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Characterization of Phenolic Acids for Incorporation into Shape Memory Polymer Foams

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Abstract

Shape memory polymer (SMP) foams provide a promising new option for hemorrhage control on the battlefield; however, they lack a mechanism to control wound infections that can delay healing processes. Adding phenolic acids (PAs) into SMP foams provides a natural, non-drug option for incorporations of antioxidant and antimicrobial functionalities that may promote healing. However, PAs lack a systemic characterization of their structure/property relationships, and their chemical incorporation into SMP foams may alter their efficacy. In this work, a library of PAs was screened in terms of antioxidant and antimicrobial properties to provide a full understanding of how their structure affects their function. Additionally, a subset of PAs was modified with SMP foam analog monomers (modified PAs, MPAs) and compared to their unmodified counterparts. PA and MPA antioxidant capabilities were tested in terms of hydrogen peroxide scavenging capacity, and their antimicrobial activities were evaluated against both gram-positive and gram-negative bacteria strains. After testing, the PA library was narrowed down from 10 to 5 candidates with the best solubility, antioxidant properties, and antimicrobial properties. These PAs will be added into SMP foams for use in antimicrobial hemostats.

Characterization of Phenolic Acids for Incorporation into Shape Memory Polymer Foams

by

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1. Introduction

1.1 Clinical Need: Uncontrolled Hemorrhage

Among the potentially survivable battlefield injuries that resulted in death, 80% of them are due to uncontrolled hemorrhage from major trauma,[1] and up to 50% of deaths occur outside of hospital.[2] Uncontrolled hemorrhage will lead to great blood loss. Losing 50% of blood volume without resuscitation is usually fatal, while patients with reduced blood pressure (those who have lost 30% to 35% of blood volume) are close to death.[3] Therefore, it is important to quickly mitigate bleeding before one can receive hospital treatment. Additionally, almost half of traumatic wounds get polymicrobial infections on the battle field.[4] After infection, the skin-bacteria inflammatory response will release collagenases and contribute to the collagen degrading, which can delay healing.[5] The current standard of care is to deliver broad spectrum oral antibiotics after injury,[6] but this approach is ineffective at eliminating infections and is complicated by the emergence of drug-resistant bacteria strains.[7] As a result, first-aid treatments for uncontrolled hemorrhage could be improved if they could provide a localized antimicrobial effect in the wound.

1.2 Current Solutions: Benefits and Limitations

Currently, the most common medical treatment to control blood loss includes the use of gauze and tourniquets. Gauze comprises a traditional wound dressing product that simply covers wounds, and it is used for bleeding from small arteries, veins, and capillaries with applied pressure. For severe conditions like large arterial bleeding, tourniquets are used when other hemostasis methods do not work. However, these

treatments are insufficient for up to 80% of bleeds. Additionally, gauze dressings do not protect against infection and adhere to the wounds, which makes it difficult and painful to remove in the subsequent treatments. As for tourniquets, their use is limited to relative short time frames (<6 hours), as prolonged tourniquet use may lead to wound complications, including nerve damage and limb loss.[8]

Due to the limitations of gauze and tourniquets, a large number of clinically-available first-aid treatments for gunshot wounds have been introduced. There are several kinds of topical hemostatic agents including gelatin foams, thrombin, chitin, chitosan, and oxidized cellulose. Gelatin foams like Gelfoam (Pfizer), provide a physical matrix for clotting. They function effectively for small vessel bleeding, and they are biological and absorbable. However, because of their swelling capability, gelatin foams could not be used in closed spaces as they can cause nerve compression around the wound; additionally, they do not protect against infection. Thrombin is able to convert fibrinogen to fibrin to help clots form. Thrombin dressings are easy to apply and fast acting, but bovine thrombin may cause an immunologic response. Chitin and chitosan are designed for emergency cases and they function as a mechanical seal. Chitin dressings are not reliable for severe wounds and chitosan dressings still have inconsistent results in the animal testing.[9] A promising new hemostat for gunshot wounds is XStat®, an oxidized cellulose material with ~96 small sponges that can be injected into a wound. After application, the cellulose micro-sponges expand quickly and apply pressure to wound walls to cut off blood flow. However, each sponge must be individually removed from the wound within four hours of application. The design significantly increases removal times, hemostat removal can cause secondary

injury and increase infection susceptibility, and this option is limited in prolonged field care scenarios when frequent dressing changes are not feasible.[10]

1.3 Shape Memory Polymer (SMP) Foams

To address the limitations of current hemostats, we propose to develop a new, antimicrobial SMP foam. SMP foams are smart materials with multiple biomedical applications. They are fabricated as expanded, open-porous foams, and they can be heated, deformed into a secondary, compressed shape, cooled down, and stored for long time frames. When the environmental temperature is increased above their glass transition temperature (Tg), SMP foams expand rapidly from the compressed secondary shape to their original shape. By tuning monomers, chemical formulations, and foaming parameters, it is possible to customize the material's thermal, mechanical, and shape memory properties.[11]

