of great significance to assess the spectrum of fungal pathogens that are responsive to myricetin treatment, especially the foodborne ones that pose urgent threats to food safety. MIC determination assays can be applied to model foodborne pathogenic fungal strains including *Alternaria, Aspergillus, Fusarium, mucormycetes, circinelloides*, and especially the antibiotic

resistant strain of *C.albicans* to test the effect of myricetin. Other flavonoids that were used in this study can also be used to compare and pinpoint the structural components that may contribute to any antifungal activities.

Another important direction would be to further elucidate the cellular mechanisms of how myricetin may act in fungal cells. In particular, ROS generation in fungus is mediated by NADPH oxidases and its onset has been implied in the plants defense mechanism [290]. It would therefore be interesting to test if this holds true for potential myricetin induced fungal inhibition. To achieve this, cytosolic NADPH level measurements can be performed via an enzymatic reporter assay first described in *S.cerevisiae* [291]. Furthermore, given the advantage of fungus cells to be genomic engineered, non-lethal deletion or point mutants of the NADPH oxidase coding genes may be introduced in the pathogenic strains to monitor its response to myricetin. A loss of drug sensitivity in the mutant strains would indicate that the MOA of myricetin is directly through NADPH mediated ROS generation. On the contrary, if the cells remain equally responsive to the compound, it would argue that myricetin acts through another metabolic pathway independent of NADHP regulation in fungus. Overall, these directions would address the question of whether myricetin would be a promising candidate in fighting broad fungal pathogens in the food industry.

5.3 *E.coli* transcriptome analysis upon myricetin treatment.

Our qRT-PCR results revealed that multiple *E.coli* genes were subject to drastic transcriptional alteration. These genes are clustered within the OxyR regulon, DNA damage repair or iron scavenging pathways that are all known directly involved in the oxidative stress response by the cells. OxyR protein is a transcription factor in the LysR family, and is responsible for the transcription of at least 20 regulon members [292]. It is highly possible that it would also impact even more downstream gene transcriptions indirectly through its target genes. However, it is unclear how many other genes are subject to myricetin mediated transcriptional regulation, and

how they may contribute to the bacteria growth inhibition. Therefore, in order to thoroughly understand how myricetin interferes with the bacterial metabolism, unbiased investigations of the transcriptome profile on the genome-scale would be profoundly helpful. Two types of studies can be of particular interest for the *E.coli* functional genomic analysis: (A) RNA sequencing and (B) mutant library screening.

For (A) RNA sequencing, published protocols that allowed directional transcript identification that was based upon an earlier DNA microarray transcription profiling approach can be applied [292, 293]. In the initial data analysis, top 1% hits among the 6,000 total annotated genes that underwent up- or down- regulation with myricetin can be selected for cluster analysis to understand their collective roles.

For (B) library based screening, a model *E.coli* K-12 strain library with single non-essential gene knockout collections (Horizon Discover, catalog OEC4988) [294, 295] can be directly exposed to myricetin at a mildly toxic concentration and colonized on plates. For genes directly involved in myricetin resistance mechanisms, the colonies with corresponding deletions are be expected to exhibit more severe growth inhibition compared to the wildtype strain. On the contrary, for genes involved in the myricetin mediated anti-bacterial pathway, their deletions are likely to reverse the growth inhibition effect and exhibit larger colonies on plates. Genes that are irrelevant to the myricetin actions in *E.coli* cells are expected to show no difference of colony size than the wildtype strain. Notably, *E.coli* O157:H7 strain may exhibit slightly biological differences the K-12 strain. To assess how well the data translates to the pathogenic strain, a similar O157:H7 strain deletion library can be analyzed for comparison. This collection is built based on the transposon-directed insertion sequencing technique and is not yet commercially available [296, 297]. Thus, it may be considered to be utilized only as a backup in the initial round of screening, and further exploited to be built for verification purposes and subsequent studies.

In both experiments, examples of some candidate genes may be worth close attention, among others: the coding genes of Glutathione reductase for disulfide reduction, MntH and Fur for iron import control and Ferrochetalase for heme synthesis. Following the high-throughput sequencing/screening, qRT-PCR analyses of selected interesting gene hits would also be helpful for verification [208]. Altogether, such genomic analyses would massively broaden our knowledge on the cellular behaviors of myricetin in *E.coli*, and may even reveal novel mechanisms of actions that were previously neglected. A fully educated understanding of these will be utterly beneficial to pave the way for myricetin to be eventually applied as an antibiotic food preservative candidate.

5.4 Evaluation of myricetin impact on gut microbiome.

The gut microbiome consists of a group of commensal bacteria residing in the gastrointestinal track of an individual. It functions to metabolize fibers and other nutrients that cannot be processed by the host. Emerging evidence shows that a healthy gut microbiome is essential and that disruption of a normal gut flora is associated with a variety of diseases, including nutrition-related chronic diseases (such as obesity and diabetes), and systemic malfunctions of immunity and brain [298]. More recently, emerging new evidence also supports that the gut microbiome is a determinant factor affecting drug efficacy as well as anticancer therapeutic outcomes for patients [299]. The composition of the gut microbiome is dynamic and highly responsive to dietary. To note, certain species in the gut are known able to metabolize myricetin into 3,4,5-trihydroxyphenylacetic acid [300].

Multiple benefits of myricetin have been described for its promising therapeutic use, including the anticarcinogenic, antithrombotic, and antidiabetic effects. Myricetin was also used for the treatment of goat diarrhea [301]. Apart from our study showing that the prooxidant character of

myricetin possesses an antibacterial activity on *E.coli* and *L. monocytogenes*, myricetin could also antagonize against a wide range of other bacteria, fungus and viruses. Intriguingly, myricetin could display most antimicrobial activities under both aerobic and anaerobic conditions, raising its potential to have a great impact on the dynamic of the gut microbiome. However, despite that myricetin has been discovered since a long time back, not many studies have looked at its potential impacts on gut microbiome. In addition, considering that myricetin is highly prevalent in daily consumable food as well as its promising usage as food preservative and in therapeutics, it would be particularly informative to examine how myricetin would alter the gut microbiome. To achieve this goal, a classic simplified human source bacteria mix with 7 defined species (SIHUMI) will be grown in their specific media [302], pre-mixed at equal density, and incubated with myricetin for 48 hours in PBS under anaerobic conditions. The pre-mix population incubated with or without myricetin will be subjected to specific 16S qPCR to measure the taxonomy alteration. Additionally, the myricetin treated population will be re-inoculate into brain-heart infusion rich media to monitor the recovery of each strain (by 16S qPCR on each specific strain). If myricetin displayed impacts on composition of the simplified bacteria mix, a more physiological relevant approach could be exerted by enriching gut microbiome from human fecal samples in lab and reproducing the myricetin treatment. In this case, 16S sequencing will be used instead of qPCR to monitor the much more complex taxonomy change upon myricetin exposure. These experiments to understand the potential impacts of myricetin on mixture of bacteria will help us design better usage of myricetin, in regards to both the dosage and period of the treatment, that can provide optimal functions while controlling to the minimal disruptions on a very important composition of our healthy lives – the gut microbiome.

5.5 Formulation of myricetin for bioavailability enhancement

Flavonoids have been long characterized as compounds with low solubility and bioavailability. Although myricetin is highly soluble in organic solvents and weakly acidic (pKa of 6.63), it is a

practical challenge to increase its bioavailability due to the low aqueous solubility (16.60 μg/ml) as well. The poor aqueous solubility of myricetin restricts its potential applications in food, pharmaceutical and cosmetic industry. New encapsulation and formulation methods are being developed to solve this problem. One potential solution is complexation with cyclodextrins. By developing a complex of myricetin/hydroxypropyl-β-cyclodextrin, the oral relative bioavailability of the myricetin/HP-β-CD inclusion complex in rats is 940% over free myricetin [303]. Another study confirmed that solid dispersions of myricetin enhanced aqueous solubility by 47-fold comparing with non-treated myricetin [304].

Moreover, flavonoids in nanocarriers are also promoted in bioavailability. Encapsulation method like nanoparticles can significantly increase compound surface area, which results in higher saturation solubility and dissolution velocity [305]. Nanoparticles are defined as particles with a size less than 1000 nm[306]. A variety of materials can be used to prepare nanoparticles including polysaccharides, proteins, and synthetic polymers. Bulk materials may exhibit new physical, chemical, and biological features after greatly decreasing the particle size. Nanoparticles are designed and prepared to achieve certain functions by controlling the size, surface property, and release profile. Drugs and bioactive substances encapsulated with nanoparticles have been increase their dispersibility and bioavailability [307]. Recent researches indicated that polymer-based nanocarriers, lipid-based nanocarriers, polymeric nanoparticles, polymeric micelles all contribute to flavonoids delivery in food processing and pharmaceutical applications [308]. These approaches of increased aqueous solubility of myricetin potentially broaden its application in food science and drug discovery.

One of the most studied plant proteins, zein is produced in large quantities as a co-product of corn wet milling. Zein is a combination of at least four proteins: a-, b-, g-, and d-zein, which differ from one another in molecular weight, solubility, and amino acid sequence[309, 310]. The water-

insoluble but alcohol-soluble characteristic of zein makes it suitable for the development of nanoparticles[311, 312]. Zein is classified as GRAS (Generally Recognized As Safe) by the USDA, which suggests its potential in the application of developing food-grade carrier system.

In our preliminary data for future experiments, zein was tested for its potential in improving the solubility of myricetin. Nanoparticles were prepared by encapsulating myricetin with zein using established procedures [313] with modifications and to be characterized in terms of particle size, zeta-potential, and morphology. We successfully prepared myricetin/zein particles and compared the particle sizes of different myricetin/zein ratio (Figure 4.1). Bacteria growth inhibition test will be performed next to evaluate the influence of zein nanoparticles on the antibacterial activity of myricetin. Altogether, this work provides new possibility for future improvement on myricetin delivery.

