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Synthesis and evaluation of gallate esters as alternatives to traditional parabens

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Synthesis and evaluation of gallate esters as alternatives to traditional parabens

by

Marcia Reeves

This thesis has been read and approved by the following supervisory committee and submitted in its final draft to the Longwood Senior Thesis Committee in the conferment of Honors towards the degree of Bachelor in Chemistry.

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Abstract

Parabens are esters of *p*-hydroxybenzoates that are commonly found in everyday consumer products as well as pharmaceuticals. They are often used in commercial products to prevent bacterial growth as well as to provide extended shelf life, but they have been shown to activate estrogen receptors in vivo, a contributing factor in human breast cancer proliferation. Our lab has previously generated a multitude of paraben derivatives that do not exhibit estrogenic activity. Many of these derivatives may have other safety concerns, but gallate esters appear to have the least hazardous properties. The estrogenic, antimicrobial, and antioxidant properties of the gallate ester family are unclear; therefore, our lab has synthesized several non-commercially available gallate esters through a DCC coupling reaction. To determine the viability of gallates as preservatives, minimum inhibitory concentrations (MIC) were obtained against *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Serratia marcescens* and were found to be comparable to traditional parabens. Estrogenic activity was compared via MTT proliferation, LDH cytotoxicity, and estradiol colorimetric competition ELISA assays, indicating gallate esters do not upregulate estrogenic activity, while traditional parabens show increased cell proliferation and upregulation of estradiol. Finally, through cyclic voltammetry and DPPH colorimetric assays, gallate esters were determined to act as antioxidants through the reduction of free radicals, where traditional parabens do not. These findings suggest gallate esters could be a suitable antioxidative alternative for traditional parabens. Further work should be completed to determine the magnitude of binding to the estrogen receptor and the extent of the associated antagonistic properties of gallate esters.

Dedication

This Senior Honors Research Thesis is dedicated to the chemistry faculty within the Department of Chemistry and Physics at Longwood University. More specifically, this document is dedicated to Dr. Andrew Yeagley for providing his guidance and support during my time at Longwood, helping me to not only discover my passion for organic chemistry, but to pursue it further through independent research. His knowledge and expertise have provided me with a solid foundation that I will expand upon during my future endeavors in graduate school and my career as a synthetic chemist. Further, this document is dedicated to my husband and best friend, Noah Reeves, who has always encouraged me to pursue my interests, supported me while doing so, and loved me unconditionally. Without his support and gravity, I would not be the confident chemist that I am today. Together, with Edgar and Faraday of course, we will overcome every challenge and pursue any adventure that comes our way.

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Chapter 1: Parabens as Preservatives

Parabens are alkyl esters of *p*-hydroxybenzoic acid and are frequently used as preservatives in a variety of consumer products (**Figure 1**). They have been used in over 22,000 cosmetic products, including makeup, soap, etc., with an allowance of up to 0.8% for a mixture of parabens or up to 0.4% of a single paraben.[1] While parabens are most prevalent in cosmetics, they have also been found in a variety of food products, with an allowance of up to 0.1%.[2] A study in 2012 found that 91% of 267 food samples contained methylparaben, followed by 63% containing propylparaben and 62% containing ethylparaben.[3] Finally, parabens even have been used in paper products, such as sanitary wipes, with concentrations up to 0.3%.[4] The frequency of paraben use can be attributed to their antimicrobial properties.

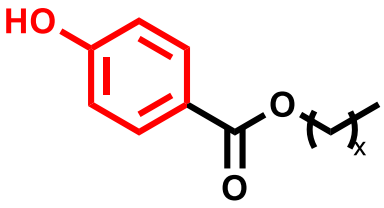


Figure 1. Structure of a traditional paraben. *Red portion represents the phenol, while the black represents the alkyl ester. X corresponds to the number of methylenes present in the alkyl chain where X=0-3 is typical for use in consumer products.*

Parabens can prevent bacterial growth in consumer products, making them a viable option for use as preservatives. There are many ways in which parabens prevent bacterial growth, including inhibition of RNA, DNA, and protein synthesis,[5] inhibition of cellular respiration,[6] and disruption of the cellular membrane (**Figure 2**).[7]

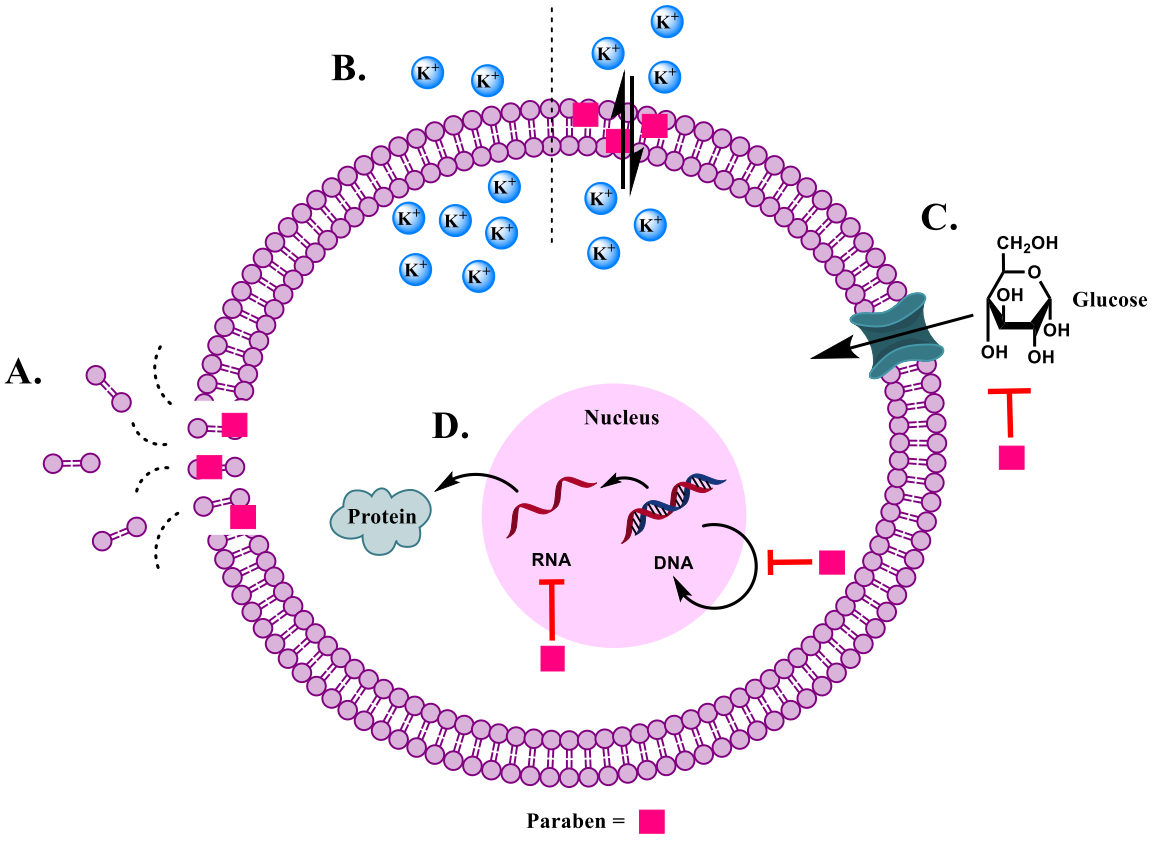


Figure 2. Paraben modes of action. *A) Increased membrane fluidity, considered primary mechanism.*[7,14] *B) Disruption of ionic gradients.*[11] *C) Inhibition of cellular respiration (damage to glucose transporters).*[6] *D) Prevention of DNA & RNA synthesis, resulting in inhibition of protein synthesis.*[5]

When some bacteria, such as *Escherichia coli,* are exposed to parabens, DNA and RNA synthesis is inhibited and likely contributes to inhibition of bacterial growth, as both mechanisms are required for replication and protein synthesis.[5] There has also been evidence of irreversible inhibition of glycolysis in bacteria, which is necessary to produce the energy needed for various vital functions within a cell. This inhibition is due in part to irreversible damage to key enzymes by parabens, such as those in the phosphotransferase system, which are responsible for transporting resources, such as sugars, into the bacteria

cells.[6] While the inhibition of DNA/RNA synthesis and glycolysis may partially contribute to the prevention of bacterial growth, the primary mode of action is agreed to be through the disruption of the cellular membrane.

Due to their overall non-polar nature, parabens become embedded in the cell membrane of bacteria cells, leading to a variety of problems that can inhibit growth or cause cell death. The alkyl ester is lipophilic and intercalates with the hydrophobic fatty acid tails of the phospholipids within the lipid bilayer, while the phenolic portion (shown in red in **Figure 1**) interacts with the polar head groups, increasing hydration of the membrane.[8] Elongation of the alkyl chain has been shown to increase the affinity of parabens for the hydrophobic region of the lipid bilayer,[9] likely contributing to a corresponding increase of overall antimicrobial activity.[10] Both of these interactions can alter the organization and functions of the cell membrane.