SMP foams have excellent biocompatibility and demonstrate rapid clotting in aneurysms and peripheral vasculature.[12][13][14][15][16] Previous work has focused on incorporating new functionalities into SMP foams, including degradability and X-ray imaging capabilities.[17][18][19][12] Due to their excellent clotting capabilities, researchers have also previously explored the use of SMP foams for hemorrhage control. A device was developed that combines SMP foams and iodine-containing poly (ethylene glycol) (PEG) hydrogels with the capacity for rapid clotting and swelling.[18] The results showed that the PEG-based SMP foams with iodine maintain shape memory behavior, increase water absorption, and reduce bacteria viability. However, inclusion of the hydrogel significantly slowed down foam expansion, which would limit this material's utility

on the battlefield. Additionally, research suggests that iodine can be detrimental to wound healing,[20] which makes exploration of other antimicrobial options a necessity.

1.4 Phenolic Acids

Due to the limitations of traditional antibiotics, there is a growing interest in nondrug-based techniques to fabricate antimicrobial medical devices. Honey has long been used to treat a variety of wounds, including burns and ulcers that are infected or chronic.[21] Honey dressings can speed up the healing of pressure wounds and they are also used to reduce wound stench.[22] These effects are due in part to two kinds of essential antimicrobial substances in honey, flavonoids and phenolic acids (PAs). It has been shown that high concentrations of flavonoids and PAs can improve honey's antimicrobial capabilities.[22]

PAs contains a carboxylic acid group on their non-active end that enables their incorporation into the polyurethane SMP foam system; thus, they were selected for further exploration in these studies. A number of studies have been performed on PA antimicrobial characterization; however, many studies focus on the use of PAs in relation to food-borne illnesses and plant pathogens rather than common wound bacteria, such as *E. coli*, *Staph. epi.*, and *Staph. aureus*. No PAs have been characterized against all strains of interest, many are lacking data on drug-resistant strains, and some of the data is conflicting (e.g. ferulic and vanillic acids were determined to be effective against *E. coli* in studies performed by Merkl et al. but not in those performed by Chatterjee et al.).[23] [24] Finally, a connection between the PA structure and antimicrobial efficacy is lacking.

Phenolic acids, as secondary metabolites of plants that could kill microorganisms or inhibit the growth of bacteria, have been involved in the plant's antimicrobial mechanisms. Inhibitory mechanisms of phenolic acids on bacteria growth include, but are not limited to, destabilizing the bacteria cytoplasmic membrane, altering the permeability of the bacteria plasma membrane, inhibition of the extracellular microbial enzymes, direct effects on microbial metabolism, and the deprivation of the substrate required for microbial growth.[25] Firstly, phenolic acids could affect bacteria cells by changing their physicochemical surface properties. For example, ferulic acid could promote the decrease of hydrophobic character of P. aeruginosa.[26] Phenolic acid treatment could also make gram-positive bacteria strains acquire polar character by measuring the values of the bacteria's surface tension components. Phenolic acids have effects on bacteria's surface electron receptor of both gram-positive (increased receptor components) and gram-negative (decreased receptor components) bacteria strains. [26] Secondly, phenolic acids have effects on the integrity of bacterial cytoplasmic membrane. As the concentrations of phenolic acids increase, the percentage of cell membrane damage shows a significant increase. But different phenolic acids have various effects on gram-positive and gram-negative bacteria strains. Last but not least, the promotion on the release of potassium in bacterial cells is also a part of phenolic acid's antimicrobial mechanisms. The control groups show more intracellular K^+ residual compared with groups after phenolic acid treatment. And the release of K^+ by gram-negative bacteria strains is more than for the gram-positive bacteria strains.[26]

Previously, cinnamic acid (CA) was incorporated into SMP foams, providing a material with tunable and clinically-relevant thermal and shape memory properties.[27] Phenolic

acids including CA don't need to be released or solubilized from the SMP network to be functional. Because all PAs are composed of a carboxylic group (for incorporating PAs into the SMP network) and a benzene ring linked with hydrogen donating groups (functional group). Therefore, PAs are still effective after tethering to the SMP network, because only carboxylic group changes during the synthesis process, their functional group and our desired properties stay. CA-based foams retained high cytocompatibility and reduced *E. coli* and *Staph. epi.* growth, indicating the feasibility of use of PAs to provide antimicrobial SMPs. In addition to their antimicrobial properties, PAs contain hydrogen donating groups that can react with oxidants to form resonance-stabilized phenoxyl radicals and provide antioxidant properties that could further improve healing outcomes.[23][28][29][30] PAs, as antioxidants, play an essential role in traumatic wound healing. Because PAs could react with reactive oxygen species (ROS) released from neutrophils and macrophages.[31] The excessive amount of ROS is always present in chronic wounds, and a great method to interrupt chronic inflammatory cycle is removing ROS with antioxidants.[31] In addition to that, ROS are able to create highly oxidizing environments for traumatic wounds, and the drastically increase of the ROS production can cause human body's oxidative stress response leading to critical illness (for example, organ dysfunction, disseminated intravascular coagulation, and the presence of an acute phase response).[30][32][33][34] Although ROS are considered to have certain beneficial antimicrobial properties, prolonged exposure of acute and chronic wounds to high levels of ROS causes cell damage and inhibits wound healing.[31] Using honey-based PAs, as free radical scavengers that could block the ROS-induced cytotoxicity, to lower the concentration of ROS could help decrease lipid peroxidation, damage to DNA, and cell

death.[31] The long-term goal of our research is to incorporate honey based phenolic acids into the SMP networks to provide antioxidant and antimicrobial scaffolds. PA-containing SMPs are expected to promote traumatic healing and lower the risk of bacterial infection of gram-positive, gram-negative, and drug-resistant bacteria strains.