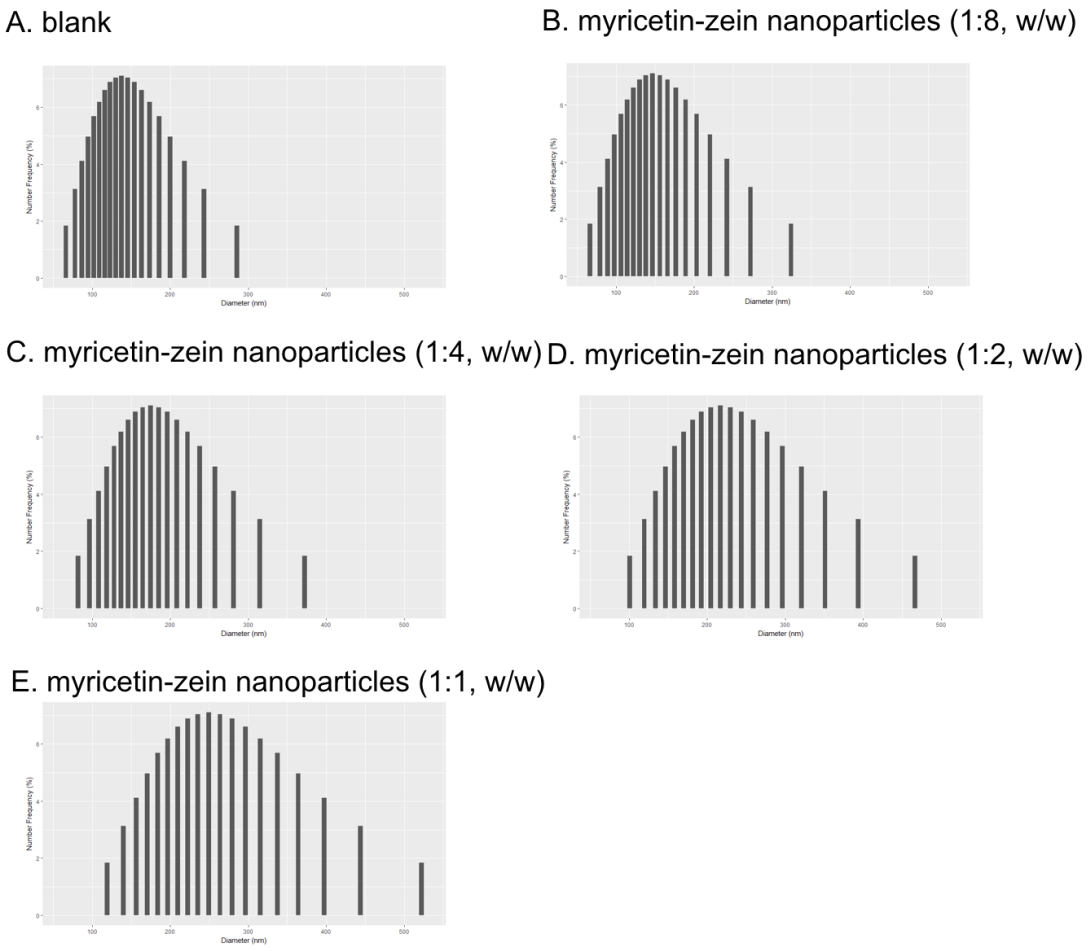

Figure 5. 1 Particle size distribution of myricetin-zein nanoparticles at various molecular ratios.

Myricetin and zein (10 mg) were dissolved in 10 mL 70% (v/v) ethanol solution at different mass ratios (1:8, 1:4, 1:2, 1:1 w/w). No myricetin was added for blank zein nanoparticles. Stock solutions were then sheared into 30 mL water under vigorous stirring. Powdered-form nanoparticles were prepared by freeze-drying the dispersions and analyzed by particle size analyzer.

Appendix A. Probiotic encapsulation with gel system

Probiotics are generally considered as the beneficiary group of bacteria within human bodies. Studies have shown that the variety of probiotics may boost the host immune system, improve lactose intolerance and even serve as potential therapeutic solutions for anxiety and depression [314]. Research focuses on the exploitation of probiotics include strain selection, pathogenicity evaluation and development of probiotic delivery approaches. Specifically, the efficiencies and safety of probiotics will directly dictate the selection spectrum and utilization efficiency of such beneficiary strains once in the host gut microbiome. Therefore, the search for a tolerant delivery system for probiotics is of utmost interest in the field of food science. In this section, two established drug delivery systems, konjac glucomannan (KGM)-Gelatin and KGM-Xanthan systems, were built to examine their function on maintaining probiotic viability and potentials to be utilized as designated probiotics packaging systems.

To prepare probiotic cells for proper microencapsulation, the well characterized strain *L. ramnosus* GG was used. Specifically, *L. ramnosus* GG was grown for 24h at 37 ˚C in MRS broth and harvested at a density of 10^9 cfu/mL by centrifugation at 5000g for 5min at room temperature. The cell were then washed in 1x PBS before centrifugation again at 5000rpm for 5min at room temperature. The washed cells were re-suspended in a total of 10 ml PBS and stored at 4 ˚C until usage. Cells were enumerated by plating on MRS agar and incubated at 37C for 24-48h.

Next, the prepared cells were subjected to encapsulation by either KGM-Gelatin or KGM-Xanthan system. For KGM-Gelatin preparation, gelatin solution (2% w/v) and KGM solution (1% w/v) were prepared and autoclaved. 0.5mL of genipin solution (100mg/mL) was added to the mixed KGM-Gelatin solution as a gelation agent. The gelation reaction was let proceed under 37 ˚C. The bacterial cell pellet was added to the finalized solution and re-suspended in 2 hours before it turned into gel. The blue gel was cooled down and stored under 4 ˚C for further use. For

KGM-Xanthan system, KGM and Xanthan gum powder were mixed at a ratio of 3:7 to achieve the maximum gel strength. A solution of 1% total polysaccharide was obtained by dissolving the powder mixture in the DI water. The bacterial cell pellet was added to the KGM-Xanthan solution at 45 ˚C before the solution turned into gel. The gel was cooled immediately and stored under 4 ˚C for further use.

The viability of encapsulated bacteria was then tested for both of the two encapsulation systems at day 1, 7 and 14. We observed that for the KGM-Gelatin system, no colony was formed on MRS agar even on day 1 test (data not shown). This result indicated that genipin may make an adverse environment for cell growth, possibly through interacting with the protein on the cell membrane. Thus, the application of KGM-Gelatin system in preserving and delivering probiotics were undermined under the investigated condition.

On the other hand, the KGM-Xanthan system has shown remarkable potentials for LGG packaging and delivery. Bacterial enumeration results demonstrated that the LGG cells were able to maintain viability for at least two weeks after encapsulation (Figure A.1A). Microscopic examinations also confirmed a physiological morphology of LGG cells at 14 days post encapsulation (Figure A.1B). Altogether, our results disproved the KGM-Gelatin as a viable LGG delivery system but instead highlighted the potentials of the KGM-Xanthan system. For future studies, it is strongly recommended the physical and chemical properties of the probiotic encapsulating gel matrix be thoroughly examined. Applying the studies to other probiotic bacterial strains will expand our knowledge of the universality of this system. Moreover, it would be ultimately valuable to investigate the viability and biological activities of such encapsulated probiotics in conditions mimicking the gastrointestinal environment, before expanding to animal studies.

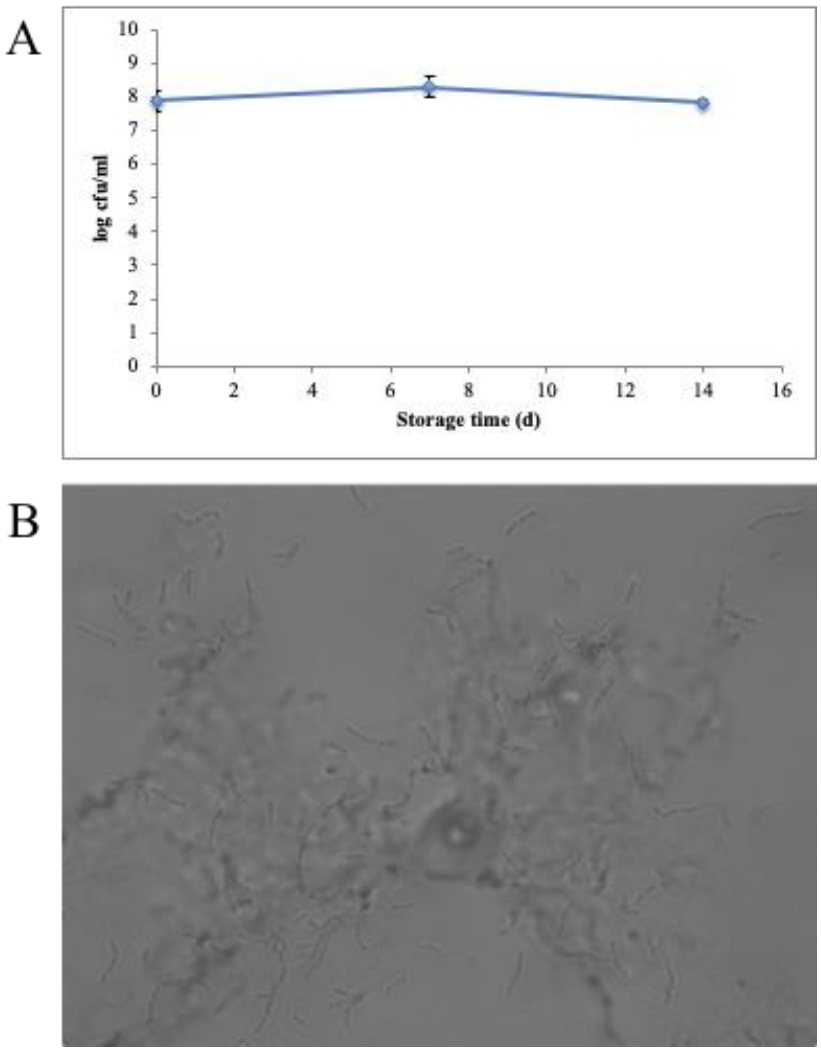

Figure A. 1 Viability of probiotic bacteria *L. ramnosus* GG encapsulated with KGM-Xanthan gel.

(**A**) Encapsulated bacteria were released in saline solution (0.9%) by vortexing. The samples then were diluted to appropriate concentrations and pour plated in MRS agar. The plates were incubated for 24-48 hours at 37 ˚C and the encapsulated bacteria enumerated as cfu ml^{-1} and plotted as a function of time. (**B**) LGG cells were observed directly under the microscope at day 14 post encapsulation for morphology examinations.

Appendix B. Antibacterial activities of essential oils

Essential oils are hydrophobic liquid extracted from aromatic herbs and spices, containing various volatile chemical compounds. Since these aromatic plants have been used as food preservatives and herbal medicine, essential oils are getting attention for their potential application in shelf-life extension and anti-infective medication. In this study, we tested the antibacterial activities of rosemary essential oil (REO) and cinnamon essential oil (CEO) against several common foodborne pathogens and probiotics.

We first tested the antibacterial activity of CEO on six bacteria strains, including *Staphylococcus aureus (S. aureus), Salmonella typhimurium (S. typhimurium), Listeria monocytogenes (L. monocytogenes), Escherichia coli (E. coli)* as the most common foodborne pathogens as well as two *Lactobacillus* probiotics: *Lactobacillus rhamnosus* (*L. rhamnosus*) and *Lactobacillus casei (L. casei).* Six bacteria strains were grown to log phase and incubated with different concentrations of CEO, and growth was measured by OD_{595} through a time course of 24 hours. Media with no CEO added serves as control, in which condition all six bacteria were able to grow (Figure B.1). Significant growth inhibition was observed for all bacteria upon treatment with CEO at 0.313 mg/ml, while visible growth was completely blocked for *S. aureus, S. typhimurium, L. monocytogenes, E. coli* and *L. casei* when CEO concentration reaches 0.625 mg/ml. *L. rhamnosus* demonstrates slightly higher resistance to CEO, wherein its minimum inhibition concentration is at 1.25 mg/ml (Figure B.1).