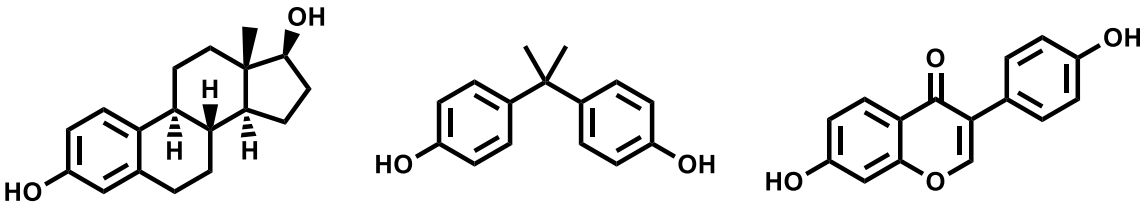
One important alteration caused by the accumulation of parabens within the cell membrane is the dissipation of important transmembrane gradients that are present across bacterial membranes. An example of such is the induction of potassium efflux in *E. coli* caused by the destabilization of the bacterial membrane.[11] Parabens may mimic the activity of pore-forming proteins, leading to increased permeability of the membrane and the induction of potassium release from the cell. They may also interact with important transmembrane proteins that control potassium release from a target cell, including OmpF porin proteins.[11] The movement of potassium out of a cell can be detrimental for survival as it is necessary for many key functions within bacteria cells, including the activity of intracellular enzymes, acting as a second messenger within signal transduction pathways, and maintenance of pH, membrane potentials, and osmotic pressure.[12] The dissipation of

transmembrane gradients in the presence of parabens is not limited to potassium, and is likely one of the main consequences of the disruption of cell membranes by parabens and other phenolic compounds.

Most of the membrane disrupting activity of parabens can be attributed to the presence of a phenol within its structure, as phenols are known contributors of cell membrane damage in bacteria cells. Phenols are able to enter the cell membrane by simple diffusion due to their amphiphilic nature, which can disturb the cell membrane structure.[13] More specifically, phenols increase the fluidity of the cell membrane, leading to leakage of important molecules, including nucleotides, amino acids, and inorganic ions.[14] While the presence of alkyl chains on parabens does aid in their affinity for the cell membrane, the presence of a phenol group grants parabens the ability to disrupt cellular membranes in order to prevent bacterial growth. The efficacy of parabens as preservatives has therefore led to its increased use in many consumer products and their FDA approval. Despite this, recent controversy regarding the safety of parabens has led to a push for paraben-free products.

Chapter 2: Estrogenic Activities of Xenoestrogens

Parabens have been heavily scrutinized for their xenoestrogenic activity, resulting in a demand for their removal from many consumer products. Xenoestrogens are a class of endocrine disruptors that specifically mimic estrogen, a natural steroid hormone found within the human body.[15] These estrogen-mimics have a wide variety of structures; however, common structural motifs include lipophilic phenolic rings and other hydrophobic components, which are shared characteristics with estrogen.[16] Xenoestrogens can be produced naturally, such as phytoestrogens produced by plants, or synthetically, such as pesticides, herbicides, and pharmaceuticals. The structures of some common xenoestrogens are shown in **Figure 3**. Despite their differences in sources or structure, all xenoestrogens have the defining ability to mimic estrogen and act as ligands for estrogen receptors, resulting in a plethora of endocrine-disrupting activities.



A. estradiol **B. bisphenol A** **C. daidzein**

Figure 3. Structures of common xenoestrogens. *A) Estradiol is an endogenous hormone. B) Bisphenol A is a synthetic chemical used in plastics. C) Daidzein is a phytoestrogen found in soy plants.*

Estrogen is a steroid hormone that is involved in many physiological processes within both reproductive and non-reproductive systems. It is one of the main hormones involved in the development of primary and secondary sexual characteristics, as well as

embryonal and fetal neural development.[17] There are three major forms of estrogen, including estrone, estradiol, and estriol.[17] Of these, estradiol is the most prevalent in premenopausal women, and therefore the focus of our interests. Estradiol is synthesized within the ovaries and is responsible for the development and maintenance of secondary sex characteristics, including breast development[18] and endometrium development.[19] It is also highly involved in regulation of the menstrual cycle and ovulation.[20]

As a hormone, estradiol acts as a messenger for a variety of receptors within the human body; however, it functions primarily through its interactions with estrogen receptors. There are two types of estrogen receptors, including estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα is primarily expressed in reproductive tissues, such as the mammary glands, uterus, and ovaries, while ERβ is found in a wider variety of tissues, including the prostate, colon, adipose tissue, and the immune system.[21] Of the two types, ERα is of the most interest for this research, as overstimulation of this receptor by estradiol and xenoestrogens can increase the risk of developing breast cancer.

ERα is activated by the binding of estradiol at the ligand binding cleft, specifically through interactions of the phenolic portions of estradiol with various amino acids present in the binding site. These interactions consist of hydrogen bonding between the phenol, two amino acids (Glu-353 and Arg-394), and a water molecule, while the second hydroxyl function interacts via hydrogen bonding with a single amino acid (His-524).[22] **Figure 4** shows the binding interactions for estradiol and xenoestrogens in general. Estrogen is the only steroidal hormone that contains a phenolic function, thus its involvement in binding is presumably the unique interaction for the estrogen receptor. Considering the interactions between ERα and estradiol, it is unsurprising that many xenoestrogen structures share the

phenolic and/or hydrophobic functions. While all xenoestrogens are categorized by their ability to mimic and trigger responses like those of estrogen, the small differences in their "fits" leads to small differences in response by the estrogen receptors. Parabens are not excluded from this, as the presence of a phenol within their structure allows them to bind and activate ERα.[15]

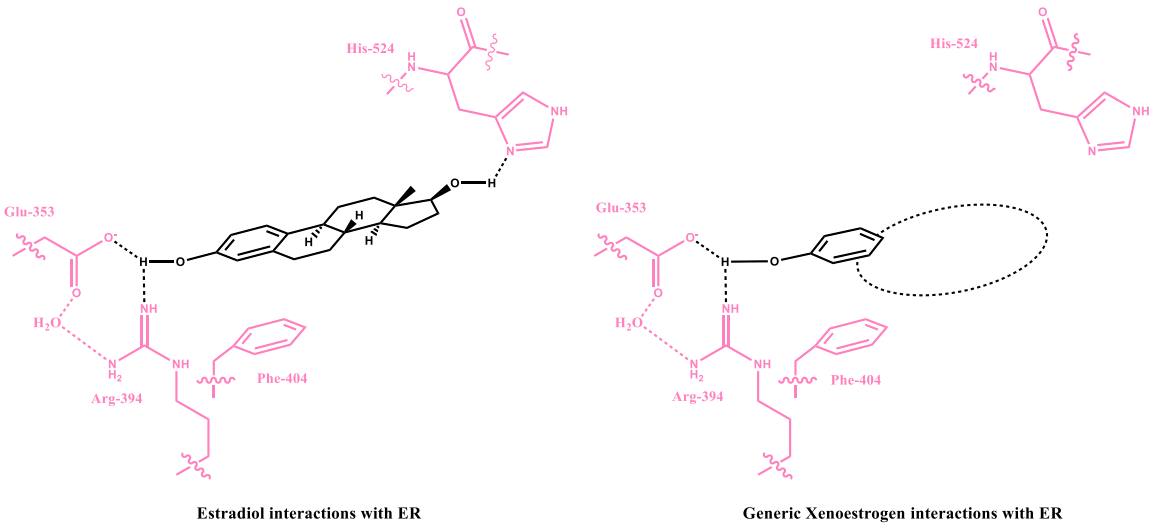


Figure 4. Specific interactions of ligands with the estrogen receptor (ER) binding pocket. *The left shows the interactions of 17β-estradiol with the ER,[22] while the right shows the interactions of a generic xenoestrogen structure with the ER.*

Parabens, as well as other xenoestrogens, can bind to ERα through phenolic binding interactions, resulting in a variety of endocrine-disrupting activities that can modulate various functions within the human body. When the ERα is activated by a ligand, a resulting conformational change occurs, in turn causing the dimerization of two estrogen receptors. This dimer is then able to bind to a specific DNA region called the Estrogen Responsive Element (ERE), which can activate gene expression in response to the presence of estradiol and/or xenoestrogens.[23] This binding leads to the transcription of genes

involved in various cellular processes, including production of estrogen and cell proliferation.[22,24] Therefore, overstimulation of ERα by estradiol and/or xenoestrogens can increase proliferation of cells within reproductive tissue, including breast tissue.

One of the main concerns of paraben use in consumer products is their potential to increase risk of developing breast cancer. This increased risk is due to their ability to overstimulate ERα at low concentrations, resulting in the overproduction of estrogen and abnormal cell proliferation within breast tissues.[25] Higher proliferation rates correspond to higher risk of developing cancer-causing mutations within DNA; therefore, exposure to parabens can be associated with higher risks of developing breast cancer. Because approximately 80% of breast cancers are hormone receptor positive (including ER), with malignant growth resulting from increased levels of estrogen, the estrogenic activity of parabens is a safety concern that cannot be ignored.[26] In addition to their estrogenic activity, other safety concerns must be considered, including their involvement in redox chemistry. There is some evidence that suggests parabens may cause oxidative stress within cells through their involvement in redox chemistry, which could further link their involvement in the development of breast cancer through damage to DNA.

Chapter 3: Redox Chemistry & Oxidative Stress

Redox reactions, or oxidation-reduction reactions, are a type of reaction that involves an exchange of electrons between the atoms involved. These types of reactions are common in biological systems, as they are often utilized to store or release energy necessary for cellular functions. One important example of redox reactions within cells is during cellular respiration, in which glucose is oxidized to produce carbon dioxide and oxygen is reduced to form water. The combination of these redox reactions allows cells to produce ATP, which is used as an energy source for many other cellular functions.

While redox reactions are important for many biological functions, they also come with a risk of producing reactive oxygen species (ROS). Some examples of ROS produced as metabolic byproducts include free radicals that have highly reactive, unpaired electrons, such as superoxide and hydroxyl radicals, as well as other reactive species, like hydrogen peroxide and singlet oxygen.[27,28] ROS production is not limited to biologically inherent redox reactions; however, as they can also be produced after exposure to environmental pollutants, heavy metals, pharmaceutical drugs, chemical solvents, alcohol, and radiation.[29,30] The degradation or metabolism of these within biological systems often produces free radical byproducts.