To reach this goal, the focus of this thesis is to characterize a library of PAs and establish their structure/property relationships. This work will aid in rational design of PA-containing biomaterials with desired functionality. Ten PAs were selected to characterize in terms of antimicrobial and antioxidant properties, **Figure 1**. The PAs without pendant hydroxyl (OH) groups on the ring structures (benzoic acid (BA) and CA) are not expected to demonstrate antioxidant properties. Additional BA- and CA-based PAs were chosen with varied pendant groups (1 OH group, 1 OH group with 1 or 2 methoxy groups, and 2 OH groups). All selected PAs in this research are expected to have varying degrees of antimicrobial efficacy, and antioxidant properties are hypothesized to be tied to pendant group chemistry.

This study fills in understanding the properties of the PAs with pendent group variations, providing relationships between structure and properties by screening the 10 selected PAs in terms of antimicrobial properties against 5 common pathogenic wound bacteria and antioxidant properties. An additional consideration is reaction of the carboxylic acid group on PAs during SMP synthesis and its potential effects on PA functionality. To address this issue, PAs that were modified with foaming monomer analogs were characterized in parallel to the unmodified PAs.

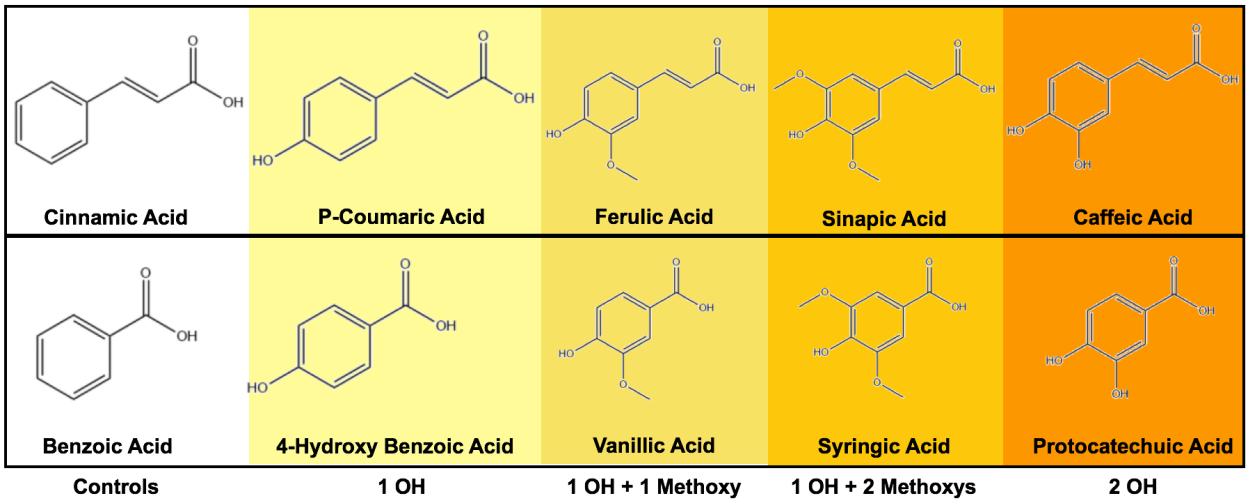


Figure 1. Ten selected PAs and their chemical structures. Top row: CA-based PAs; Bottom row: BA-based PAs.

2. Methods and Materials

2.1 Modified Phenolic Acid (MPA) Synthesis

Approximately 3 g of each PA was dried overnight under vacuum and dissolved in anhydrous dimethyl sulfoxide (DMSO) in a reaction flask at ~0.1 g/ml. Hexyl isocyanate (HI) (1.1 molar equivalents) was added dropwise to the reaction under anhydrous conditions. The solution was reacted under nitrogen at 65°C for ~72 hours, or until the isocyanates were fully reacted, as indicated by a disappearance of the isocyanate peak at ~2250 cm^{-1} in the Fourier transform infrared spectroscopy (FTIR) spectra of the reaction contents.

Complete reaction resulted in separation of the reaction contents into a 'viscous' and 'liquid' portion. Gravity filtration was applied to the solution with a short stem funnel and filter paper to separate the viscous and liquid portions. The product was precipitated from the filtered liquid portion in cold water at a 1:5 volume ratio (liquid portion: cold water). Then, the solution was centrifuged in 4000 rpm for 15 minutes. The target MPA product was separated from the solvent and dried under vacuum overnight. The MPA structure was analyzed using FTIR and nuclear magnetic resonance (NMR) spectroscopy.