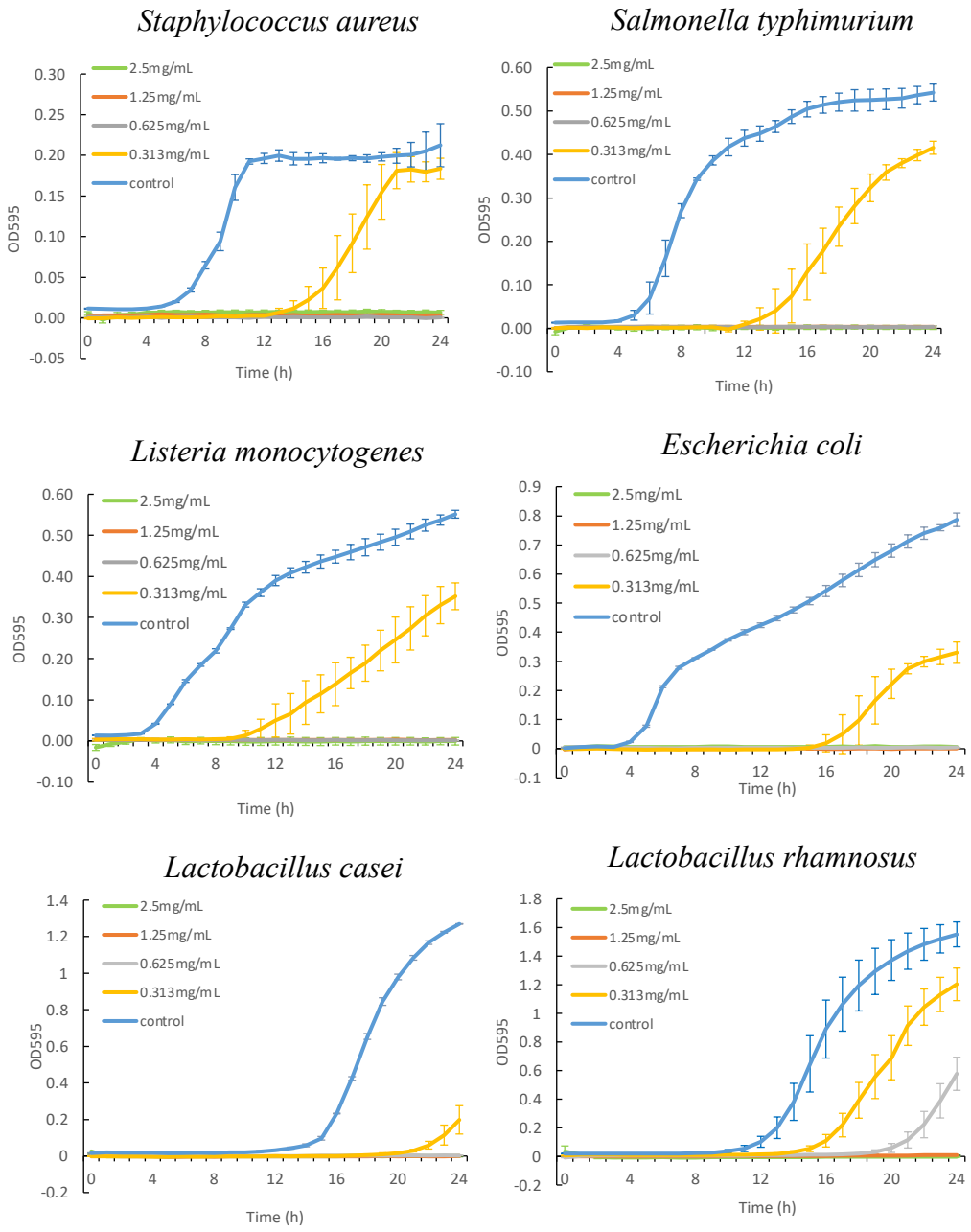

Figure B. 1 Antibacterial activity of cinnamon essential oil.

Staphylococcus aureus, *Salmonella typhimurium*, *Listeria monocytogenes*, and *Escherichia coli* were grown in TSB media, while *Lactobacillus casei* and *Lactobacillus rhamnosus* were grown in MRS media for overnight and cells were re-inoculated into fresh media to a concentration of 10^5 CFU/ml. Growth curves of each bacteria under the treatment of cinnamon essential oil at various concentrations were monitored over the course of 24 hours by OD_{595}.

We went onto examine the antibacterial activity of REO on *E. coli, L. rhamnosus* and *L. casei.* For two *Lactobacillus,* we observed a dose dependent inhibition of bacterial growth supplemented with REO starting from 0.313 mg/ml for *L. casei* and 0.625 mg/ml for *L. rhamnosus.* Interestingly, REO is not effective while inhibiting the growth of *E. coli* (Figure B.2). In addition, the highest concentration of REO tested here (2.5 mg/ml) did not completely block the growth of any of the three bacteria, demonstrating a weaker antibacterial function compared to CEO.

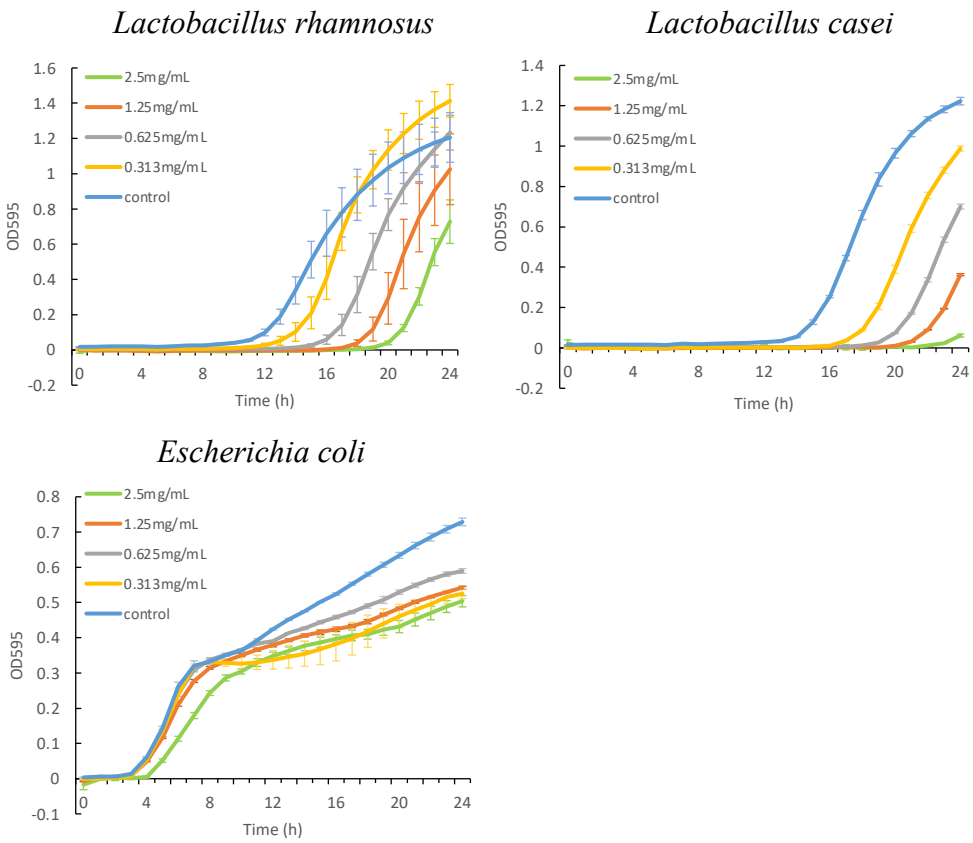

Figure B. 2 Antibacterial activity of rosemary essential oil.

Escherichia coli was grown in TSB media, while *Lactobacillus casei* and *Lactobacillus rhamnosus* were grown in MRS media for overnight and cells were re-inoculated into fresh media to a concentration of 10^5 CFU/ml. Growth curves of each bacteria under the treatment of rosemary essential oil at various concentrations was monitored over the course of 24 hours by OD_{595}.

Overall, we observed an effective antibacterial activity of CEO, which inhibits a wide spectrum of bacteria including both Gram-positive (*S. aureus, L. monocytogenes, L. rhamnosus* and *L. casei*) and Gram-negative (*S. typhimurium* and *E. coli*) bacteria. REO has less efficient antibacterial functions but provides dose dependent inhibition on *L. rhamnosus* and *L. casei.* Interestingly, REO does not inhibit *E. coli* at the tested concentrations. One explanation could be that the existence of outer membrane in Gram-negative bacteria provides extra protection, thus rendering the bacteria less susceptible. This is in agreement with the discovery that essential oil targets the cytoplasmic membrane for disruption as its mode of action [315]. Examinations on additional Gram-negative bacteria strains could provide more insights on this possible hypothesis. In summary, we evaluated two essential oils, CEO and REO, for their antibacterial activities. We found that CEO is more effective than REO on all bacterial strains tested, spanning both Gram-positive and Gram-negative bacteria. However, both essential oils inhibit two *Lactobacillus* probiotics as effectively as they inhibit foodborne pathogens, indicating that such broad-spectrum antibacterial reagents should be used depending on occasions and with caution. Additional investigation should focus on effective constituents of the antibacterial essential oils and their detailed mode of actions.

Reference

1. Joardder, M.U. and M.H. Masud, *A brief history of food preservation*, in *Food Preservation in Developing Countries: Challenges and Solutions*. 2019, Springer. p. 57-66.
2. Ohlsson, T. and N. Bengtsson, *Minimal processing technologies in the food industries*. 2002: Elsevier.
3. Tauxe, R.V., *Emerging foodborne diseases: an evolving public health challenge.* Emerging infectious diseases, 1997. **3**(4): p. 425.
4. Endtz, H.P., et al., *Quinolone resistance in Campylobacter isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine.* Journal of Antimicrobial Chemotherapy, 1991. **27**(2): p. 199-208.
5. Anand, S. and N. Sati, *Artificial preservatives and their harmful effects: looking toward nature for safer alternatives.* International journal of pharmaceutical sciences and research, 2013. **4**(7): p. 2496.
6. Tajkarimi, M.M., S.A. Ibrahim, and D.O. Cliver, *Antimicrobial herb and spice compounds in food.* Food Control, 2010. **21**(9): p. 1199-1218.
7. Hartmann, M., et al., *Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy.* Antimicrobial agents and chemotherapy, 2010. **54**(8): p. 3132-3142.
8. Mohammed, M., *Phage typing or CRISPR typing for epidemiological surveillance of Salmonella Typhimurium?* BMC research notes, 2017. **10**(1): p. 578.
9. DuPont, H.L., *The growing threat of foodborne bacterial enteropathogens of animal origin.* Clinical infectious diseases, 2007. **45**(10): p. 1353-1361.
10. Griffin, P., *Escherichia coli O157: H7 and other enterohemorrhagic Escherichia coli.* Infections of the gastrointestinal tract, 1995: p. 739-761.
11. Dramsi, S. and P. Cossart, *Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite.* The Journal of cell biology, 2002. **156**(6): p. 943-946.
12. Marroquín-Cardona, A., et al., *Mycotoxins in a changing global environment–a review.* Food and Chemical Toxicology, 2014. **69**: p. 220-230.
13. Lee, S.C., et al., *Analysis of a food-borne fungal pathogen outbreak: virulence and genome of a Mucor circinelloides isolate from yogurt.* MBio, 2014. **5**(4).
14. Tomsikova, A., *Risk of fungal infection from foods, particularly in immunocompromised patients.* Epidemiologie, mikrobiologie, imunologie: casopis Spolecnosti pro epidemiologii a mikrobiologii Ceske lekarske spolecnosti JE Purkyne, 2002. **51**(2): p. 78-81.
15. Brenier-Pinchart, M., et al., *Ten-year surveillance of fungal contamination of food within a protected haematological unit.* Mycoses, 2006. **49**(5): p. 421-425.
16. Pitt, J.I. and A.D. Hocking, *Fungi and food spoilage*. Vol. 519. 2009: Springer.
17. Kapoor, G., S. Saigal, and A. Elongavan, *Action and resistance mechanisms of antibiotics: A guide for clinicians.* Journal of anaesthesiology, clinical pharmacology, 2017. **33**(3): p. 300.
18. Liu, Y. and E. Breukink, *The membrane steps of bacterial cell wall synthesis as antibiotic targets.* Antibiotics, 2016. **5**(3): p. 28.
19. Binda, E., F. Marinelli, and G.L. Marcone, *Old and new glycopeptide antibiotics: action and resistance.* Antibiotics, 2014. **3**(4): p. 572-594.
20. Mingeot-Leclercq, M.-P., Y. Glupczynski, and P.M. Tulkens, *Aminoglycosides: activity and resistance.* Antimicrobial agents and chemotherapy, 1999. **43**(4): p. 727-737.
21. Grossman, T.H., *Tetracycline antibiotics and resistance.* Cold Spring Harbor Perspectives in Medicine, 2016. **6**(4): p. a025387.