Low levels of ROS are necessary for cellular processes, including but not limited to protein phosphorylation, activation of various transcription factors, apoptosis, immunity, and even cell differentiation.[31] An accumulation of excess ROS, however, can lead to a phenomenon called oxidative stress.[32,33] Oxidative stress is characterized as an imbalance

between ROS and antioxidants, during which high levels of ROS can cause damage to all cell structures, including proteins, lipids, and nucleic acids (**Figure 5**).[34] The damage to cellular structures from oxidative stress can be responsible for the development of various diseases, such as cancer, diabetes, metabolic disorders, atherosclerosis, and cardiovascular disease.[35]

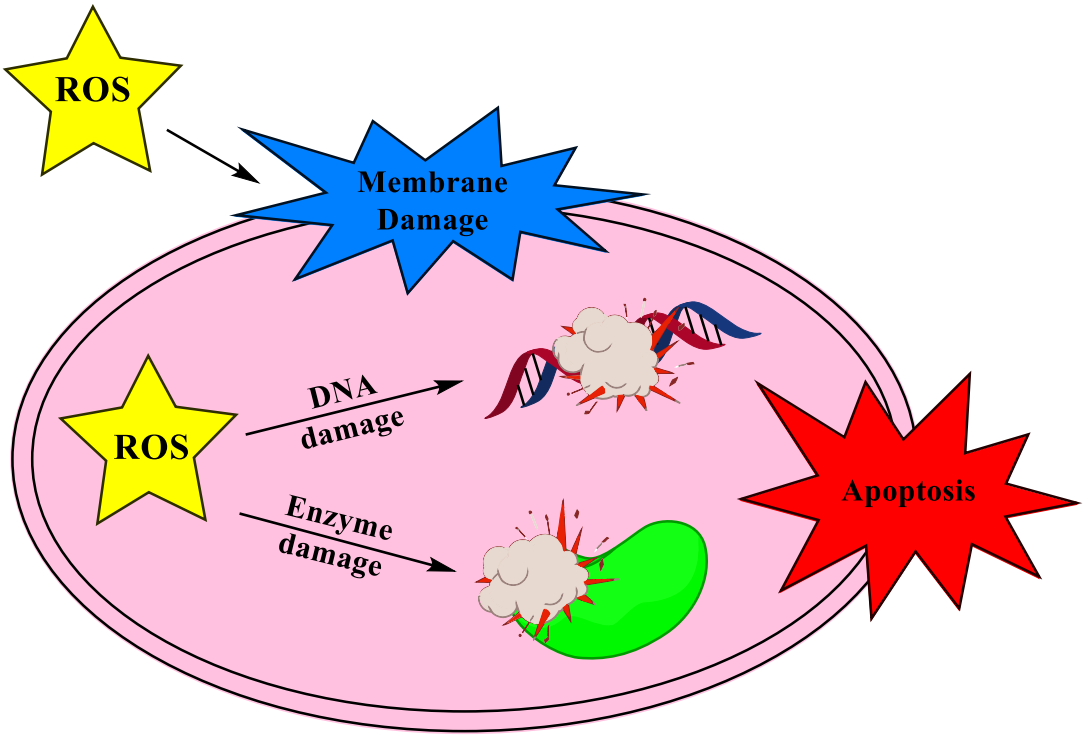


Figure 5. Oxidative stress as a result of reactive oxygen species (ROS) accumulation. *High levels of ROS causes damage to all cellular structures, including cell membrane, DNA, and proteins.*[34]

One known source of oxidative stress and its associated complications is through exposure to phenolic compounds. For example, skin exposure to phenol has been found to be toxic, causing skin rash, dermal inflammation, contact dermatitis, depigmentation, and even cancer promotion.[36,37] The presence of a hydroxyl group within the phenolic function allows phenolic compounds to participate in redox chemistry and contribute to oxidative

stress. More specifically, one-electron oxidation of phenolic compounds by metabolic enzymes produces their free radical intermediates that can cause cytotoxic and genotoxic effects (**Figure 6**).[37] Phenolic compounds are even suspected to be involved in redox-cycling, in which their free radical intermediates are reduced by intracellular reductants, such as thiols. This results in regeneration of the parent phenolic compounds, which may continue cycling through the enzymatic redox reactions and contributing to oxidative stress.

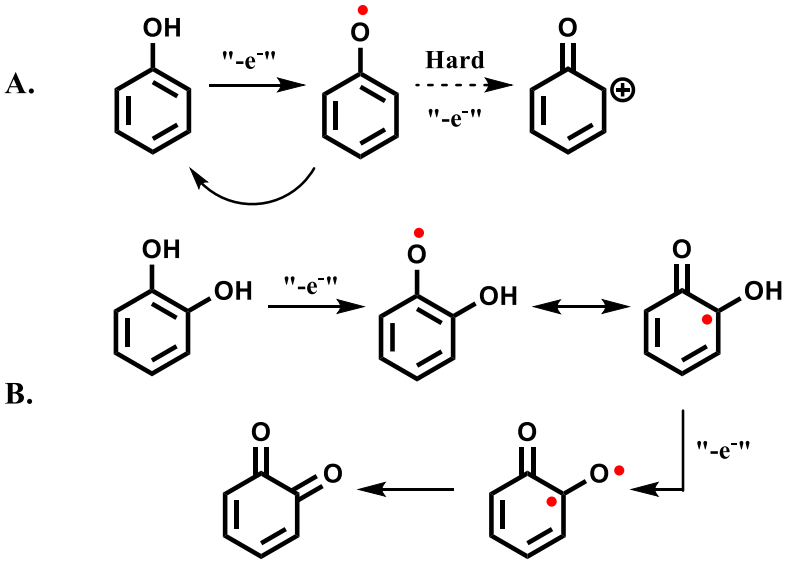


Figure 6. Oxidation of phenolic compounds by hydrogen abstraction. *A) Monophenols undergo a single, one-electron oxidation to form a free radical (in red). Multiple oxidations are very difficult and unlikely to happen; however, redox-cycling is possible and can regenerate the parent compound.[37] B) Polyphenols can undergo multiple, one-electron oxidations to stabilize a free radical. The final quinone product is much less reactive, leading to antioxidant behavior.[45]*

The phenolic function within parabens is a major safety concern, as it allows them to participate in redox chemistry that can cause oxidative stress. The contribution of methylparaben to oxidative stress has been studied in rats, showing that it can cause lipid peroxidation, a source of oxidative stress in which the free radical metabolites of

methylparaben "steal" electrons from lipids within cell membranes.[38] Lipid peroxidation is associated with damage to cell membranes and is associated with various pathologies and diseases, such as cancer.[39] Butylparaben also has been discovered to promote the production of intracellular ROS in human trophoblasts, a type of cell that is important in fetal development, which can in turn induce cell apoptosis through the release of calcium ions and mitochondrial dysfunction.[40] The ability to form free radical species may further link the involvement of parabens in the development of breast cancer, as oxygen free radicals can cause oxidative damage and mutations within DNA, a known cause of cancer development;[41] however, this link has not yet been confirmed.

Considering cells are highly susceptible to oxidative stress, there are natural biological mechanisms in which accumulation of excess ROS can be prevented. One such mechanism is through the production and use of antioxidants, a class of molecules that protect cells from oxidative damage caused by ROS. Antioxidants can provide this protection by either indirectly preventing the oxidation of molecules that usually form free radicals through the regulation of free radical generating enzymes, or through free radical neutralization by accepting or donating electrons to eliminate the highly reactive, existing free radicals.[42] The body produces a few antioxidants on its own, one of which is glutathione, a tripeptide that is able to reduce ROS through a thiol-containing cysteine group.[43] While glutathione is an extremely powerful antioxidant, it cannot maintain oxidative homeostasis on its own, making it very important to obtain antioxidants from the diet.

There are many dietary antioxidants, including important vitamins and phytochemicals. For example, vitamin C and E both accept a stable free radical state to

prevent oxidative damage.[42] Flavonoids, a class of plant-derived phenolic compounds, are another important source of antioxidants, whose antiradical feature is strongly dependent on its chemical structure.[44] The structures of many flavonoids include polyphenolic functions, in which multiple hydroxyl groups are bound to the aromatic rings. The presence of multiple hydroxyl groups has been found to increase the antioxidant activity, likely due to the ability to undergo several oxidations to form a stable quinone radical (**Figure 6**).[45]

While polyphenols can be beneficial if they are able to act as antioxidants, they still have the potential to act as pro-oxidants. Their ability to participate in redox chemistry allows them to be involved in the generation of ROS, as well as the scavenging of free radicals.[46] The balance between antioxidant and pro-oxidant activity heavily depends on the feasibility of multiple oxidations and the stability of the radicals formed. This makes the redox chemistry of phenols and polyphenols quite complicated; however, the potential benefits of polyphenols as antioxidants could be applied to parabens to minimize the pro-oxidant activities that have been observed and improve their overall safety.

Chapter 4: Substituted Parabens as a Lead

The rising concern regarding the safety of paraben use has not only led to a push for paraben-free products by consumers, but has also inspired scientific interest in discovering methods to improve paraben safety. One strategy has been through 3,5-substitution of the phenolic function, shown in **Figure 7**. Initial interest in substituted parabens resulted from the detection of parabens in aquatic environments, including river water[47] and sewage treatment plants.[48] Further investigation of parabens in chlorinated water, such as tap water, revealed the kinetically favorable chlorination of parabens at the 3- and 5- positions on the phenolic ring in the presence of free chlorine.[49] This coincidental discovery of 3,5-dichlorinated parabens resulted in a new lead for improving the safety of parabens through substitution.