2.2 PA and MPA Antioxidant Test

The antioxidant capacity of the 10 PAs and corresponding MPAs was measured based on their H_2O_2 scavenging capabilities as previously described. All selected PAs and MPAs were dissolved in DMSO at 5 mg/ml. A 0.002% H_2O_2 solution was prepared in water, a 0.1M phosphate-buffered saline (PBS) solution was prepared in water, dying agents (phenol red dye and horseradish peroxidase) were dissolved in PBS at 0.2 mg/ml

and 0.1 mg/ml, respectively, and a 1M sodium hydroxide solution was prepared in water. Each PA was serially diluted from 5 to 0.078 mg/ml in 10 µl DMSO in wells of a 96-well plate, as shown in **Figure 2**. Blank solutions (10 ul DMSO) were also included in each plate. Then, 10 µl of the prepared H_2O_2 solution and 80 µl of PBS were added to each sample well. After 10 minutes incubation at 37°C, 100 µl of the prepared dying agents was added. Subsequently, the plate was incubated for 15 minutes in at 37°C, and 5 µl of the prepared sodium hydroxide solution was added to each sample well. Hydrogen peroxide scavenging was analyzed immediately after addition of sodium hydroxide using a plate reader at an absorbance of 610 nm (O.D. 610). Each sample was tested in triplicate, and all selected PAs and MPAs were tested with this method.

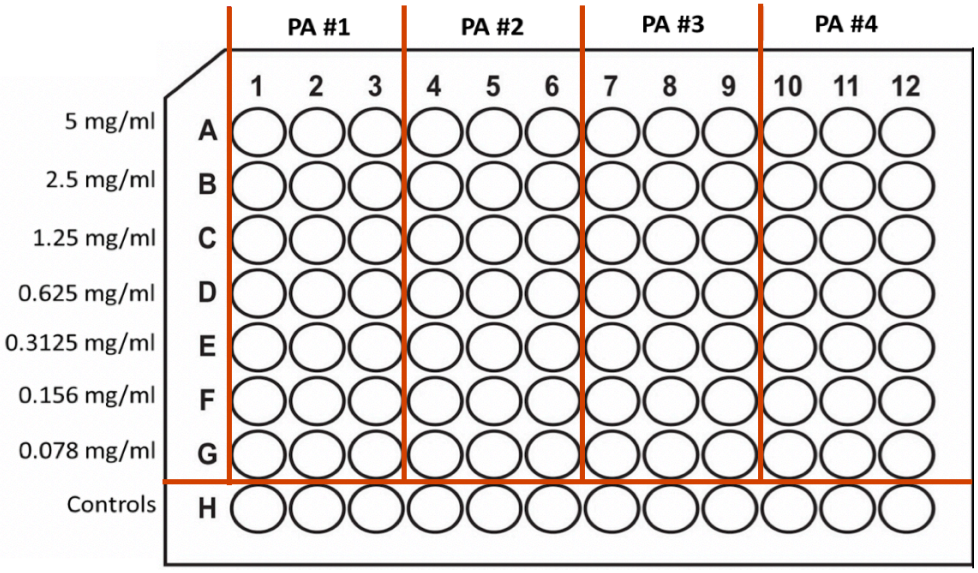


Figure 2. General test setup for antioxidant and antimicrobial analysis on 96 well plate. Each acid was tested in triplicate in columns 1-3, 4-6, 7-9, and 10-12 (4 acids per plate). Samples were serially diluted from A to G; row H served as a control with no PA or MPA.

The antioxidant properties of PAs were quantified in terms of H_2O_2 scavenging activity (Hs) using **Equation 1**:

$$H_s = 100\% \times [C_0 - (C_{0.05} \times C_0)] \quad \textbf{Equation 1}$$

where C_0 is the absorbance value of the control group with no PA and $C_{0.05}$ is the absorbance value of the PA sample at the specific concentration.

2.3 PA and MPA Antimicrobial Tests

2.3.1 Preparation of Bacteria Strains

In this study, we used *Escherichia coli* (*E. coli*), *Staphylococcus epidermidis* (*Staph. epi*., native and drug-resistant) and *Staphylococcus aureus* (*Staph. aureus*, native and drug-resistant) to test the efficacy of PA antimicrobial properties. Before incubating with PAs, bacteria strains were grown in 5 ml of fresh LB broth (prepared at 25 g/L of deionized water and autoclaved) at 37°C for ~16-17 hours. Subsequently, 1ml of the 5ml bacteria medium was taken and cultured in 9 ml of fresh LB broth until bacteria reached the logarithmic growth period when optical density at an absorbance of 600 nm (O.D. 600) value equals 0.6. The O.D. value was measured using a plate reader.