22. Bozdogan, B. and P.C. Appelbaum, *Oxazolidinones: activity, mode of action, and mechanism of resistance*. International journal of antimicrobial agents, 2004. **23**(2): p. 113-119.
23. Bermingham, A. and J.P. Derrick, *The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery.* Bioessays, 2002. **24**(7): p. 637-648.
24. Yoneyama, H. and R. Katsumata, *Antibiotic resistance in bacteria and its future for novel antibiotic development.* Bioscience, biotechnology, and biochemistry, 2006. **70**(5): p. 1060-1075.
25. Pankey, G.A. and L. Sabath, *Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections.* Clinical infectious diseases, 2004. **38**(6): p. 864-870.
26. Tato, M., et al., *Characterization of variables that may influence ozenoxacin in susceptibility testing, including MIC and MBC values.* Diagnostic microbiology and infectious disease, 2014. **78**(3): p. 263-267.
27. Kohanski, M.A., et al., *A common mechanism of cellular death induced by bactericidal antibiotics.* Cell, 2007. **130**(5): p. 797-810.
28. Poehlsgaard, J. and S. Douthwaite, *The bacterial ribosome as a target for antibiotics.* Nature Reviews Microbiology, 2005. **3**(11): p. 870-881.
29. Walsh, C., *Molecular mechanisms that confer antibacterial drug resistance.* Nature, 2000. **406**(6797): p. 775-781.
30. Lewis, T. and J. Cook, *Fluoroquinolones and tendinopathy: a guide for athletes and sports clinicians and a systematic review of the literature.* Journal of athletic training, 2014. **49**(3): p. 422-427.
31. Berendsen, B., et al., *Evidence of natural occurrence of the banned antibiotic chloramphenicol in herbs and grass.* Analytical and Bioanalytical Chemistry, 2010. **397**(5): p. 1955-1963.
32. Long, K.S., et al., *The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics.* Antimicrobial agents and chemotherapy, 2006. **50**(7): p. 2500-2505.
33. Gill, E.E., O.L. Franco, and R.E. Hancock, *Antibiotic adjuvants: diverse strategies for controlling drug-resistant pathogens.* Chemical biology & drug design, 2015. **85**(1): p. 56-78.
34. Tiwari, B.K., et al., *Application of natural antimicrobials for food preservation.* J Agric Food Chem, 2009. **57**(14): p. 5987-6000.
35. Leleu, S., et al., *Effects on Salmonella shell contamination and trans-shell penetration of coating hens' eggs with chitosan.* Int J Food Microbiol, 2011. **145**(1): p. 43-8.
36. Juneja, V.K., H.P. Dwivedi, and X. Yan, *Novel natural food antimicrobials.* Annu Rev Food Sci Technol, 2012. **3**: p. 381-403.
37. Demirel, Z., et al., *Antimicrobial and antioxidant activity of brown algae from the Aegean sea.* Journal of the Serbian Chemical Society, 2009. **74**(6): p. 619-628.
38. Willis, W.L., O.S. Isikhuemhen, and S.A. Ibrahim, *Performance assessment of broiler chickens given mushroom extract alone or in combination with probiotics.* Poult Sci, 2007. **86**(9): p. 1856-60.
39. Kalyoncu, F., et al., *Antimicrobial and antioxidant activities of mycelia of 10 wild mushroom species.* J Med Food, 2010. **13**(2): p. 415-9.
40. Negi, P.S., *Plant extracts for the control of bacterial growth: efficacy, stability and safety issues for food application.* Int J Food Microbiol, 2012. **156**(1): p. 7-17.
41. Balasundram, N., K. Sundram, and S. Samman, *Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses.* Food Chemistry, 2006. **99**(1): p. 191-203.

42. Engels, C., et al., *Antimicrobial activity of gallotannins isolated from mango (Mangifera indica L.) kernels.* J Agric Food Chem, 2009. **57**(17): p. 7712-8.
43. Ayala-Zavala, J.F., et al., *Agro-industrial potential of exotic fruit byproducts as a source of food additives.* Food Research International, 2011. **44**(7): p. 1866-1874.
44. Gorinstein, S., et al., *Comparison of some biochemical characteristics of different citrus fruits.* Food Chemistry, 2001. **74**(3): p. 309-315.
45. Gyawali, R. and S.A. Ibrahim, *Natural products as antimicrobial agents.* Food Control, 2014. **46**: p. 412-429.
46. Savoia, D., *Plant-derived antimicrobial compounds: alternatives to antibiotics.* Future Microbiology, 2012. **7**(8): p. 979-990.
47. Xue, J., P.M. Davidson, and Q. Zhong, *Thymol nanoemulsified by whey protein-maltodextrin conjugates: the enhanced emulsifying capacity and antilisterial properties in milk by propylene glycol.* J Agric Food Chem, 2013. **61**(51): p. 12720-6.
48. Ultee, A., M. Bennik, and R. Moezelaar, *The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus.* Applied and environmental microbiology, 2002. **68**(4): p. 1561-1568.
49. Farag, R., et al., *Antimicrobial activity of some Egyptian spice essential oils.* Journal of Food Protection®, 1989. **52**(9): p. 665-667.
50. Dorman, H. and S. Deans, *Antimicrobial agents from plants: antibacterial activity of plant volatile oils.* Journal of applied microbiology, 2000. **88**(2): p. 308-316.
51. Alcaraz, L., et al., *Antibacterial activity of flavonoids against methicillin-resistant Staphylococcus aureus strains.* Journal of Theoretical Biology, 2000. **205**(2): p. 231-240.
52. Chusnie, T. and A. Lamb, *Antimicrobial activity of flavonoid.* Int. J. Antimicrob. Agent, 2005. **26**: p. 343-356.
53. Havsteen, B., *Flavonoids, a class of natural products of high pharmacological potency.* Biochemical pharmacology, 1983. **32**(7): p. 1141-1148.
54. Górniak, I., R. Bartoszewski, and J. Króliczewski, *Comprehensive review of antimicrobial activities of plant flavonoids.* Phytochemistry Reviews, 2019. **18**(1): p. 241-272.
55. Harborne, J.B. and C.A. Williams, *Advances in flavonoid research since 1992.* Phytochemistry, 2000. **55**(6): p. 481-504.
56. Anderson, E.R., et al., *Multiple Plantago species (Plantaginaceae) modify floral reflectance and color in response to thermal change.* American Journal of Botany, 2013. **100**(12): p. 2485-2493.
57. Hatier, J. and K. Gould, *Anthocyanins: biosynthesis, functions, and applications.* Anthocyanin function in vegetative organs. Editors: Gould, K., Davies, K., Winefield, C.(1), 2009: p. 1-19.
58. Groot, H.d. and U. Rauen, *Tissue injury by reactive oxygen species and the protective effects of flavonoids.* Fundamental & clinical pharmacology, 1998. **12**(3): p. 249-255.
59. Ginwala, R., et al., *Potential role of flavonoids in treating chronic inflammatory diseases with a special focus on the anti-inflammatory activity of apigenin.* Antioxidants, 2019. **8**(2): p. 35.
60. Chahar, M.K., et al., *Flavonoids: A versatile source of anticancer drugs.* Pharmacognosy reviews, 2011. **5**(9): p. 1.
61. Kaul, T., *Middleton E Jr, Ogra PL.* Antiviral effect of flavonoids on human viruses. J Med Virol, 1985. **15**: p. 71-79.
62. Bitencourt, T.A., et al. *Antifungal activity of flavonoids and modulation of expression of genes of fatty acid synthesis in the dermatophyte Trichophyton rubrum*. in *BMC Proceedings*. 2014. BioMed Central.
63. Xie, Y., et al., *Antibacterial activities of flavonoids: structure-activity relationship and mechanism.* Current medicinal chemistry, 2015. **22**(1): p. 132-149.

64. Tsuchiya, H., et al., *Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant Staphylococcus aureus.* Journal of ethnopharmacology, 1996. **50**(1): p. 27-34.
65. Suksamrarn, A., et al., *Antimycobacterial activity and cytotoxicity of flavonoids from the flowers ofChromolaena odorata.* Archives of Pharmacal Research, 2004. **27**(5): p. 507-511.
66. Zhao, X., et al., *Antibacterial activity of the flavonoids from Dalbergia odorifera on Ralstonia solanacearum.* Molecules, 2011. **16**(12): p. 9775-9782.
67. Yao, X., et al., *Antimicrobial activity of nobiletin and tangeretin against Pseudomonas.* Food Chemistry, 2012. **132**(4): p. 1883-1890.
68. Salvatore, M.J., et al., *Antibacterial activity of Lonchocarpol A.* Journal of Natural Products, 1998. **61**(5): p. 640-642.
69. Wächter, G.A., et al., *Antibacterial and antifungal flavanones from Eysenhardtia texana.* Phytochemistry, 1999. **52**(8): p. 1469-1471.
70. Nanayakkara, N.D., C.L. Burandt Jr, and M.R. Jacob, *Flavonoids with activity against methicillin-resistant Staphylococcus aureus from Dalea scandens var. paucifolia.* Planta medica, 2002. **68**(06): p. 519-522.
71. Sohn, H.-Y., et al., *Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: Morus alba L., Morus mongolica Schneider, Broussnetia papyrifera (L.) Vent, Sophora flavescens Ait and Echinosophora koreensis Nakai.* Phytomedicine, 2004. **11**(7-8): p. 666-672.
72. Yin, S., et al., *Antibacterial prenylflavone derivatives from Psoralea corylifolia, and their structure–activity relationship study.* Bioorganic & medicinal chemistry, 2004. **12**(16): p. 4387-4392.
73. Babu, K.S., et al., *Synthesis and in vitro study of novel 7-O-acyl derivatives of Oroxylin A as antibacterial agents.* Bioorganic & medicinal chemistry letters, 2005. **15**(17): p. 3953-3956.
74. Babu, K.S., et al., *Synthesis and biological evaluation of novel C (7) modified chrysin analogues as antibacterial agents.* Bioorganic & medicinal chemistry letters, 2006. **16**(1): p. 221-224.
75. Li, H.-Q., et al., *Synthesis of C (7) modified chrysin derivatives designing to inhibit β-ketoacyl-acyl carrier protein synthase III (FabH) as antibiotics.* Bioorganic & medicinal chemistry, 2009. **17**(17): p. 6264-6269.
76. Kuroyanagi, M., et al., *Antibacterial and Antiandrogen Flavonoids from Sophora f lavescens.* Journal of natural products, 1999. **62**(12): p. 1595-1599.
77. Dueñas, M., et al., *Antioxidant evaluation of O-methylated metabolites of catechin, epicatechin and quercetin.* Journal of pharmaceutical and biomedical analysis, 2010. **51**(2): p. 443-449.
78. Nabavi, S.F., et al., *Role of quercetin as an alternative for obesity treatment: you are what you eat!* Food Chemistry, 2015. **179**: p. 305-310.
79. Rattanachaikunsopon, P. and P. Phumkhachorn, *Contents and antibacterial activity of flavonoids extracted from leaves of Psidium guajava.* Journal of Medicinal Plants Research, 2010. **4**(5): p. 393-396.
80. Kleemann, R., et al., *Anti-inflammatory, anti-proliferative and anti-atherosclerotic effects of quercetin in human in vitro and in vivo models.* Atherosclerosis, 2011. **218**(1): p. 44-52.
81. Fang, D., et al., *Chemopreventive mechanisms of galangin against hepatocellular carcinoma: A review.* Biomedicine & Pharmacotherapy, 2019. **109**: p. 2054-2061.
82. Semwal, D.K., et al., *Myricetin: A dietary molecule with diverse biological activities.* Nutrients, 2016. **8**(2): p. 90.