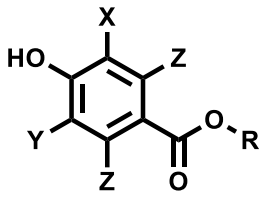


Figure 7. Structure of 3,5-substituted parabens. *X and Y are on the 3,5-position of the phenolic function. Z groups are on the 4,6-positions of the phenolic function. R represents the alkyl chain of the derivatives.*

While it became clear that parabens are favorably chlorinated in chlorine-containing bodies of water, the effects of this chlorination on the properties of parabens had not been investigated. In an initial lead study, Terasaki and co-workers were interested in investigating the effect of mono- and dichlorination of parabens on the estrogenic

activity of parabens.[50] An estradiol competition enzyme-linked immunosorbent assay (ELISA) was used to compare the estrogenic activity of the chlorinated parabens compared to traditional parabens. The results of this study revealed that chlorinated derivatives of parabens exhibited considerably weaker ERα activity or no activity at all. These findings were significant, as they presented a potential avenue for minimizing the estrogenic activity and improving the safety of parabens through substitution.

After discovering this initial lead, our lab became interested in further exploring the potential of 3,5-substitution of parabens. It was hypothesized that 3,5-substitution sterically blocked binding to ERα, resulting in the observed weakening of estrogenic activity. Bergquist and co-workers synthesized a library of 3,5-substituted parabens, included in **Figure 7**, to determine the effect of various substituent types on the estrogenic and antimicrobial activities of parabens.[51] Microdilution assays against *Staphylococcus aureus* revealed improved antimicrobial abilities for many of the derivatives when compared to butylparaben, a commonly used paraben derivative (**Table 1**). An estradiol competition ELISA failed to show estrogenic activity for the substituted derivatives, while both ethylparaben and butylparaben showed estrogenic stimulation. Finally, a terbium-based time-resolved fluorescence resonance energy transfer (TR-FRET) binding assay revealed that the substituted derivatives were still able to bind to ERα, though weakly compared to butylparaben and estradiol (**Table 1**). These findings suggest that the 3,5-substituted parabens are still able to bind to ERα but are unable to cause activation.

Table 1. Minimum inhibitory concentration and TR-FRET Analysis data from Bergquist et. al.[51] *X,Y,Z represent the substituents present on the phenolic function, while R represents the alkyl ester chain. MIC values represent the lowest concentration necessary for inhibition of bacteria growth, while IC_{50} values represent the concentration necessary to bind 50% of the estrogen receptors through displacement of a fluorescent tracer.*

X, Y, Z	R	*S. aureus* MIC µg/mL (µM)	TR-FRET IC_{50} (nM)
F, F, F	butyl	128 (483)	29400
Cl, Cl, H	butyl	64 (245)	55300
Br, Br, H	butyl	32 (91)	39400
Br, H, H	butyl	64 (236)	8210
I, I, H	butyl	16 (36)	34600
I, H, H	butyl	64 (199)	7980
CH_3, CH_3, H	butyl	>256 (>1045)	32500
^{t}Bu, ^{t}Bu, H	butyl	>256 (834)	>200000
OH, OH, H	butyl	512 (2055)	8970
OMe, OMe, H	butyl	>256 (924)	>200000
NO_2, NO_2, H	butyl	512 (1809)	>200000
I, I, H	octyl	16 (31.8)	13200
CH_3, CH_3, H	octyl	64 (213)	16200
OH, OH, H	octyl	64 (210)	8740
NO_2, NO_2, H	octyl	16 (47)	60400
H, H, H	butyl	256 (1320)	1420

The findings of our lab's previous studies were surprising, as they revealed the possible antagonistic ERα activity of 3,5-substituted parabens.[51] A further study by Sasaki and Terasaki studied both the agonistic and antagonistic properties of mono- and dibrominated parabens.[52] The agonistic studies revealed that bromination of parabens prevented agonistic activity, or activation of ERα. On the other hand, the antagonistic studies revealed higher antagonistic activity with increased bromination, meaning the dibrominated parabens showed greater antagonistic activity than the monobrominated derivatives. These findings elucidated that brominated parabens are able to bind to ERα and prevent its activity to some extent. While this type of testing has not been completed for the other 3,5-substituted derivatives that our lab has previously studied, the antagonistic

properties of the brominated parabens could explain the binding and estrogenic activities for the other substituted derivatives as well.

The 3,5-substituted parabens that our lab has synthesized could potentially be safer for use in consumer products due to their decreased estrogenic activity; however, other safety concerns must be considered to determine which of the derivatives might be the best option. In general, halogenated organic compounds, such as halogenated phenols, are toxic and should be avoided. Many halogenated phenols are used commercially as flame retardants and wood preservatives, including tribromophenol (tri-BPh)[53] and pentachlorophenol (PCP).[54] PCP is extremely toxic, with a known ability to cause cancer and birth defects in laboratory animal studies, as well as blood disorders and nerve damage in humans.[55] While these dangers have not been assessed for the brominated and chlorinated parabens, it is best to consider alternative derivatives that are not as likely to be toxic. Further investigation into the potential benefits of polyphenols and their ability to act as antioxidants has led to interest in evaluating gallate esters as a potentially safer alternative to traditional parabens.

Chapter 5: Preliminary Work & Research Goals

The use of parabens in consumer products has been an ongoing safety concern. This is due to their ability to act as xenoestrogens, as well as their ability to participate in redox chemistry and contribute to oxidative stress. An ideal alternative would be one that does not exhibit either of these properties, making it much safer for use in consumer products. Therefore, the focus of this research was to evaluate the safety of gallate esters, including their estrogenic and antioxidant/pro-oxidant activities.

While gallate esters have been studied in the past to some extent, there are many gaps in the literature regarding their viability as preservatives. This is likely due to the limited accessibility of gallate ester derivatives through commercial means, particularly those consisting of odd-numbered carbon chains. Previous studies have attempted to evaluate their antimicrobial properties; however, they have only performed the necessary biological assays on the commercially available derivatives, providing limited information about the full range of gallate ester derivatives.

Before beginning studies regarding the safety of gallate esters, a broader range of derivatives needed to be available. Therefore, our lab synthesized several derivatives that were not commercially available, including pentyl, hexyl, heptyl, and decyl gallate esters, shown in **Figure 8**.

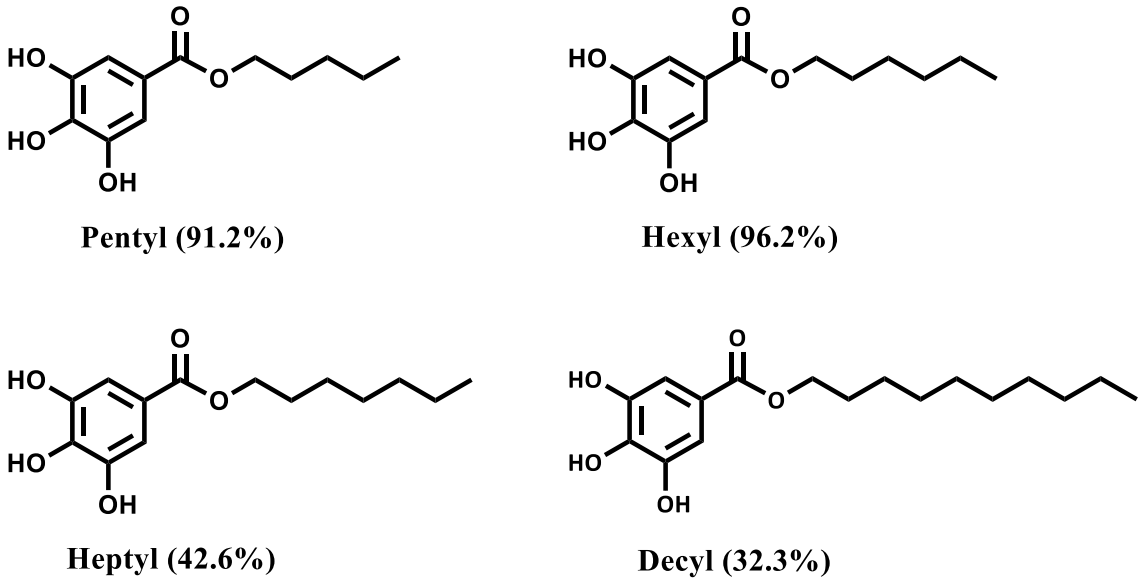
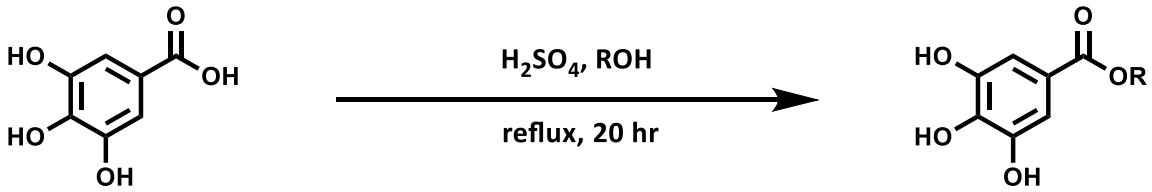


Figure 8. Non-commercially available gallate esters previously synthesized by our lab. *Percent yields are included as parentheticals for each compound.*

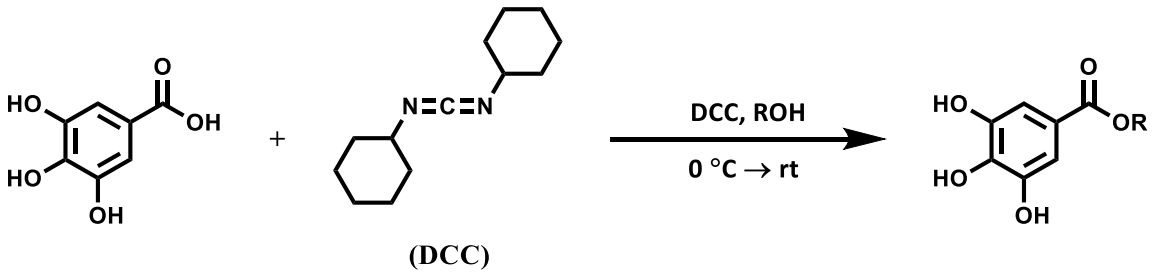
Initially, synthesis was completed using a Fischer esterification reaction (**Scheme 1**); however, the oxidative nature of sulfuric acid and high reflux temperatures led to products that were dark-colored and potentially quinones.