2.3.2 Multi-Well Plate Assay

2.3.2.1 DMSO Bacterial toxicity

Before testing PAs antimicrobial performance, each bacteria strain was cultured with a range of concentrations of DMSO in multi-well plates. This testing was to ensure that DMSO does not affect the growth of bacteria strains and interfere with measurements of PA antimicrobial efficacy, because PAs are dissolved in DMSO prior to being added to

bacteria. Different volumes (0, 1, 2, 4, 8, 16, 32, 64, and 128 µl) of DMSO were added to LB broth in a well; each well contained a total of 200 µl of DMSO/LB broth solution. Each concentration of DMSO was tested in triplicate. Then, 20 µl of the prepared log-phase growth bacteria medium was added into each well. The plate was placed in a 37°C shaker table for 24 hours. Bacteria growth was analyzed via plate reader absorbance readings at O.D. 600 at 0, 1, 2, 4, and 24 hours with a goal of finding the maximum volume of DMSO that could be used in PA testing without affecting the growth of bacteria (comparable O.D. 600 value to 0 µl samples).

2.3.2.2 PA Characterization

From the DMSO test, it was found that *E. coli* could be exposed to 5 µl DMSO, and *Staph. epi.* and *Staph. aureus* could be exposed to 10 µl DMSO without negatively affecting growth. Therefore, PAs were serially diluted from 5 to 0.078 mg/ml in 5 µl DMSO and 95 µl of fresh LB broth for *E. coli* tests and in 10 µl DMSO and 90 µl of fresh LB broth for *Staph. epi.* and *Staph. aureus* tests. Controls included wells with 5 or 10 µl DMSO in 95 or 90 µl of fresh LB broth (DMSO control), 100 µl LB broth (LB control), and a 1% penicillin-streptomycin solution in 5 or 10 µl DMSO and LB broth (drug control). For the drug-resistant strains, 1% methicillin or oxacillin solution was prepared in DMSO and LB broth as additional drug controls. After that, 20 µl of prepared log-phase growth bacteria medium and 100 µl fresh LB broth was added to all wells in the plate. Bacterial growth was tested via absorbance readings at O.D. 600 at 0, 1, 2, 4, and 24 hours. Every sample was tested in triplicate, and all 10 selected PAs were tested using this method. Results were analyzed in terms of IC50 value and the log reduction values.

IC50 is the measure of the concentration of antimicrobial agent required to kill 50% of bacteria. It illustrates potency of inhibiting bacteria growth and provides information on minimal concentrations required for future incorporation into antimicrobial SMP scaffolds. IC50 was calculated with Using **Equations 2** and **3**:

$$Half\ concentration = \frac{C_0 - C_1}{2}$$ **Equation 2**

$$IC50 = \frac{Half\ concentration - b}{a}$$ **Equation 3**

where C_0 is the O.D. value of DMSO group; C_1 is the O.D. value of the drug control group; b is the y-intercept of the bacteria's growth curve relative to PA concentration; and a is the slope of the bacteria's growth curve relative to PA concentration.

Log reduction is the measure of how a specified concentration of antimicrobial reduces bacteria concentration and was calculated using **Equation 4**:

$$Log\ reduction = Log_{10}(\frac{C_0}{C_1})$$ **Equation 4**

where C_0 is the O.D. value of the DMSO control and C_1 is the O.D. value of a PA or MPA.

2.3.2.3 Petri Dish Assay

Due to low solubility of PAs in aqueous solvents, sediment was observed in some wells with higher concentration of PA samples, which affected absorbance readings in the multi-well plate assay. Currently synthesized MPAs (modified benzoic acid, syringic

acid, cinnamic acid, and p-coumaric acid) also had low solubility, which affected absorbance readings. To address this issue, a Petri dish assay was applied to quantify the antimicrobial properties of MPAs and PAs with low solubility.

LB-agar (LB at 20 g/L, agar at 15 g/L) was prepared in deionized water, autoclaved, poured into Petri dishes, and allowed to gel overnight. Each PA for which sediment was observed in a study with a specific bacteria strain was selected. Every selected PA was dissolved at 5 mg/ml in 20 µl DMSO and mixed with 380 µl fresh LB broth in a 24 well plate. Fresh LB broth (400 µl) and 20 µl DMSO in 380 µl LB broth were used as controls. Subsequently, 40 µl of prepared log-phase growth bacteria medium was added to each well in the plate. Each sample was tested in triplicate.

At 2, 4, and 24 hours, 10 µl of the bacteria medium was extracted from each sample well and diluted by 10,000 in fresh LB. Then, 100 µl of the diluted solution was applied to the surface of Petri dishes and cultured for 24 hours at 37°C. Photographs were obtained of each plate surface after culturing, and ImageJ software was used to quantify colony forming units (CFUs) and analyze bacteria growth.