83. M Calderon-Montano, J., et al., *A review on the dietary flavonoid kaempferol.* Mini reviews in medicinal chemistry, 2011. **11**(4): p. 298-344.
84. Sasaki, H., et al., *Prenylated flavonoids from Desmodium caudatum and evaluation of their anti-MRSA activity.* Phytochemistry, 2012. **82**: p. 136-142.
85. Zhang, Y., et al., *Antioxidation properties and mechanism of action of dihydromyricetin from Ampelopsis grossedentata.* Yao xue xue bao= Acta pharmaceutica Sinica, 2003. **38**(4): p. 241-244.
86. Du, Q., et al., *Purification of (+)-dihydromyricetin from leaves extract of Ampelopsis grossedentata using high-speed countercurrent chromatograph with scale-up triple columns.* Journal of Chromatography A, 2002. **973**(1-2): p. 217-220.
87. Sunil, C. and B. Xu, *An insight into the health-promoting effects of taxifolin (dihydroquercetin).* Phytochemistry, 2019. **166**: p. 112066.
88. Yamada, H., *Natural products of commercial potential as medicines.* Current Opinion in Biotechnology, 1991. **2**(2): p. 203-210.
89. Balde, A., et al., *Plant antiviral agents. VII. Antiviral and antibacterial proanthocyanidins from the bark of Pavetta owariensis.* Phytotherapy research, 1990. **4**(5): p. 182-188.
90. Gupta, A., et al., *Inhibition of adherence of multi-drug resistant E. coli by proanthocyanidin.* Urological research, 2012. **40**(2): p. 143-150.
91. Al-Habib, A., et al., *Bactericidal effect of grape seed extract on methicillin-resistant Staphylococcus aureus (MRSA).* The Journal of toxicological sciences, 2010. **35**(3): p. 357-364.
92. Mayer, R., et al., *Proanthocyanidins: target compounds as antibacterial agents.* Journal of agricultural and food Chemistry, 2008. **56**(16): p. 6959-6966.
93. Idowu, T.O., et al., *Doubly linked, A-type proanthocyanidin trimer and other constituents of Ixora coccinea leaves and their antioxidant and antibacterial properties.* Phytochemistry, 2010. **71**(17-18): p. 2092-2098.
94. Bandgar, B.P., et al., *Synthesis and biological evaluation of a novel series of 2, 2-bisaminomethylated aurone analogues as anti-inflammatory and antimicrobial agents.* European journal of medicinal chemistry, 2010. **45**(7): p. 3223-3227.
95. Venkateswarlu, S., et al., *Synthesis, structural revision, and biological activities of 4'-chloroaurone, a metabolite of marine brown alga Spatoglossum variabile.* Tetrahedron, 2007. **63**(29): p. 6909-6914.
96. Ferreira, E.O., et al., *A new heptasubstituted (E)-aurone glucoside and other aromatic compounds of Gomphrena agrestis with biological activity*. Zeitschrift für Naturforschung C, 2004. **59**(7-8): p. 499-505.
97. Mahabusarakam, W., et al., *A benzil and isoflavone derivatives from Derris scandens Benth.* Phytochemistry, 2004. **65**(8): p. 1185-1191.
98. He, J., et al., *Antibacterial Compounds from Glycyrrhiza u ralensis.* Journal of natural products, 2006. **69**(1): p. 121-124.
99. Gupta, V.K., et al., *Antimicrobial potential of Glycyrrhiza glabra roots.* Journal of ethnopharmacology, 2008. **116**(2): p. 377-380.
100. Sato, M., et al., *Growth inhibition of oral bacteria related to denture stomatitis by anti-candidal chalcones.* Australian Dental Journal, 1997. **42**(5): p. 343-346.
101. Sufian, A.S., et al., *Isolation and identification of antibacterial and cytotoxic compounds from the leaves of Muntingia calabura L.* Journal of Ethnopharmacology, 2013. **146**(1): p. 198-204.
102. Tran, T.-D., et al., *Synthesis and anti Methicillin resistant Staphylococcus aureus activity of substituted chalcones alone and in combination with non-beta-lactam antibiotics.* Bioorganic & medicinal chemistry letters, 2012. **22**(14): p. 4555-4560.

103. Ávila, H.P., et al., *Structure–activity relationship of antibacterial chalcones.* Bioorganic & medicinal chemistry, 2008. **16**(22): p. 9790-9794.
104. Cushnie, T.T. and A.J. Lamb, *Detection of galangin-induced cytoplasmic membrane damage in Staphylococcus aureus by measuring potassium loss.* Journal of ethnopharmacology, 2005. **101**(1): p. 243-248.
105. Pietta, P.-G., *Flavonoids as antioxidants.* Journal of natural products, 2000. **63**(7): p. 1035-1042.
106. Rice-Evans, C., N. Miller, and G. Paganga, *Antioxidant properties of phenolic compounds.* Trends in plant science, 1997. **2**(4): p. 152-159.
107. Heim, K.E., A.R. Tagliaferro, and D.J. Bobilya, *Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships.* The Journal of nutritional biochemistry, 2002. **13**(10): p. 572-584.
108. Halliwell, B., J. Rafter, and A. Jenner, *Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not?* The American journal of clinical nutrition, 2005. **81**(1): p. 268S-276S.
109. Amic, D., et al., *SAR and QSAR of the antioxidant activity of flavonoids.* Current medicinal chemistry, 2007. **14**(7): p. 827-845.
110. Voskresensky, O.N. and A.P. Levitsky, *QSAR aspects of flavonoids as a plentiful source of new drugs.* Current medicinal chemistry, 2002. **9**(14): p. 1367-1383.
111. Zhang, H.-Y., *Structure-activity relationships and rational design strategies for radical-scavenging antioxidants.* Current Computer-Aided Drug Design, 2005. **1**(3): p. 257-273.
112. Benavente-García, O., et al., *Uses and properties of citrus flavonoids.* J Agric Food Chem, 1997. **45**(12): p. 4505-4515.
113. Huang, X., et al., *3D-QSAR model of flavonoids binding at benzodiazepine site in GABAA receptors.* Journal of medicinal chemistry, 2001. **44**(12): p. 1883-1891.
114. de Wet, H., et al., *Sequence requirements of the ATP-binding site within the C-terminal nucleotide-binding domain of mouse P-glycoprotein: Structure− activity relationships for flavonoid binding.* Biochemistry, 2001. **40**(34): p. 10382-10391.
115. Williams, R.J., J.P. Spencer, and C. Rice-Evans, *Flavonoids: antioxidants or signalling molecules?* Free radical biology and medicine, 2004. **36**(7): p. 838-849.
116. Wei, D.-G., et al., *Binding model construction of antifungal 2-aryl-4-chromanones using CoMFA, CoMSIA, and QSAR analyses.* J Agric Food Chem, 2005. **53**(5): p. 1604-1611.
117. Rice-Evans, C.A. and L. Packer, *Flavonoids in health and disease*. 2003: CRC Press.
118. Silva, M.M., et al., *Structure-antioxidant activity relationships of flavonoids: a re-examination.* Free radical research, 2002. **36**(11): p. 1219-1227.
119. Sichel, G., et al., *In vitro scavenger activity of some flavonoids and melanins against O2− dot.* Free radical biology and medicine, 1991. **11**(1): p. 1-8.
120. Chen, Z., et al., *Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups.* Chemistry and Physics of Lipids, 1996. **79**(2): p. 157-163.
121. Rice-Evans, C.A., N.J. Miller, and G. Paganga, *Structure-antioxidant activity relationships of flavonoids and phenolic acids.* Free radical biology and medicine, 1996. **20**(7): p. 933-956.
122. Cao, G., E. Sofic, and R.L. Prior, *Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships.* Free radical biology and medicine, 1997. **22**(5): p. 749-760.
123. Yokozawa, T., et al., *Study on the inhibitory effect of tannins and flavonoids against the 1, 1-diphenyl-2-picrylhydrazyl radical.* Biochemical pharmacology, 1998. **56**(2): p. 213-222.

124. McPhail, D.B., et al., *Kinetic and stoichiometric assessment of the antioxidant activity of flavonoids by electron spin resonance spectroscopy.* J Agric Food Chem, 2003. **51**(6): p. 1684-1690.
125. Woodman, O.L., W.F. Meeker, and M. Boujaoude, *Vasorelaxant and antioxidant activity of flavonols and flavones: structure-activity relationships.* Journal of cardiovascular pharmacology, 2005. **46**(3): p. 302-309.
126. Haenen, G.R., et al., *Structure and activity in assessing antioxidant activity in vitro and in vivo: A critical appraisal illustrated with the flavonoids.* Environmental toxicology and pharmacology, 2006. **21**(2): p. 191-198.
127. Bors, W., et al., *[36] Flavonoids as antioxidants: Determination of radical-scavenging efficiencies.* Methods in enzymology, 1990. **186**: p. 343-355.
128. Furusawa, M., et al., *Antioxidant activity of hydroxyflavonoids.* Journal of health science, 2005. **51**(3): p. 376-378.
129. Sroka, Z., *Antioxidative and antiradical properties of plant phenolics.* Zeitschrift für Naturforschung C, 2005. **60**(11-12): p. 833-843.
130. van Acker, S.A., A. Bast, and W.J. van der Vijgh, *Structural aspects of antioxidant activity of flavonoids.* 1998.
131. de Araújo, M.E.M.B., et al., *Enzymatic de-glycosylation of rutin improves its antioxidant and antiproliferative activities.* Food chemistry, 2013. **141**(1): p. 266-273.
132. Trouillas, P., et al., *A DFT study of the reactivity of OH groups in quercetin and taxifolin antioxidants: the specificity of the 3-OH site.* Food Chemistry, 2006. **97**(4): p. 679-688.
133. Middleton Jr, E. and C. Kandaswami, *The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer.* The flavonoids. London: Chapman and Hall, 1994.
134. Rietjens, I.M., *Structure-activity study on the quinone/quinone methide chemistry of flavonoids.* STUDIES ON THE PRO-OXIDANT CHEMISTRY OF FLAVONOIDS, 2001. **14**(4): p. 35.
135. Galati, G. and P.J. O'brien, *Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties.* Free radical biology and medicine, 2004. **37**(3): p. 287-303.
136. Galati, G., et al., *Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics.* Toxicology, 2002. **177**(1): p. 91-104.
137. Middleton, E., *The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer.* The flavonoids, advances in research since 1986, 1994: p. 619-645.
138. Murty, V.S. and T.M. Penning, *Polycyclic aromatic hydrocarbon (PAH) ortho-quinone conjugate chemistry: kinetics of thiol addition to PAH ortho-quinones and structures of thioether adducts of naphthalene-1, 2-dione.* Chemico-biological interactions, 1992. **84**(2): p. 169-188.
139. Ohshima, H., et al., *Antioxidant and pro-oxidant actions of flavonoids: effects on DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion.* Free radical biology and medicine, 1998. **25**(9): p. 1057-1065.
140. Yoshino, M., et al., *Prooxidant activity of flavonoids: copper-dependent strand breaks and the formation of 8-hydroxy-2'-deoxyguanosine in DNA.* Molecular Genetics and Metabolism, 1999. **68**(4): p. 468-472.
141. Van Acker, S.A., et al., *Structural aspects of antioxidant activity of flavonoids.* Free radical biology and medicine, 1996. **20**(3): p. 331-342.
142. Carlioz, A. and D. Touati, *Isolation of superoxide dismutase mutants in Escherichia coli: is superoxide dismutase necessary for aerobic life?* The EMBO journal, 1986. **5**(3): p. 623-630.