Scheme 1. Fischer esterification reaction. **ROH denotes the alcohol used with varying carbon chain lengths.*

To combat this, synthetic methods were changed to a DCC coupling reaction (**Scheme 2**), in which heat was not required and the resulting products were white powders, like the

commercially available derivatives. The yields of this reaction were not as high, especially for the longer-chained derivatives like decyl gallate, likely explaining the limited commercial availability of gallate esters.



***Scheme 2**. **DCC coupling reaction.** *ROH denotes the alcohol used with varying carbon chain lengths.*

After completing the synthesis, the full range of gallate esters needed to be evaluated for their ability to act as preservatives. Our lab completed microdilution assays to obtain the minimum inhibitory concentrations (MIC) against three different bacteria, including *Staphylococcus epidermidis* and *Staphylococcus aureus* as Gram-positive representatives, and *Serratia marcescens* as a Gram-negative representative. The MIC represents the lowest concentration of each compound required to prevent bacterial growth. The results, found in **Table 2,** indicate that increasing the alkyl chain length improves the MIC against gram-positive bacteria, with dodecyl gallate being the most effective antimicrobial agent of the derivatives. Overall, the findings further support the viability of gallate esters as preservative agents, justifying the evaluation of their safety to a greater extent.

Table 2. Minimum inhibitory concentration (MIC) of gallate esters against bacterial growth. *S. epidermidis and S. aureus are gram-positive, while S. marcescens is gram-negative.*

Compound	Source	MIC (*S. epidermidis*) (µg/mL)	MIC (*S. marcescens*) (µg/mL)	MIC (*S. aureus*) (µg/mL)
Methyl gallate	purchased	>512	>512	512
Ethyl gallate	purchased	>512	>512	>512
Propyl gallate	purchased	>512	>512	512
Butyl gallate	purchased	512	>512	512
Pentyl gallate	synthesized	256	512	128
Hexyl gallate	synthesized	128	256	128
Heptyl gallate	synthesized	64	----[b]	32
Octyl gallate	purchased	128	256	32
Decyl gallate	synthesized	64	----[b]	64
Dodecyl gallate	purchased	32	>512	32-128[a]
Ampicillin	purchased	>512	>512	8
Erythromycin	purchased	>512	----[b]	16

[a] Solubility leading to inconsistent results.
[b] Not yet tested.

With the knowledge that gallate esters prevent bacterial growth, the evaluation of their estrogenic and redox safety concerns could finally be completed. This began with the use of an estradiol colorimetric competition enzyme-linked immunosorbent assay (ELISA) to determine whether gallate esters act as xenoestrogens. The estradiol colorimetric competition ELISA provides information about the estrogenic activities of a potential xenoestrogen by detecting the upregulation of estrogenic activity. More specifically, the production of estradiol by cells is determined using absorbance spectroscopy at 405 nm to calculate the concentration of estradiol. If a compound upregulates estrogenic activity, the estradiol production increases, suggesting the ability to bind and activate the estrogen receptor. This method has been previously used for 3,5-substituted parabens,[50,51] including a few gallate esters,[51] making it a suitable method for evaluating the estrogenic activity of the full range of gallate ester derivatives.

Next, the redox behavior and antioxidant properties of gallate esters were evaluated through two different methods. The first method used was a 2,2-diphenyl-1-picrylhydrazyl (DPPH) colorimetric assay, which detects the ability to scavenge a free radical and is an accepted method for screening the antioxidant activity of phenolic compounds.[56] In this assay, DPPH begins as a free radical of a purple color and will become yellow when exposed to an antioxidant as a result of hydrogen abstraction (**Figure 9**). Absorbance spectroscopy at 490 nm can be used over time to measure this color change and calculate anti-radical power of a compound. The DPPH assay can directly measure the antioxidant capabilities of a compound relative to the DPPH radical with a known oxidation potential.

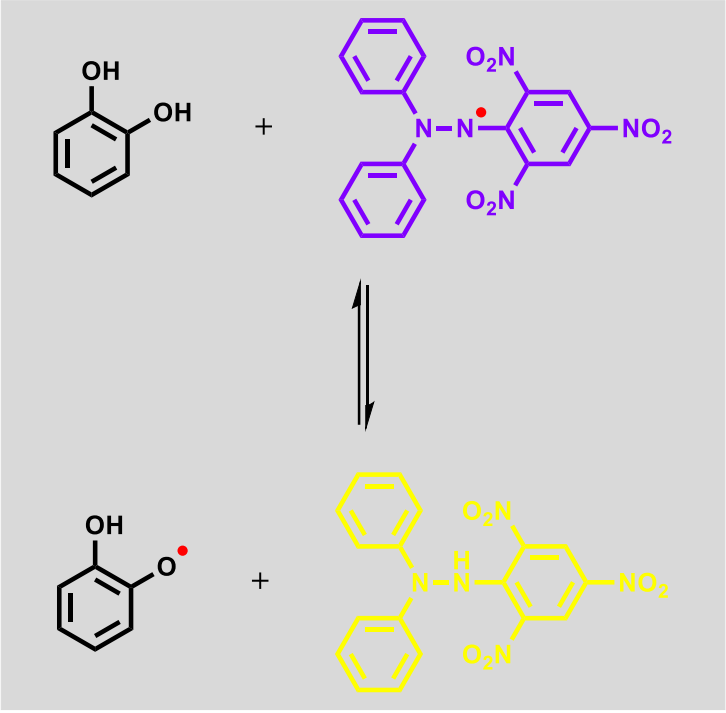


Figure 9. Mechanism of DPPH radical scavenging assay. *Absorbance at 490 nm is used to measure the color change after exposure to an antioxidant.*

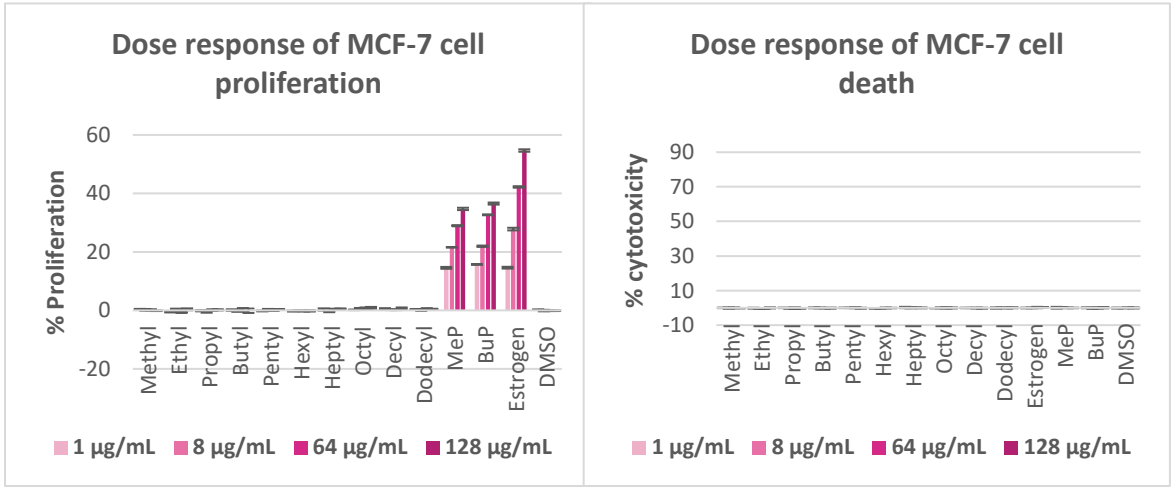
The redox behavior of gallate esters was more generally evaluated by determining the redox potential of the compounds through cyclic voltammetry (CV). This

electrochemical method was used to measure the oxidation potentials of gallate esters, which in turn provides insight into their antioxidant properties. CV has been used for the determination of antioxidant capabilities in the same manner as the more widely used DPPH assay because of the correlation between low oxidation potentials and anti-radical power.[57] Additionally, there have been studies in which CV has been successfully used to evaluate the antioxidant properties of parabens,[58] gallic acid,[57] and dodecyl gallate.[59] The results from CV can provide more general information regarding the ability to oxidize a molecule, which can contribute to whether a molecule will act as an antioxidant through free radical exchange.