3. Results and Discussion

3.1 PA and MPA Antioxidant Properties

In general, reaction of hydroxyl groups with oxidizing agents (e.g. reactive oxygen species) forms resonance-stable phenoxy radicals, which enables phenols to have antioxidant properties.[29] Therefore, we hypothesized that PAs that do not contain pendant hydroxyl (OH) groups (cinnamic and benzoic acid controls) would not demonstrate antioxidant capability. In this experiment, hydrogen peroxide (H_2O_2) was utilized as an oxidizing agent to quantify the antioxidant properties of 10 selected PAs. Then, the antioxidant properties of PAs were quantified in terms of H_2O_2 scavenging activity. Successful H_2O_2 scavenging was taken to be greater than 50%.[28]

Figure 3 shows the H_2O_2 scavenging capabilities of both benzoic acid (BA) and cinnamic acid (CA) groups. From these results, the relative antioxidant capacity of PAs can be seen in terms of H_2O_2 scavenging capabilities. For BA group, **Figure 3A**, BA does not demonstrate antioxidant capacity, and 4-hydroxy benzoic acid (4-HBA) with only one OH group has the second lowest antioxidant capacity. These results are in line with the hypothesis. With the addition of methoxy and additional hydroxyl groups in protocatechuic acid (PA), syringic acid (SYA) and vanillic acid (VA), their antioxidant efficacy is significantly increased. The addition of functional pendant groups on PA, SYA, and VA enables more effective formation of resonance-stabilized phenoxy radicals, which imparts stronger antioxidant capacity. As for CA group, **Figure 3B**, CA does not demonstrate antioxidant capabilities, as expected. The addition of hydrogen donating groups onto the

other CA-based PAs resulted in significantly improved antioxidant capabilities compared with CA.

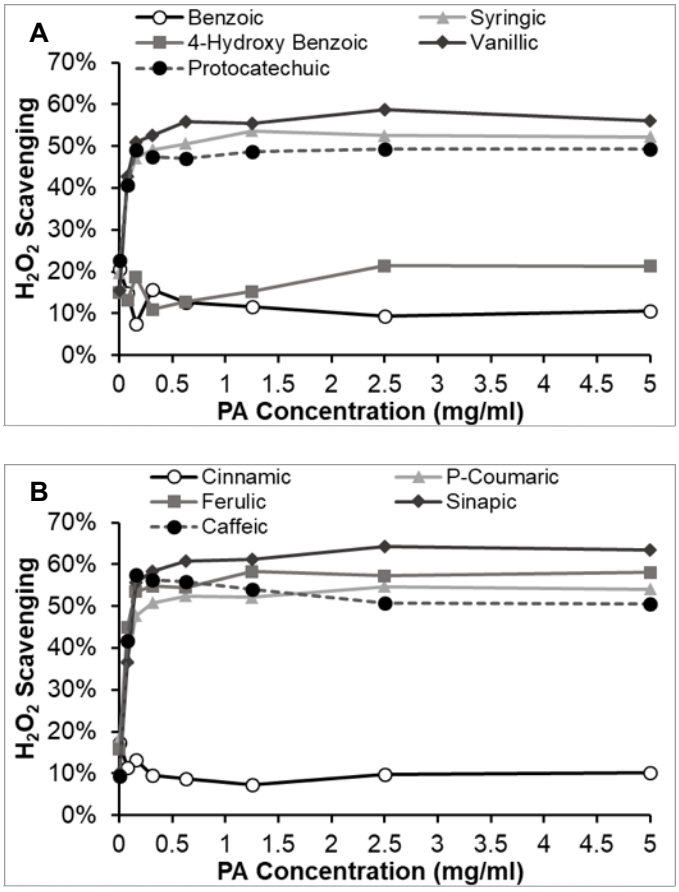


Figure 3. Hydrogen peroxide scavenging capabilities of PAs from the (A) Benzoic acid group and (B) Cinnamic acid group.

Upon characterization of unmodified PA antioxidant capabilities, the antioxidant properties of modified PAs (MPAs) were characterized to test whether PAs maintain their antioxidant capacity after the chemical modification required for incorporation into SMP foams. **Figure 4** shows the H_2O_2 scavenging capabilities of currently synthesized MPAs, modified benzoic and syiringic acids from the BA group (**Figure 4A**) and modified cinnamic and p-coumaric acids from the CA group (**Figure 4B**). From **Figure 4**, it can be

seen that modified BA retains low antioxidant capacity at levels that are similar to those of BA. Similarly, syringic acid and modified syringic acid have comparable antioxidant efficacy. The CA group shows the same trend in that CA and modified CA both have low H_2O_2 scavenging capabilities, while p-coumaric acid (P-CA) and modified P-CA have similarly increased antioxidant properties. These results indicate that the chemical modification of PAs that is required for their incorporation into SMP foams will not alter their antioxidant capacity, enabling future synthesis of antioxidant SMP scaffolds. Antioxidant SMP scaffold, as polymeric medical devices with extended biostability, have various potential applications. Additives in SMPs that have oxygen radical scavenging capabilities could inhibit the oxidative mechanisms of degradation, which can cause scission and crosslinking of polyurethane chains.[35] With the presence of antioxidants in the network, it is possible to control the SMP's degradation. Therefore, with controlled degrading rate, the SMPs could be made as microparticles in the drug delivery system. For example, the SMPs with antioxidants could deliver superoxide dismutase to a model of lung fibrosis without inflammatory response.[36] Except for the applications in the aspect of degradation, the antioxidant SMP scaffolds also have healing benefits. Studies have shown that the onset of varieties of diseases such as rheumatoid arthritis, atherosclerosis, and cancer is related to the presence of free radicals and excess reactive oxygen species. The development of SMP scaffolds with antioxidants could help lower the concentration of these compounds. Moreover, the excess amount of reactive oxygen species is able to prevent the process of wound healing, the antioxidants-present SMPs could help overcome this problem.[37]