143. Chang, E., et al., *Genetic and biochemical characterization of Cu, Zn superoxide dismutase mutants in Saccharomyces cerevisiae.* Journal of Biological Chemistry, 1991. **266**(7): p. 4417-4424.
144. Van Loon, A., B. Pesold-Hurt, and G. Schatz, *A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen.* Proceedings of the National Academy of Sciences, 1986. **83**(11): p. 3820-3824.
145. González-Flecha, B. and B. Demple, *Metabolic sources of hydrogen peroxide in aerobically growing Escherichia coli.* Journal of Biological Chemistry, 1995. **270**(23): p. 13681-13687.
146. Imlay, J.A., *A metabolic enzyme that rapidly produces superoxide, fumarate reductase of Escherichia coli.* Journal of Biological Chemistry, 1995. **270**(34): p. 19767-19777.
147. Imlay, J.A. and I. Fridovich, *Assay of metabolic superoxide production in Escherichia coli.* Journal of Biological Chemistry, 1991. **266**(11): p. 6957-6965.
148. Messner, K.R. and J.A. Imlay, *The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of Escherichia coli.* Journal of Biological Chemistry, 1999. **274**(15): p. 10119-10128.
149. Messner, K.R. and J.A. Imlay, *Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase.* Journal of Biological Chemistry, 2002. **277**(45): p. 42563-42571.
150. Park, S., X. You, and J.A. Imlay, *Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx-mutants of Escherichia coli.* Proceedings of the National Academy of Sciences, 2005. **102**(26): p. 9317-9322.
151. Jang, S. and J.A. Imlay, *Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes.* Journal of Biological Chemistry, 2007. **282**(2): p. 929-937.
152. Kuo, C., T. Mashino, and I.α. Fridovich, *alpha, beta-Dihydroxyisovalerate dehydratase. A superoxide-sensitive enzyme.* Journal of Biological Chemistry, 1987. **262**(10): p. 4724-4727.
153. Flint, D.H., J. Tuminello, and M. Emptage, *The inactivation of Fe-S cluster containing hydro-lyases by superoxide.* Journal of Biological Chemistry, 1993. **268**(30): p. 22369-22376.
154. Farr, S.B., R. D'Ari, and D. Touati, *Oxygen-dependent mutagenesis in Escherichia coli lacking superoxide dismutase.* Proceedings of the National Academy of Sciences, 1986. **83**(21): p. 8268-8272.
155. Levin, D.E., et al., *A new Salmonella tester strain (TA102) with AXT base pairs at the site of mutation detects oxidative mutagens.* Proceedings of the National Academy of Sciences, 1982. **79**(23): p. 7445-7449.
156. Lynch, R.E. and I. Fridovich, *Permeation of the erythrocyte stroma by superoxide radical.* Journal of Biological Chemistry, 1978. **253**(13): p. 4697-4699.
157. Korshunov, S.S. and J.A. Imlay, *A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of Gram-negative bacteria.* Molecular microbiology, 2002. **43**(1): p. 95-106.
158. DS, P.D.Y., *Sarma GN. Wood ZA. Karplus PA. Poole LB. Analysis of the link between enzymatic activity and oligomeric state in AhpC, a bacterial peroxiredoxin.* Biochemistry, 2005. **44**: p. 10583-10592.
159. Falcone Ferreyra, M.L., S. Rius, and P. Casati, *Flavonoids: biosynthesis, biological functions, and biotechnological applications.* Frontiers in plant science, 2012. **3**: p. 222.
160. Hatier, J.-H.B. and K.S. Gould, *Anthocyanin function in vegetative organs*, in *Anthocyanins*. 2008, Springer. p. 1-19.

161. Anderson, M.E., *[70] Determination of glutathione and glutathione disulfide in biological samples*, in *Methods in enzymology*. 1985, Elsevier. p. 548-555.
162. Cushnie, T.T. and A.J. Lamb, *Antimicrobial activity of flavonoids.* International journal of antimicrobial agents, 2005. **26**(5): p. 343-356.
163. Cushnie, T., et al., *Aggregation of Staphylococcus aureus following treatment with the antibacterial flavonol galangin.* Journal of applied microbiology, 2007. **103**(5): p. 1562-1567.
164. Plaper, A., et al., *Characterization of quercetin binding site on DNA gyrase.* Biochemical and biophysical research communications, 2003. **306**(2): p. 530-536.
165. Li, B.-H., et al., *Inactivation mechanism of the β-ketoacyl-[acyl carrier protein] reductase of bacterial type-II fatty acid synthase by epigallocatechin gallate.* Biochemistry and cell biology, 2006. **84**(5): p. 755-762.
166. Sanver, D., et al., *Experimental modeling of flavonoid–biomembrane interactions.* Langmuir, 2016. **32**(49): p. 13234-13243.
167. Demetzos, C., et al., *Structure elucidation, conformational analysis and thermal effects on membrane bilayers of an antimicrobial myricetin ether derivative.* Journal of Heterocyclic Chemistry, 2001. **38**(3): p. 703-710.
168. Mori, A., et al., *Antibacterial activity and mode of plant flavonoids against Proteus vulgaris and Staphylococcus aureus.* Phytochem, 1987. **26**: p. 223-224.
169. Ohemeng, K., et al., *DNA gyrase inhibitory and antibacterial activity of some flavones (1).* Bioorganic & medicinal chemistry letters, 1993. **3**(2): p. 225-230.
170. Chinnam, N., et al., *Dietary bioflavonoids inhibit Escherichia coli ATP synthase in a differential manner.* International journal of biological macromolecules, 2010. **46**(5): p. 478-486.
171. Elmasri, W.A., et al., *Multitargeted flavonoid inhibition of the pathogenic bacterium Staphylococcus aureus: A Proteomic characterization.* Journal of Proteome Research, 2017. **16**(7): p. 2579-2586.
172. Firuzi, O., et al., *Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant power" assay and cyclic voltammetry.* Biochimica et Biophysica Acta (BBA)-General Subjects, 2005. **1721**(1-3): p. 174-184.
173. Amić, D., et al., *Structure-radical scavenging activity relationships of flavonoids.* Croatica chemica acta, 2003. **76**(1): p. 55-61.
174. Bors, W., et al., *[36] Flavonoids as antioxidants: determination of radical-scavenging efficiencies*, in *Methods in enzymology*. 1990, Elsevier. p. 343-355.
175. Asmi, K.S., et al., *Therapeutic aspects of taxifolin–An update.* Journal of Advanced Pharmacy Education & Research| Jul-Sep, 2017. **7**(3).
176. Kohri, S., et al., *An oxygen radical absorbance capacity-like assay that directly quantifies the antioxidant's scavenging capacity against AAPH-derived free radicals.* Analytical biochemistry, 2009. **386**(2): p. 167-171.
177. Chen, X.-M., A.R. Tait, and D.D. Kitts, *Flavonoid composition of orange peel and its association with antioxidant and anti-inflammatory activities.* Food Chemistry, 2017. **218**: p. 15-21.
178. Raffa, D., et al., *Recent discoveries of anticancer flavonoids.* European journal of medicinal chemistry, 2017. **142**: p. 213-228.
179. Spagnuolo, C., S. Moccia, and G.L. Russo, *Anti-inflammatory effects of flavonoids in neurodegenerative disorders.* European journal of medicinal chemistry, 2018. **153**: p. 105-115.
180. Gil, E.S. and R.O. Couto, *Flavonoid electrochemistry: a review on the electroanalytical applications.* Revista Brasileira de Farmacognosia, 2013. **23**(3): p. 542-558.

181. Ramírez-Flandes, S., B. González, and O. Ulloa, *Redox traits characterize the organization of global microbial communities.* Proceedings of the National Academy of Sciences, 2019. **116**(9): p. 3630-3635.
182. Simić, A., et al., *Electrochemical behavior and antioxidant and prooxidant activity of natural phenolics.* Molecules, 2007. **12**(10): p. 2327-2340.
183. Nijveldt, R.J., et al., *Flavonoids: a review of probable mechanisms of action and potential applications.* The American journal of clinical nutrition, 2001. **74**(4): p. 418-425.
184. Tsao, R., *Chemistry and biochemistry of dietary polyphenols.* Nutrients, 2010. **2**(12): p. 1231-1246.
185. Yang, W.-Y., et al., *Streptococcus mutans sortase A inhibitory metabolites from the flowers of Sophora japonica.* Bioorganic & medicinal chemistry letters, 2015. **25**(7): p. 1394-1397.
186. He, M., et al., *Antimicrobial mechanism of flavonoids against Escherichia coli ATCC 25922 by model membrane study.* Applied Surface Science, 2014. **305**: p. 515-521.
187. Chang, Q., et al., *Antibiotics in agriculture and the risk to human health: how worried should we be?* Evolutionary applications, 2015. **8**(3): p. 240-247.
188. Abraham, E.P., *Fleming's Discovery.* Reviews of Infectious Diseases, 1980. **2**(1): p. 140-140.
189. Croft, A.C., A.V. D'Antoni, and S.L. Terzulli, *Update on the antibacterial resistance crisis.* Medical science monitor, 2007. **13**(6): p. RA103-RA118.
190. Shah, P., *The need for new therapeutic agents: what is in the pipeline?* Clinical Microbiology and infection, 2005. **11**: p. 36-42.
191. Fan, L., et al., *Content determination of dihydromyricetin and myricetin in leaves of Ampelopsis grossedentata by UPLC and investigation of their thermal stability.* China Pharmacy, 2012. **23**(35): p. 3316-3319.
192. Zhang, J., et al., *Recent update on the pharmacological effects and mechanisms of dihydromyricetin.* Frontiers in pharmacology, 2018. **9**: p. 1204.
193. Chobot, V. and F. Hadacek, *Exploration of pro-oxidant and antioxidant activities of the flavonoid myricetin.* Redox Report, 2011. **16**(6): p. 242-247.
194. Xiao, X.-N., et al., *Antibacterial activity and mode of action of dihydromyricetin from ampelopsis grossedentata leaves against food-borne bacteria.* Molecules, 2019. **24**(15): p. 2831.
195. Åslund, F., et al., *Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol—disulfide status.* Proceedings of the National Academy of Sciences, 1999. **96**(11): p. 6161-6165.
196. Yeiser, B., et al., *SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness.* Proceedings of the National Academy of Sciences, 2002. **99**(13): p. 8737-8741.
197. Michán, C., et al., *In vivo transcription of the Escherichia coli oxyRregulon as a function of growth phase and in response to oxidative stress.* Journal of Bacteriology, 1999. **181**(9): p. 2759-2764.
198. Liu, Y. and J.A. Imlay, *Cell death from antibiotics without the involvement of reactive oxygen species.* Science, 2013. **339**(6124): p. 1210-1213.
199. Pourcel, L., et al., *Flavonoid oxidation in plants: from biochemical properties to physiological functions.* Trends in plant science, 2007. **12**(1): p. 29-36.
200. Yuan, X., et al., *Copper-catalyzed hydroquinone oxidation and associated redox cycling of copper under conditions typical of natural saline waters.* Environmental science & technology, 2013. **47**(15): p. 8355-8364.
201. Canada, A.T., et al., *The production of reactive oxygen species by dietary flavonols.* Free radical biology and medicine, 1990. **9**(5): p. 441-449.