Chapter 6: Results & Discussion

Evaluation of estrogenic activities

Prior to completing the estradiol colorimetric competition ELISA, it was necessary to determine the ability of gallate esters to stimulate cellular proliferation and/or trigger cell death. To do so, lactate dehydrogenase (LDH) cytotoxicity and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation assays were completed using MCF-7 breast cancer cells, a human cell line that is positive for estrogen receptor expression. During these assays, methyl- and butylparaben were used as examples of traditional parabens, as they are both widely used in consumer products, and estradiol was used as a control. Both assays were completed in biological triplicate over 24 and 72 hours, and the results of one representative biological replicate are shown in **Figure 10**.



A. **B.**

Figure 10. Preliminary viability and cytotoxicity assays using MCF-7 cells. *A) LDH cytotoxicity assay of gallate esters. B) MTT proliferation of gallate esters.*

The results of the MTT proliferation assays demonstrated that gallate esters did not stimulate cellular proliferation compared to MCF-7 cells alone, while both paraben derivatives and estradiol stimulated MCF-7 proliferation to a similar degree (**Figure 10A**). It was unsurprising that both paraben derivatives stimulated proliferation of MCF-7 cells, as stimulation of the ER may contribute to increased proliferation. The results were consistent with our lab's previous work, in which neither butyl nor octyl gallate stimulated MCF-7 proliferation, while butylparaben did.[51] Despite this, it was still necessary to further examine the estrogenic activity of each compound using a more specified approach, such as an estradiol colorimetric competition ELISA, as cellular proliferation can be caused by biological processes other than ER stimulation. Additionally, LDH cytotoxicity assays revealed that no significant cell death was caused by any of the compounds studied, including all gallate ester derivatives and paraben derivatives, in comparison to MCF-7 cells alone (**Figure 10B**). This was also consistent with our previous work, in which none of the 3,5-substituted parabens caused cell death. The results of the LDH assays demonstrated that all compounds did not affect the viability of MCF-7 cells. In summary, the results of both assays confirmed that gallate esters were suitable for ELISA testing and ensured the data provided from the ELISA would not be skewed by changes in either cell proliferation or cell death caused by exposure to the compounds studied.

Once the MTT proliferation and LDH cytotoxicity assays were completed, an estradiol colorimetric competition ELISA was used to evaluate the estrogenic activity of gallate esters compared to parabens. As before, the ELISA assays were completed in triplicate using the MCF-7 cell line cultures exposed to the studied compounds for 24 hours, and the results of one representative biological replicate are shown in **Figure 11**.

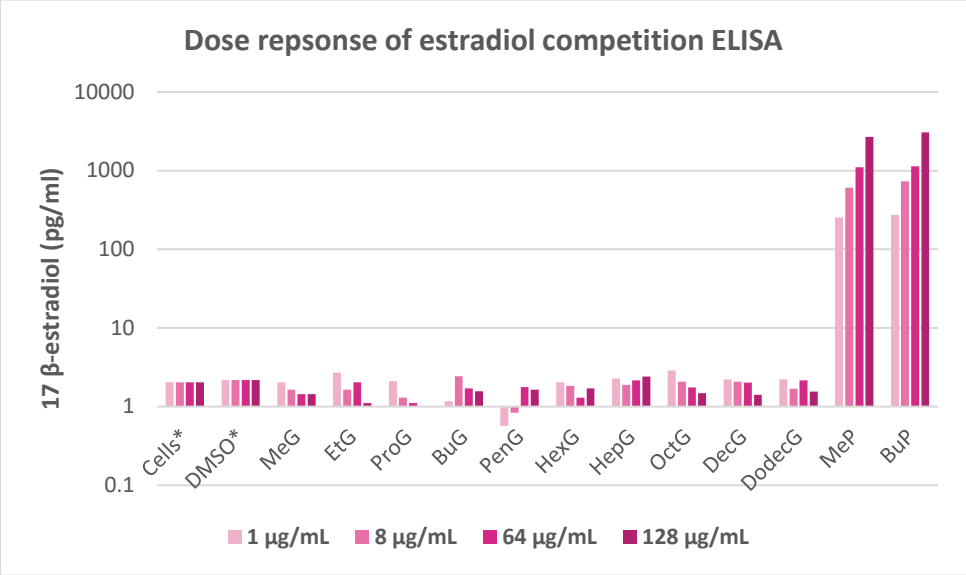
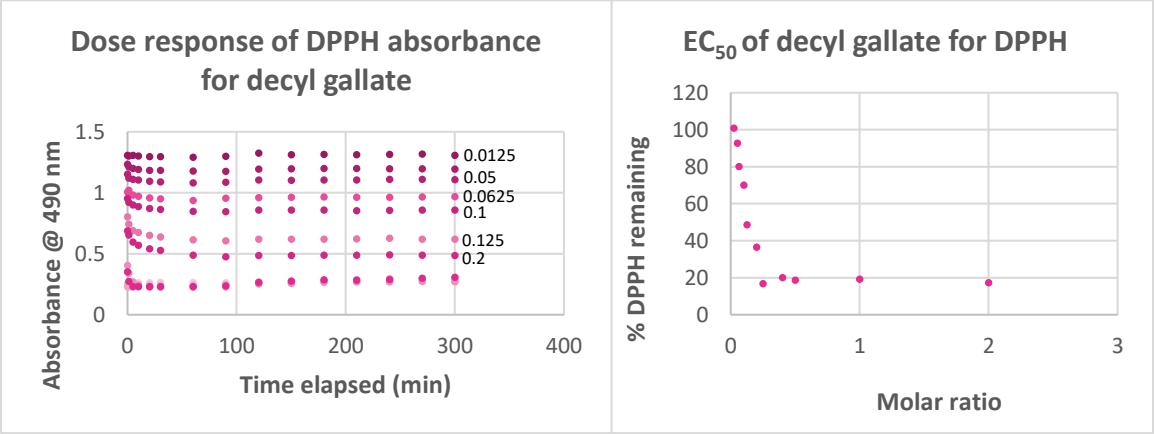


Figure 11. Estradiol colorimetric competition ELISA using MCF-7 cells. *Represents one biological replicate of three completed. *Concentration constant in all wells.*

The results of the ELISA revealed no upregulation of estrogenic activity by gallate esters, as estradiol production was comparable to that of MCF-7 cells alone. In contrast, both paraben derivatives showed upregulation of estradiol activity, with exponentially higher amounts of estradiol produced. Both results are consistent with our previous work, in which butyl and octyl gallate did not show estrogenic activity, while butyl paraben did. Our previous work also consisted of TR-FRET binding assays, in which butyl and octyl gallate showed increased binding affinities compared to the other 3,5-substituted parabens, with half-maximal inhibitory concentration (IC_{50}) values of 8970 nM and 8740 nM, respectively.[51] The results of these ELISA assays, in combination with previous the binding data, suggest that gallate esters likely act as antagonists for the ER, binding and preventing the activation of the ER by estradiol and other xenoestrogens.

Evaluation of antioxidant capabilities

The antioxidant properties of gallate esters were measured in two ways, the first being through a set of DPPH radical scavenging assays. To do so, the DPPH radical was exposed to each compound at various molar ratios, including gallate esters, methyl- and butylparaben, and vitamin C as a control. The absorbances were then collected over time to determine the dose response of DPPH absorbance and half-maximal effective concentration (EC_{50}) for each compound. An example of a dose response plot and EC_{50} plot for decyl gallate is shown in **Figure 12**.



A. **B.**

Figure 12. DPPH radical scavenging assay for decyl gallate. *A) Dose response of DPPH absorption. B) EC_{50} plot for DPPH reduction.*

These assays were once again completed in triplicate and the resulting sets of data were averaged to produce **Table 3**, which shows the EC_{50} and anti-radical powers (ARP), or the inverse of the EC_{50}, for all compounds studied.

Table 3. Determined EC_{50} for DPPH reduction and antiradical power (ARP) of gallate esters. *DPPH assays were completed in triplicate for all compounds and averaged to produce the following results.*

Compound	Avg EC_{50}	Stdev EC_{50}	ARP
Ascorbic Acid (Vit C)	0.497	0.04	2.01
Methyl gallate	0.0323	0.02	30.9
Ethyl gallate	0.0728	0.01	13.7
Propyl gallate	0.0803	0.01	12.5
Butyl gallate	0.0804	0.003	12.4
Pentyl gallate	0.0540	0.01	18.5
Hexyl gallate	0.0950	0.01	10.5
Heptyl gallate	0.0957	0.02	10.4
Octyl gallate	0.0838	0.01	11.9
Decyl gallate	0.127	0.02	7.88
Dodecyl gallate	0.0988	0.01	10.1
Methyl paraben	-	-	-
Butyl paraben	-	-	-

During all trials of the DPPH assays, gallate esters were able to reduce the free radical form of DPPH, resulting in the yellow, reduced form of DPPH. The gallate esters were also able to do this with minimal EC_{50} values and high ARP values compared to vitamin C, a known antioxidant. For the most part, the EC_{50} values and ARP values were similar for all derivatives, ranging between 7.8 and 18.5, with methyl gallate being an exception. For methyl gallate, there was a larger range of EC_{50} values between trials, ranging from 0.00767 to 0.0472, leading to a much higher average ARP value of 30.9 and a standard deviation almost twice that of the other gallate derivatives. This suggests that methyl gallate may exhibit stronger antioxidant properties than the other derivatives; however, more replicates would be necessary to provide a better understanding of the antioxidant capability of methyl gallate. In contrast to gallate esters, both paraben derivatives were unable to reduce DPPH, even at higher molar ratios. Therefore, the results of the DPPH scavenging assays demonstrated that gallate esters act as antioxidants through free radical reduction, while parabens do not.