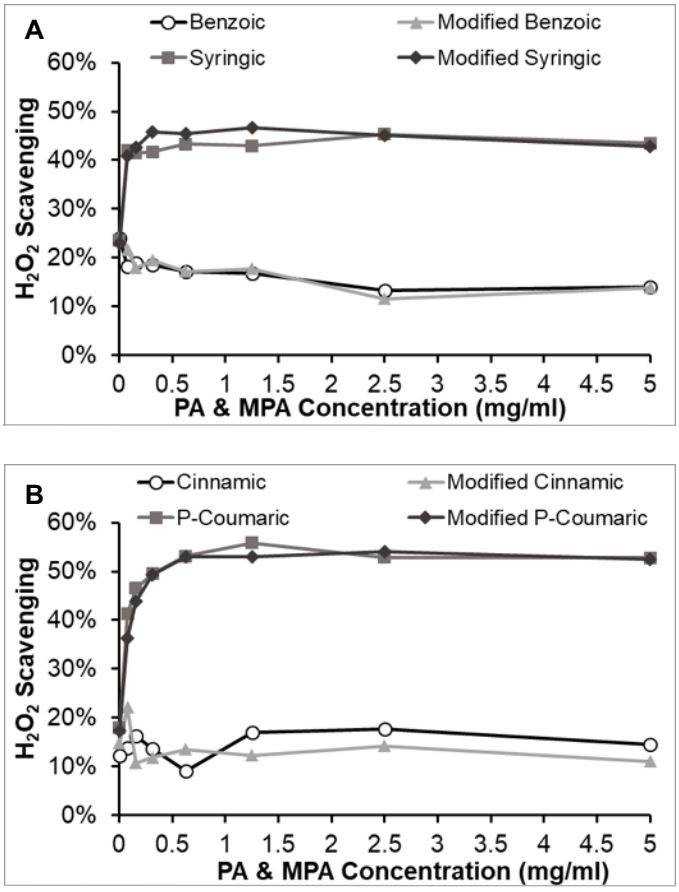


Figure 4. Hydrogen peroxide scavenging capabilities of MPAs from the (A) Benzoic acid group and (B) Cinnamic acid group in comparison to their unmodified controls.

3.2 PA and MPA Antimicrobial Properties

3.2.1 DMSO Bacterial Toxicity

Before testing PA and MPA antimicrobial properties, a test was run with each bacteria strain to find the maximum volume of DMSO that could be used to dissolve PAs and MPAs without affecting bacteria growth. **Figure 5** shows the DMSO test results over 24 hours with the 5 selected bacteria strains (*E. coli*; *Staph. epi.*, native and drug-resistant;

Staph. aureus, native and drug-resistant). From the data, we could see that the different bacteria strains have variable maximum DMSO volume tolerance. For *E. coli* in **Figure 5A**, the O.D. values (i.e. bacteria density) with exposure to 8 µl DMSO are comparable to those of the control group (0 µl DMSO) over 24 hours; however, the O.D. values were greatly decreased compared to the control group with exposure to 16 µl DMSO. Therefore, each concentration of PAs was dissolved in 5 µl DMSO for *E. coli* tests to ensure that DMSO did not affect PA characterization. In **Figure 5B**, it can be seen that the O.D. values for *Staph. epi*. native strain did not show great decrease compared with the control group over 24 hours with exposure to up to 16 µl DMSO. In **Figure 5C**, 16 µl DMSO did not affect *Staph. epi*. drug-resistant strain growth at 4 hours, and a slight increase in growth can be observed at 24 hours with 16 µl DMSO. These results were attributed to the fact that different organisms will respond individually to the same amount and concentration of one particular organic solvent.[38] Therefore, PAs were dissolved in 10 µl DMSO for both *Staph. epi*. native and drug-resistant strains. From **Figure 5D**, 16 µl DMSO did not greatly affect the O.D. value for *Staph. aureus* over 4 hours compared with the control group, but bacteria growth was decreased at 24 hours for all concentrations of DMSO. From **Figure 5E**, 16 µl DMSO slightly decreased the O.D. values for drug-resistant *Staph. aureus* over 24 hours, to the lesser extent than the 32 µl DMSO. In consideration of PA's solubility and these results, PAs were dissolved in 10 µl DMSO for testing with *Staph. aureus* native and drug-resistant strains.