202. Abrams, J.J. and D.A. Webster, *Purification, partial characterization, and possible role of catalase in the bacterium Vitreoscilla.* Archives of biochemistry and biophysics, 1990. **279**(1): p. 54-59.
203. Treutter, D., *Significance of flavonoids in plant resistance: a review.* Environmental Chemistry Letters, 2006. **4**(3): p. 147.
204. Mochizuki, M., et al., *Kinetic analysis and mechanistic aspects of autoxidation of catechins.* Biochimica et Biophysica Acta (BBA)-General Subjects, 2002. **1569**(1-3): p. 35-44.
205. Tatsumi, H., et al., *Mechanistic study of the autoxidation of reduced flavin and quinone compounds.* Journal of Electroanalytical Chemistry, 1998. **443**(2): p. 236-242.
206. Sang, S., et al., *Autoxidative quinone formation in vitro and metabolite formation in vivo from tea polyphenol (-)-epigallocatechin-3-gallate: studied by real-time mass spectrometry combined with tandem mass ion mapping.* Free Radical Biology and Medicine, 2007. **43**(3): p. 362-371.
207. Schweigert, N., A.J. Zehnder, and R.I. Eggen, *Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals: minireview.* Environmental microbiology, 2001. **3**(2): p. 81-91.
208. Imlay, J.A., *Cellular defenses against superoxide and hydrogen peroxide.* Annu. Rev. Biochem., 2008. **77**: p. 755-776.
209. Cornelis, P., et al., *Iron homeostasis and management of oxidative stress response in bacteria.* Metallomics, 2011. **3**(6): p. 540-549.
210. Zeth, K., *Dps biomineralizing proteins: multifunctional architects of nature.* Biochemical Journal, 2012. **445**(3): p. 297-311.
211. Pandey, A. and S. Kumar, *Perspective on plant products as antimicrobial agents: A review.* Pharmacologia, 2013. **4**(7): p. 469-480.
212. Cushnie, T.T. and A.J. Lamb, *Recent advances in understanding the antibacterial properties of flavonoids.* International journal of antimicrobial agents, 2011. **38**(2): p. 99-107.
213. Wu, T., et al., *A structure–activity relationship study of flavonoids as inhibitors of E. coli by membrane interaction effect.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2013. **1828**(11): p. 2751-2756.
214. Dzoyem, J.P., et al., *Antimicrobial action mechanism of flavonoids from Dorstenia species.* Drug discoveries & therapeutics, 2013. **7**(2): p. 66-72.
215. Cushnie, T.T. and A.J. Lamb, *Detection of galangin-induced cytoplasmic membrane damage in Staphylococcus aureus by measuring potassium loss.* Journal of ethnopharmacology, 2005. **101**(1-3): p. 243-248.
216. Fathima, A. and J.R. Rao, *Selective toxicity of Catechin—a natural flavonoid towards bacteria.* Applied microbiology and biotechnology, 2016. **100**(14): p. 6395-6402.
217. Cai, L. and C.D. Wu, *Compounds from Syzygium aromaticum possessing growth inhibitory activity against oral pathogens.* J Nat Prod, 1996. **59**(10): p. 987-90.
218. Lee, J.-H., et al., *Apple flavonoid phloretin inhibits Escherichia coli O157: H7 biofilm formation and ameliorates colon inflammation in rats.* Infection and immunity, 2011. **79**(12): p. 4819-4827.
219. Awolola, G.V., et al., *Antibacterial and anti-biofilm activity of flavonoids and triterpenes isolated from the extracts of Ficus sansibarica Warb. subsp. sansibarica (Moraceae) extracts.* African Journal of Traditional, Complementary and Alternative Medicines, 2014. **11**(3): p. 124-131.
220. Christena, L.R., et al., *Dual role of pinostrobin-a flavonoid nutraceutical as an efflux pump inhibitor and antibiofilm agent to mitigate food borne pathogens.* Rsc Advances, 2015. **5**(76): p. 61881-61887.

221. Mori, A., et al., *Antibacterial activity and mode of action of plant flavonoids against Proteus vulgaris and Staphylococcus aureus.* Phytochemistry, 1987. **26**(8): p. 2231-2234.
222. Selvaraj, S., et al., *Flavonoid–metal ion complexes: a novel class of therapeutic agents.* Medicinal Research Reviews, 2014. **34**(4): p. 677-702.
223. SR Husain, J Cillard, and P. Cillard, *Hydroxyl radical scavenging activity of flavonoids.* phytochemistry, 1987. **Volume 26**(Issue 9): p. Pages 2489-2491
.
224. Wang, Z.H., et al., *Myricetin suppresses oxidative stress-induced cell damage via both direct and indirect antioxidant action.* Environ Toxicol Pharmacol, 2010. **29**(1): p. 12-8.
225. Bennett, C.J., et al., *Potential therapeutic antioxidants that combine the radical scavenging ability of myricetin and the lipophilic chain of vitamin E to effectively inhibit microsomal lipid peroxidation.* Bioorg Med Chem, 2004. **12**(9): p. 2079-98.
226. Said Ahmad, M., et al., *Activities of flavonoids for the cleavage of DNA in the presence of Cu(II): correlation with generation of active oxygen species.* Carcinogenesis, 1992. **13**(4): p. 605-8.
227. Hodnick, W.F., et al., *Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. A structure-activity study.* Biochem Pharmacol, 1986. **35**(14): p. 2345-57.
228. Laughton, M.J., et al., *Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA.* Biochem Pharmacol, 1989. **38**(17): p. 2859-65.
229. Chobot, V. and F. Hadacek, *Exploration of pro-oxidant and antioxidant activities of the flavonoid myricetin.* Redox Rep, 2011. **16**(6): p. 242-7.
230. Canada, A.T., et al., *The production of reactive oxygen species by dietary flavonols.* Free Radic Biol Med, 1990. **9**(5): p. 441-9.
231. Franklin, S.J. and P.B. Myrdal, *Solid-State and Solution Characterization of Myricetin.* AAPS PharmSciTech, 2015. **16**(6): p. 1400-8.
232. Amitesh Anand, et al., *Adaptive evolution reveals a tradeoff between growth rate and oxidative stress during naphthoquinone-based aerobic respiration.* Proceedings of the National Academy of Sciences, 2019. **116**(50): p. 25287-25292.
233. N. Bartlett, P., *Bioenergetics and Biological Electron Transport*, in *Bioelectrochemistry*. John Wiley & Sons, Ltd. p. 1-37.
234. Holzerová, E. and H. Prokisch, *Mitochondria: much ado about nothing? How dangerous is reactive oxygen species production?* The international journal of biochemistry & cell biology, 2015. **63**: p. 16-20.
235. Huang, Z. and E. London, *Cholesterol lipids and cholesterol-containing lipid rafts in bacteria.* Chemistry and physics of lipids, 2016. **199**: p. 11-16.
236. Farmer, T., N. Naslavsky, and S. Caplan, *Tying trafficking to fusion and fission at the mighty mitochondria.* Traffic, 2018. **19**(8): p. 569-577.
237. Garcia, I., et al., *The little big genome: The organization of mitochondrial DNA.* Frontiers in bioscience (Landmark edition), 2017. **22**: p. 710.
238. Shadel, G.S. and T.L. Horvath, *Mitochondrial ROS signaling in organismal homeostasis.* Cell, 2015. **163**(3): p. 560-569.
239. Boguszewska, K., et al., *The Similarities between Human Mitochondria and Bacteria in the Context of Structure, Genome, and Base Excision Repair System.* Molecules, 2020. **25**(12): p. 2857.
240. R. Vinothkumar, K., Jiapeng Zhu, and Judy Hirst, *Architecture of mammalian respiratory complex I.* Nature, 2014. **515**(7525): p. 80-84.
241. Dawson, E.R., et al., *Stable retention of chloramphenicol-resistant mtDNA to rescue metabolically impaired cells.* Scientific reports, 2020. **10**(1): p. 1-14.

242. Thangapazham, R.L., et al., *Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells in vitro and in vivo.* Cancer letters, 2007. **245**(1-2): p. 232-241.
243. Tang, Y., et al., *Epigallocatechin-3 gallate induces growth inhibition and apoptosis in human breast cancer cells through survivin suppression.* International journal of oncology, 2007. **31**(4): p. 705-711.
244. Hagen, R.M., et al., *Epigallocatechin-3-gallate promotes apoptosis and expression of the caspase 9a splice variant in PC3 prostate cancer cells.* International journal of oncology, 2013. **43**(1): p. 194-200.
245. Shankar, S., G. Suthakar, and R.K. Srivastava, *Epigallocatechin-3-gallate inhibits cell cycle and induces apoptosis in pancreatic cancer.* Front Biosci, 2007. **12**(12): p. 5039-51.
246. Chalah, A. and R. Khosravi-Far, *The mitochondrial death pathway*, in *Programmed cell death in cancer progression and therapy*. 2008, Springer. p. 25-45.
247. Satoh, M., et al., *EGCG induces human mesothelioma cell death by inducing reactive oxygen species and autophagy.* Cancer cell international, 2013. **13**(1): p. 1-8.
248. Havsteen, B.H., *The biochemistry and medical significance of the flavonoids.* Pharmacology & therapeutics, 2002. **96**(2-3): p. 67-202.
249. Ooi, N., et al., *XF-73, a novel antistaphylococcal membrane-active agent with rapid bactericidal activity.* Journal of antimicrobial chemotherapy, 2009. **64**(4): p. 735-740.
250. Imlay, J.A., *Diagnosing oxidative stress in bacteria: not as easy as you might think.* Current opinion in microbiology, 2015. **24**: p. 124-131.
251. Van Acker, H. and T. Coenye, *The role of reactive oxygen species in antibiotic-mediated killing of bacteria.* Trends in microbiology, 2017. **25**(6): p. 456-466.
252. Cotelle, N., et al., *Scavenger and antioxidant properties of ten synthetic flavones.* Free Radic Biol Med, 1992. **13**(3): p. 211-9.
253. Hong, S., et al., *Pyrogallol 2-Aminoethane: A Plant Flavonoid-Inspired Molecule for Material-Independent Surface Chemistry.* Advanced Materials Interfaces, 2014. **1**(4).
254. Sanz Del Olmo, N., et al., *Antioxidant and Antibacterial Properties of Carbosilane Dendrimers Functionalized with Polyphenolic Moieties.* Pharmaceutics, 2020. **12**(8).
255. Vinh, P.T., et al., *Baicalein Inhibits Stx1 and 2 of EHE: Effects of Baicalein on the Cytotoxicity, Production, and Secretion of Shiga Toxins of Enterohaemorrhagic Escherichia coli.* Toxins (Basel), 2019. **11**(9).
256. Kaczmarek, B., *Tannic Acid with Antiviral and Antibacterial Activity as A Promising Component of Biomaterials-A Minireview.* Materials (Basel), 2020. **13**(14).
257. Pinheiro Silva, L., et al., *Terminalia catappa L.: a medicinal plant from the Caribbean pharmacopeia with anti-Helicobacter pylori and antiulcer action in experimental rodent models.* J Ethnopharmacol, 2015. **159**: p. 285-95.
258. Betts, J.W., et al., *Restoring the activity of the antibiotic aztreonam using the polyphenol epigallocatechin gallate (EGCG) against multidrug-resistant clinical isolates of Pseudomonas aeruginosa.* J Med Microbiol, 2019. **68**(10): p. 1552-1559.
259. Kitichalermkiat, A., et al., *Effect of epigallocatechin gallate on gene expression of Staphylococcus aureus.* J Glob Antimicrob Resist, 2020.
260. Friedman, M., et al., *Antimicrobial activities of tea catechins and theaflavins and tea extracts against Bacillus cereus.* J Food Prot, 2006. **69**(2): p. 354-61.
261. Pannala, A.S., et al., *Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics.* Biochemical and biophysical research communications, 2001. **282**(5): p. 1161-1168.
262. Tsimogiannis, D. and V. Oreopoulou, *Free radical scavenging and antioxidant activity of 5, 7, 3', 4'-hydroxy-substituted flavonoids.* Innovative Food Science & Emerging Technologies, 2004. **5**(4): p. 523-528.