To further support the findings of the DPPH assays and gain information about the redox behavior of gallate esters in a more general sense, cyclic voltammetry (CV) was completed for gallate esters, methyl- and butylparaben. **Figure 13** shows the resulting, combined voltammograms of all compounds studied, with gallate esters in shades of red/pink and parabens in shades of blue.

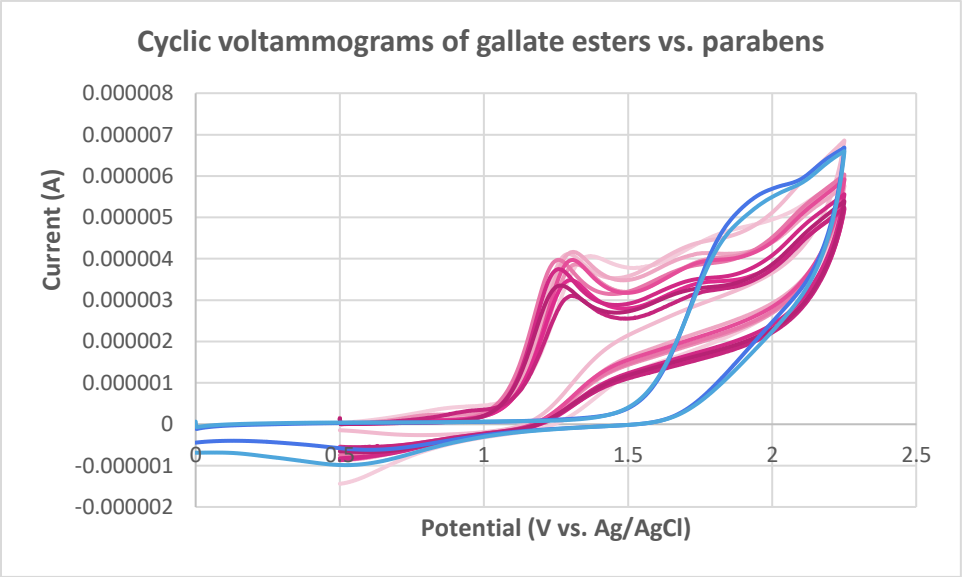


Figure 13. Combined cyclic voltammograms for gallate esters vs. parabens. *Gallate esters are in pink shades, while parabens are in blue. Electrodes included: Pt working, Ag/AgCl (1M KCl) reference, and Pt metal counter. TBAP in acetonitrile (0.1M) was used as the supporting electrolyte. All compounds were tested at 1 mM concentrations. Parameters included an initial potential of +0.50 V for gallate esters, an initial potential of +0.00 V for parabens, a vertex potential of +2.25 V for all compounds, and a scan rate of 100 mV/s for all compounds*

The cyclic voltammograms were then used to determine the oxidation potential(s) for each compound studied, shown in **Table 4**, by finding the local maxima for each oxidation peak identified.

Table 4. Oxidation potentials of gallate esters using cyclic voltammetry.

Compound	Peak 1 (V)	Peak 2 (V)	Peak Diff (V)
Ferrocene	0.426	-	-
Methyl gallate	1.360	1.660	0.300
Ethyl gallate	1.292	1.650	0.358
Propyl gallate	1.308	1.628	0.320
Butyl gallate	1.318	1.648	0.330
Pentyl gallate	1.258	0.638	0.380
Hexyl gallate	1.308	1.658	0.350
Heptyl gallate	1.298	1.618	0.320
Octyl gallate	1.258	1.610	0.352
Decyl gallate	1.302	1.626	0.324
Dodecyl gallate	1.262	1.620	0.358
Methyl paraben	1.842	-	-
Butyl paraben	1.846	-	-

The CV data revealed that each gallate ester derivative undergoes two stable and irreversible one-electron oxidations, showing two oxidation peaks and no reduction peaks. On the other hand, parabens only exhibited one irreversible one-electron oxidation, with only one oxidation peak and no reduction peaks. The first oxidation peaks for most gallate ester derivatives ranged between 1.258 V and 1.318 V, an exception being methyl gallate with a peak at 1.360 V, suggesting that methyl gallate may be slightly harder to oxidize initially compared to the other derivatives. In contrast to gallates, the oxidation peaks for methyl- and butylparaben were found to be 1.842 V and 1.846 V, respectively. The potentials for parabens were higher overall than those of gallates, showing that more voltage (or energy) is required to oxidize parabens relative to gallate esters, making oxidation more difficult or unfavorable for parabens. The presence of two peaks relatively close together for gallate esters also suggests that the second oxidation quickly follows the first. This further supports the antioxidant properties of gallate esters, as the second oxidation peak allows the radical to be eliminated through the formation of a quinone.

Interestingly, the distance between peaks for methyl gallate was the lowest of all derivatives tested with a value of 0.300 V, compared to a range of 0.320 – 0.380 V for all other derivatives. While the first oxidation is likely harder for methyl gallate due to an increased oxidation potential, the decreased difference between peaks could make the second oxidation easier. This could explain the improved ARP values observed for methyl gallate in the DPPH assays. Finally, the stabilization of a free radical through a second oxidation is difficult for parabens as they only have one hydroxyl group, making radical formation unfavorable and requiring a higher oxidation potential.

Overall, the oxidation potentials of gallates and parabens are consistent with the results of the DPPH assay. Since low oxidation potentials correlate to higher ARP values,[57] the lower oxidation potentials of gallate esters compared to parabens explains the ability of gallates to reduce DPPH and the inability of parabens to do so. Additionally, the oxidation potential of DPPH is likely lower than that of parabens, making it the preferable radical of the two. The combination of both DPPH and CV demonstrates that gallate esters are able to act as antioxidants due to their ability to undergo multiple oxidations in order to scavenge and stabilize free radicals.

Conclusions & Future Directions

This research was conducted to evaluate the safety of gallate esters, including their estrogenic and redox activities. Before doing so, synthesis of four non-commercially available gallate derivatives was completed via Fischer esterification and DCC coupling methods. The full range of gallate esters were then evaluated for their antimicrobial activities through microdilution assays to confirm their viability as preservative agents. The results demonstrated that gallates can prevent bacterial growth comparable to parabens, supporting the continuation of safety evaluation.

After confirmation of antimicrobial activity, the estrogenic activity was evaluated through an estradiol colorimetric competition ELISA, with preliminary LDH cytotoxicity and MTT proliferation assays. These assays revealed that gallate esters did not increase cell proliferation, cause cell death, or upregulate estrogen activity of MCF-7 breast cancer cells, while parabens both increased proliferation and upregulated estradiol production. Additionally, this data, in combination with past work, further supports the role of gallates as antagonists for the estrogen receptor. Finally, the antioxidant and redox properties of gallate esters were evaluated using DPPH radical scavenging assays and cyclic voltammetry. The results of both assays support that gallate esters can act as antioxidants by reducing free radicals, while parabens are unable to.

In conclusion, gallate esters have shown significantly reduced estrogenic activity and increased antioxidant properties, both of which should be considered when evaluating their viability as paraben replacements. Provided this, gallate esters are not only a

potentially safer alternative for traditional parabens but have an added benefit of acting as antioxidants; therefore, they could one day be used in consumer products to eliminate the risks associated with paraben-use. Future work includes TR-FRET binding assays for the entire range of gallate esters to determine the magnitude of binding to the estrogen receptor, as well as ELISA assays to determine the extent of antagonistic activity for the estrogen receptor. It would also be valuable to further investigate whether parabens directly cause oxidative DNA damage through free radical formation using biological assays, which would confirm their involvement in breast cancer development via oxidative means. Finally, other safety concerns of gallate esters should be evaluated, including environmental toxicity and overall organismal toxicity. This information, in combination with the results of this research, would provide even more reason to use gallate esters as a replacement for traditional parabens in consumer products.

Experimental Methods

Synthesis of gallate esters by Fischer esterification

Fischer esterification was performed within a 50 mL round bottom flask containing approximately 2.00 g of gallic acid dissolved in 4 mL (excess) of the desired alcohol. Then, 20 drops of 18 M sulfuric acid were added to the solution. The resulting solution was then heated at reflux overnight (~20 hours). Thin layer chromatography was used to monitor the reaction progress. The crude reaction material was purified by flash column chromatography on silica gel with approximately 400 mL of 15% ethyl acetate in hexanes with 1% acetic acid, followed by approximately 350 mL of 50% ethyl acetate in hexanes. The resulting fractions containing the final product were concentrated in vacuo to afford an off-white, sometimes brown, solid as the final product.

Synthesis of gallate esters by DCC coupling

The DCC coupling reaction was performed in a 25 mL round bottom flask containing approximately 340 mg of gallic acid and an equimolar volume of the desired alcohol dissolved in 6 mL of THF. Then, approximately 870 mg (2.1 equiv.) of N,N'-dicyclohexylcarbodiimide (DCC) was separately dissolved in 6 mL of THF and added to the solution in the round bottom flask and cooled to 0º C. The reaction was stirred on ice for approximately 8 hours and allowed to warm to room temperature for the remaining 12 hours. The crude reaction material was washed with ethyl acetate in triplicate and filtered. The filtrate was then washed successively with 10% aqueous citric acid, saturated aqueous

$NaHCO_3$, and saturated NaCl, each in triplicate. The resulting organic layer was then evaporated with $MgSO_4$ and evaporated via rotary. Finally, the reaction was purified by flash column chromatography on silica gel using approximately 400 mL of 30% ethyl acetate in hexanes with 1% acetic acid, followed by approximately 350 mL of 50% ethyl acetate in hexanes. The resulting fractions containing the final product were concentrated in vacuo to afford a white solid as the final product.