263. Nasr, B., et al., *Electrochemical oxidation of hydroquinone, resorcinol, and catechol on boron-doped diamond anodes.* Environmental science & technology, 2005. **39**(18): p. 7234-7239.
264. Enache, T.A. and A.M. Oliveira-Brett, *Phenol and para-substituted phenols electrochemical oxidation pathways.* Journal of Electroanalytical Chemistry, 2011. **655**(1): p. 9-16.
265. Han, R.-M., et al., *Comparison of flavonoids and isoflavonoids as antioxidants.* J Agric Food Chem, 2009. **57**(9): p. 3780-3785.
266. Sakihama, Y., et al., *Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants.* Toxicology, 2002. **177**(1): p. 67-80.
267. Rødtjer, A., L.H. Skibsted, and M.L. Andersen, *Antioxidative and prooxidative effects of extracts made from cherry liqueur pomace.* Food Chemistry, 2006. **99**(1): p. 6-14.
268. Kumamoto, M., et al., *Effects of pH and metal ions on antioxidative activities of catechins.* Bioscience, biotechnology, and biochemistry, 2001. **65**(1): p. 126-132.
269. Michael Schieber and Navdeep S. Chandel, *ROS Function in Redox Signaling and Oxidative Stress.* Current Biology, 2014. **24**(10): p. R453-R462.
270. Di Zhang, et al., *Comparative study on antioxidant capacity of flavonoids and their inhibitory effects on oleic acid-induced hepatic steatosis in vitro.* European Journal of Medicinal Chemistry, 2011. **46**(9): p. 4548-4558.
271. Ong, K.C. and H.E. Khoo, *Biological effects of myricetin.* Gen Pharmacol, 1997. **29**(2): p. 121-6.
272. N. Paiva, C. and M. T. Bozza, *Are Reactive Oxygen Species Always Detrimental to Pathogens?* Antioxidants & Redox Signaling, 2014. **20**(6): p. 1000-1037.
273. Price, L.B., et al., *Staphylococcus aureus CC398: host adaptation and emergence of methicillin resistance in livestock.* MBio, 2012. **3**(1).
274. Ewnetu, D. and A. Mihret, *Prevalence and antimicrobial resistance of Campylobacter isolates from humans and chickens in Bahir Dar, Ethiopia.* Foodborne pathogens and disease, 2010. **7**(6): p. 667-670.
275. Lin, R.D., Y.P. Chin, and M.H. Lee, *Antimicrobial activity of antibiotics in combination with natural flavonoids against clinical extended-spectrum beta-lactamase (ESBL)-producing Klebsiella pneumoniae.* Phytother Res, 2005. **19**(7): p. 612-7.
276. Xu, H.X. and S.F. Lee, *Activity of plant flavonoids against antibiotic-resistant bacteria.* Phytother Res, 2001. **15**(1): p. 39-43.
277. Naz, S., et al., *Antibacterial activity directed isolation of compounds from Punica granatum.* J Food Sci, 2007. **72**(9): p. M341-5.
278. Yadav, A.K., et al., *Screening of flavonoids for antitubercular activity and their structure–activity relationships.* Medicinal Chemistry Research, 2012. **22**(6): p. 2706-2716.
279. Ono, K., et al., *Differential inhibitory effects of various flavonoids on the activities of reverse transcriptase and cellular DNA and RNA polymerases.* Eur J Biochem, 1990. **190**(3): p. 469-76.
280. Daino, G.L., et al., *Identification of Myricetin as an Ebola Virus VP35-Double-Stranded RNA Interaction Inhibitor through a Novel Fluorescence-Based Assay.* Biochemistry, 2018. **57**(44): p. 6367-6378.
281. Yu, M.S., et al., *Identification of myricetin and scutellarein as novel chemical inhibitors of the SARS coronavirus helicase, nsP13.* Bioorg Med Chem Lett, 2012. **22**(12): p. 4049-54.
282. Keum, Y.S. and Y.J. Jeong, *Development of chemical inhibitors of the SARS coronavirus: viral helicase as a potential target.* Biochem Pharmacol, 2012. **84**(10): p. 1351-8.

283. Dilis, V., E. Vasilopoulou, and A. Trichopoulou, *The flavone, flavonol and flavan-3-ol content of the Greek traditional diet.* Food Chemistry, 2007. **105**(2): p. 812-821.
284. Khezerlou, A., et al., *Nanoparticles and their antimicrobial properties against pathogens including bacteria, fungi, parasites and viruses.* Microbial pathogenesis, 2018. **123**: p. 505-526.
285. Haghighi, F., et al., *Antifungal activity of TiO2 nanoparticles and EDTA on Candida albicans biofilms.* Infection, Epidemiology and Microbiology, 2013. **1**(1): p. 33-38.
286. Wu, T., et al., *Antifungal action and inhibitory mechanism of polymethoxylated flavones from Citrus reticulata Blanco peel against Aspergillus niger.* Food Control, 2014. **35**(1): p. 354-359.
287. Liu, L., et al., *Structure–activity relationship of citrus polymethoxylated flavones and their inhibitory effects on Aspergillus niger.* J Agric Food Chem, 2012. **60**(17): p. 4336-4341.
288. Salazar-Aranda, R., et al., *Activity of polyphenolic compounds against Candida glabrata.* Molecules, 2015. **20**(10): p. 17903-17912.
289. Cassetta, A., et al., *Structural basis for inhibition of 17β-hydroxysteroid dehydrogenases by phytoestrogens: the case of fungal 17β-HSDcl.* The Journal of Steroid Biochemistry and Molecular Biology, 2017. **171**: p. 80-93.
290. Egan, M.J., et al., *Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease.* Proceedings of the National Academy of Sciences, 2007. **104**(28): p. 11772-11777.
291. Zhang, J., et al., *Determination of the cytosolic NADPH/NADP ratio in Saccharomyces cerevisiae using shikimate dehydrogenase as sensor reaction.* Scientific reports, 2015. **5**: p. 12846.
292. Zheng, M., et al., *DNA microarray-mediated transcriptional profiling of the Escherichia coli response to hydrogen peroxide.* Journal of Bacteriology, 2001. **183**(15): p. 4562-4570.
293. Li, S., X. Dong, and Z. Su, *Directional RNA-seq reveals highly complex condition-dependent transcriptomes in E. coli K12 through accurate full-length transcripts assembling.* BMC genomics, 2013. **14**(1): p. 520.
294. Baba, T., et al., *Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection.* Molecular systems biology, 2006. **2**(1): p. 2006.0008.
295. Yamamoto, N., et al., *Update on the Keio collection of Escherichia coli single-gene deletion mutants.* Molecular systems biology, 2009. **5**(1): p. 335.
296. Eckert, S.E., et al., *Retrospective application of transposon-directed insertion site sequencing to a library of signature-tagged mini-Tn5Km2 mutants of Escherichia coli O157: H7 screened in cattle.* Journal of Bacteriology, 2011. **193**(7): p. 1771-1776.
297. Warr, A.R., et al., *Transposon-insertion sequencing screens unveil requirements for EHEC growth and intestinal colonization.* PLoS pathogens, 2019. **15**(8): p. e1007652.
298. Hughes, R.L., *A review of the role of the gut microbiome in personalized sports nutrition.* Frontiers in nutrition, 2020. **6**: p. 191.
299. Gopalakrishnan, V., et al., *The influence of the gut microbiome on cancer, immunity, and cancer immunotherapy.* Cancer cell, 2018. **33**(4): p. 570-580.
300. Smith, G. and L. Griffiths, *Metabolism of myricitrin and 3, 4, 5-trihydroxyphenylacetic acid.* Biochemical Journal, 1970. **118**(3): p. 53P-54P.
301. Ong, K.C. and H.-E. Khoo, *Biological effects of myricetin.* General Pharmacology: The Vascular System, 1997. **29**(2): p. 121-126.
302. Becker, N., et al., *Human intestinal microbiota: characterization of a simplified and stable gnotobiotic rat model.* Gut microbes, 2011. **2**(1): p. 25-33.

303. Yao, Y., et al., *Development of a myricetin/hydroxypropyl-β-cyclodextrin inclusion complex: Preparation, characterization, and evaluation.* Carbohydrate polymers, 2014. **110**: p. 329-337.
304. Mureşan-Pop, M., et al., *Solid dispersions of Myricetin with enhanced solubility: Formulation, characterization and crystal structure of stability-impeding Myricetin monohydrate crystals.* Journal of Molecular Structure, 2017. **1141**: p. 607-614.
305. Gupta, R., *Fundamentals of drug nanoparticles.* DRUGS AND THE PHARMACEUTICAL SCIENCES, 2006. **159**: p. 1.
306. Buzea, C., I.I. Pacheco, and K. Robbie, *Nanomaterials and nanoparticles: sources and toxicity.* Biointerphases, 2007. **2**(4): p. MR17-MR71.
307. Roger, E., et al., *Biopharmaceutical parameters to consider in order to alter the fate of nanocarriers after oral delivery.* Nanomedicine, 2010. **5**(2): p. 287-306.
308. Bilia, A.R., et al., *Flavonoids loaded in nanocarriers: an opportunity to increase oral bioavailability and bioefficacy.* Food and Nutrition Sciences, 2014. **2014**.
309. Shukla, R. and M. Cheryan, *Zein: the industrial protein from corn.* Industrial crops and products, 2001. **13**(3): p. 171-192.
310. Zhu, F., A.V. Kale, and M. Cheryan, *Fractionation of zein by size exclusion chromatography.* J Agric Food Chem, 2007. **55**(10): p. 3843-3849.
311. Parris, N., P.H. Cooke, and K.B. Hicks, *Encapsulation of essential oils in zein nanospherical particles.* J Agric Food Chem, 2005. **53**(12): p. 4788-4792.
312. Zhong, Q. and M. Jin, *Zein nanoparticles produced by liquid–liquid dispersion.* Food Hydrocolloids, 2009. **23**(8): p. 2380-2387.
313. Zou, T., et al., *Fabrication, characterization, and cytotoxicity evaluation of cranberry procyanidins-zein nanoparticles.* Food hydrocolloids, 2012. **27**(2): p. 293-300.
314. Shi, L.H., et al., *Beneficial properties of probiotics.* Tropical life sciences research, 2016. **27**(2): p. 73.
315. Lopez-Romero, J.C., et al., *Antibacterial effects and mode of action of selected essential oils components against Escherichia coli and Staphylococcus aureus.* Evidence-Based Complementary and Alternative Medicine, 2015. **2015**.