Microdilution assay for Minimum Inhibitory Concentration (MIC) of gallate esters

Sterilized MHB was inoculated to 5 x 10^5 CFU mL^{-1} with *S. aureus* (OD_{600} = 0.00983; Carolina Biologicals 155554A), *S. epidermidis* (OD_{600} = 0.0570; Carolina Biologicals 155556A), and *S. marcescens* (OD_{600} = 0.0790; Carolina Biologicals 155450A). Each inoculum was aliquoted (1 mL) into separate sterilized test tubes and the resulting gallate ester derivative (from 16 mg/mL DMSO stocks) was added to give the highest concentration to be tested (512 μg/mL). The resulting sample solutions were then aliquoted (200 μL) into the top row of a 96-well plate while retaining the final well (column) for untreated bacteria cells to act as the control. Rows 2 – 12 of the plate were then aliquoted (100 μL) with the original, untreated inoculant. Row 1 wells were mixed 6 to 8 times, and then 100 μL was transferred to row 2. Row 2 wells were mixed 6 to 8 times, followed by a 100 μL transfer from row 2 to row 3. This procedure was repeated to serially dilute the rest of the rows of the microtiter plate, excluding the control column. The serially diluted plate was then sealed with GLAD Press n' Seal® and incubated under stationary conditions at 37º C. After 16 hours of incubation, MIC values were recorded as the lowest concentration of each test compound at which no visible growth of bacteria was observed.

This process was repeated for a minimum of three days with each experiment consisting of a pair of trials originating from two differing day cultures for a minimum of six biological replicates.

MTT cellular proliferation assays

MCF-7 cells (human breast cancer call line with hormone receptor expression) were used in this study. The cells were grown in complete RPMI media (Gibco Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin, 1 nM sodium pyruvate, 10 mM Hepes, and 0.1 mM non-essential amino acids in 5% CO_2 atmosphere at 37º C. To determine the effect of the gallate ester derivatives on cell proliferation, 2 x 10^5 cells were plated into flat-bottom 96-well plates (Thermo Scientific Nunc, Waltham, MA) in 0.2 mL aliquots of complete medium and treated with different concentrations of each compound (from 16 mg/mL DMSO stocks). For comparison, ethylparaben, butylparaben and estradiol were used as controls. Proliferation was determined after 24 and 72 hours of culture with the compounds using a CellTiter 96 Non-radioactive Cell Proliferation Assay (MTT) according to the manufacturer's protocol (Promega Madison, WI). These protocols were then performed in triplicate with three biological replicates on each plate. The percent proliferation was determined using the following formula: ((Experimental optical density – untreated optical density) / (Untreated optical density)) x 100.

LDH cellular cytotoxicity assays

To determine the cytotoxicity of the gallate ester derivatives, 2 x 10^5 MCF-7 cells were plated into flat-bottom 96-well plates in 0.2 mL aliquots of complete medium and treated with different concentrations of each compound (from 16 mg/mL DMSO stocks). For comparison, ethylparaben, butylparaben and estradiol were used as controls. Cytotoxicity was determined after 24 and 72 hours of culture with the compounds using an LDH cytotoxicity assay according to the manufacturer's protocol (Thermo Scientific Pierce). These protocols were then performed in triplicate with three biological replicates on each plate. The percent cell death was determined by using the formula: ((Experimental sample release – spontaneous release) / (Maximum release – spontaneous release)) x 100.

Estradiol colorimetric competition enzyme-linked immunosorbent assays (ELISA)

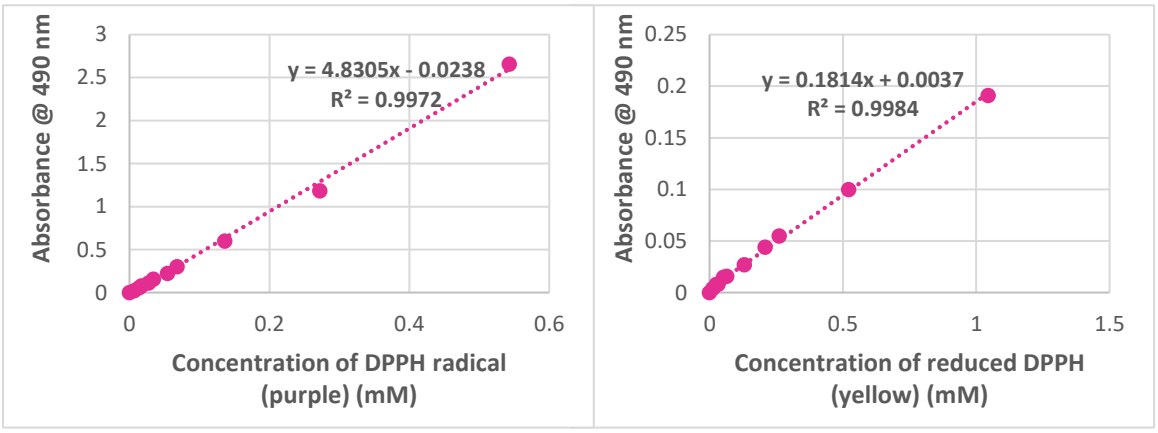
To determine if the gallate ester derivatives stimulated estradiol secretion from MCF-7 cells, 2 x 10^5 cells were plated into flat-bottom 96-well plates in 0.2 mL aliquots of complete medium and treated with different concentrations of each compound (from 16 mg/mL DMSO stocks). For comparison, ethylparaben and butylparaben were used as controls. Estradiol secretion was determined after 24 hours of culture with the compounds using a 17β – estradiol colorimetric competition ELISA kit according to the manufacturer's protocols (Enzo Life Sciences Farmingdale, NY). These protocols were performed in triplicate with three biological replicates on each plate.

DPPH radical scavenging assays

The methods for this assay were modified from those used in Brand-Williams et al[60] and are described as follows:

Antioxidant activities were determined using DPPH as a free radical. Each gallate ester derivative was tested at different concentrations, expressed as the molar ratio of compound/DPPH (ranged from 0.0125 to 2.00). For comparison, ethylparaben, butylparaben, and ascorbic acid were used as controls. Each compound was accurately diluted in HPLC-grade methanol prior to completion of the assays (from 10 mM in methanol stocks), and a DPPH stock solution was accurately diluted to 0.5 mM in methanol. All assays were performed in flat-bottom 96-well plates, in which all wells received a 100 μL methanol aliquot. Then, 100 μL aliquots of 1.0 mM and 0.1 mM for each compound were added to columns 1 and 7, respectively, excluding the top two rows. Column 1 was then mixed 6 to 8 times, and then 100 μL was transferred from column 1 to column 2. Column 2 was then mixed 6 to 8 times, followed by a 100 μL transfer from column 2 to column 3. This process was repeated to serially dilute through column 6, after which 100 μL was discarded. Then, the serial dilution procedure began again at column 7 through the remaining columns on the plate. At this point, a 100 μL aliquot of 0.5 mM DPPH in methanol was added to all wells, excluding the top row of wells that acted as a blank. The plate was covered with clear tape to prevent evaporation and the absorbance of the solutions were measured at 0, 1, 5, 10, 20, 30, and 60 minutes using a Biotek ELx808 Microplate Reader with a 490 nm filter. These protocols were repeated in triplicate and averaged for analysis.

Before analysis, calibration curves were prepared for the DPPH radical (purple) and the reduced DPPH (yellow) to account for the absorbance of both compounds at 490 nm. The DPPH radical solutions were created by accurately diluting a 0.5 mM stock solution in methanol to the appropriate concentrations, while the reduced DPPH solutions were created by accurately diluting a 0.5 mM of DPPH with 1.0 mM ascorbic acid stock solution in methanol. **Figure 14** shows the resulting calibration curves, which were used to determine the absorption coefficients of both compounds. These coefficients were then used to calculate the percent of DPPH remaining with the following equation: [((Abs – blank) – (yellow coefficient x starting [DPPH])) / (purple coefficient – yellow coefficient)] / starting [DPPH] x 100.



A. **B.**

Figure 14. Calibration curves for DPPH radical scavenging assays. *A) Calibration curve for purple-colored DPPH radical prior to antioxidant exposure. B) Calibration curve for yellow-colored reduced DPPH radical after exposure to ascorbic acid, a known antioxidant.*

To determine the EC_{50} molar ratios for each compound, the molar ratio of the compound/DPPH and 1 / percent of DPPH remaining after one hour were plotted to produce linear plots. The trendline of each plot provided an equation that was used to

determine the ratio at which 50% of DPPH would be remaining, or the EC_{50} molar ratios. The ARP was then determined using the EC_{50} values in the following equation: $1 / EC_{50}$.

Cyclic voltammetry (CV)

The methods for cyclic voltammetry were modified from those used in Masek et al[59] and are described as follows:

Cyclic voltammetry was carried out with a Gamry Interface 1010T potentiostat/galvanostat using platinum working, platinum metal counter, and Ag/AgCl reference electrodes. All measurements were carried out at room temperature in acetonitrile, with 0.1M tetrabutylammonium perchlorate (TBAP) as the supporting electrolyte. The gallate ester derivatives were evaluated in acetonitrile at 1.0 mM concentrations. For comparison, ethylparaben, butylparaben, and ferrocene were used as controls at 1.0 mM concentrations in acetonitrile. The voltammetric parameters included an initial potential of +0.50 V for gallate esters, an initial potential of +0.00 V for parabens, a vertex potential of +2.25 V for all compounds, and a scan rate of 100 mV/s for all compounds. The oxidation potential(s) for each compound was determined by subtracting the voltammogram for TBAP alone and locating the local maxima for each oxidation peak.

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