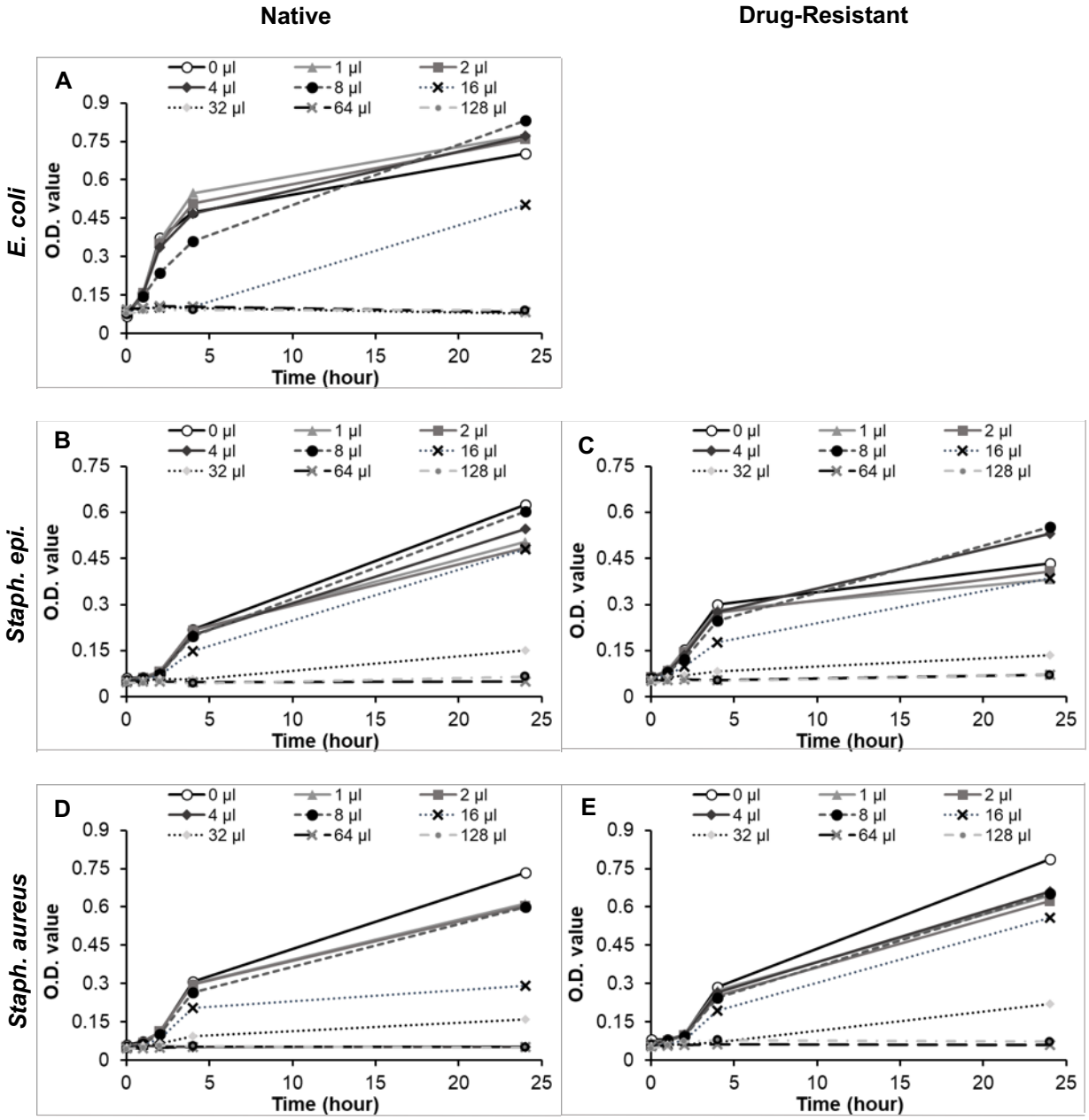


Figure 5. DMSO maximum volume test over 24 hours with (A) *E. coli*, (B) *Staph. epi.* native strain, (C) *Staph. epi.* drug-resistant strain, (D) *Staph. aureus* native strain, and (E) *Staph. aureus* drug-resistant strain.

3.2.2 PA IC50

Upon determination of appropriate amounts of DMSO for each test, the antimicrobial properties of PAs and MPAs were characterized in terms of IC50 value (concentration required to kill 50% of cells) and log reduction value (number of cells killed in comparison with media control).

The IC50 results are shown in **Figures 6-7**. The lack of data for CA and CAA is because of limited solubility and color change that affected absorbance measurements. From **Figure 6A** and **6B**, it can be seen that the IC50 value of each PA have increased from 4 to 24 hours, which means PA antimicrobial capabilities generally decrease with time for *E. coli* and *Staph. epi.*, In particular, protocatechuic acid (PA) and syringic acid (SYA) in the BA group and ferulic acid (FA) and sinapic acid (SA) in the CA group show large decreases in antimicrobial properties with time. These reductions in efficacy over time are likely due to a combination of bacterial uptake of PA to reduce effective concentration during live bacterial growth. As shown in **Figure 6C**, the IC50 value of each PA have decreased between 4 to 24 hours, which means PA's antimicrobial capabilities increase with time for *Staph. aureus*. From **Figure 7A**, we can see that the IC50 value of each PA decreased from 4 to 24 hours for drug-resistant *Staph. epi.*, indicating that PAs have increased antimicrobial capabilities over time with this bacterial strain. The opposite trend can be viewed in **Figure 7B**, where PA IC50s increase from 4 to 24 hours, indicating decreased antimicrobial capacity over time with drug-resistant *Staph. aureus*.

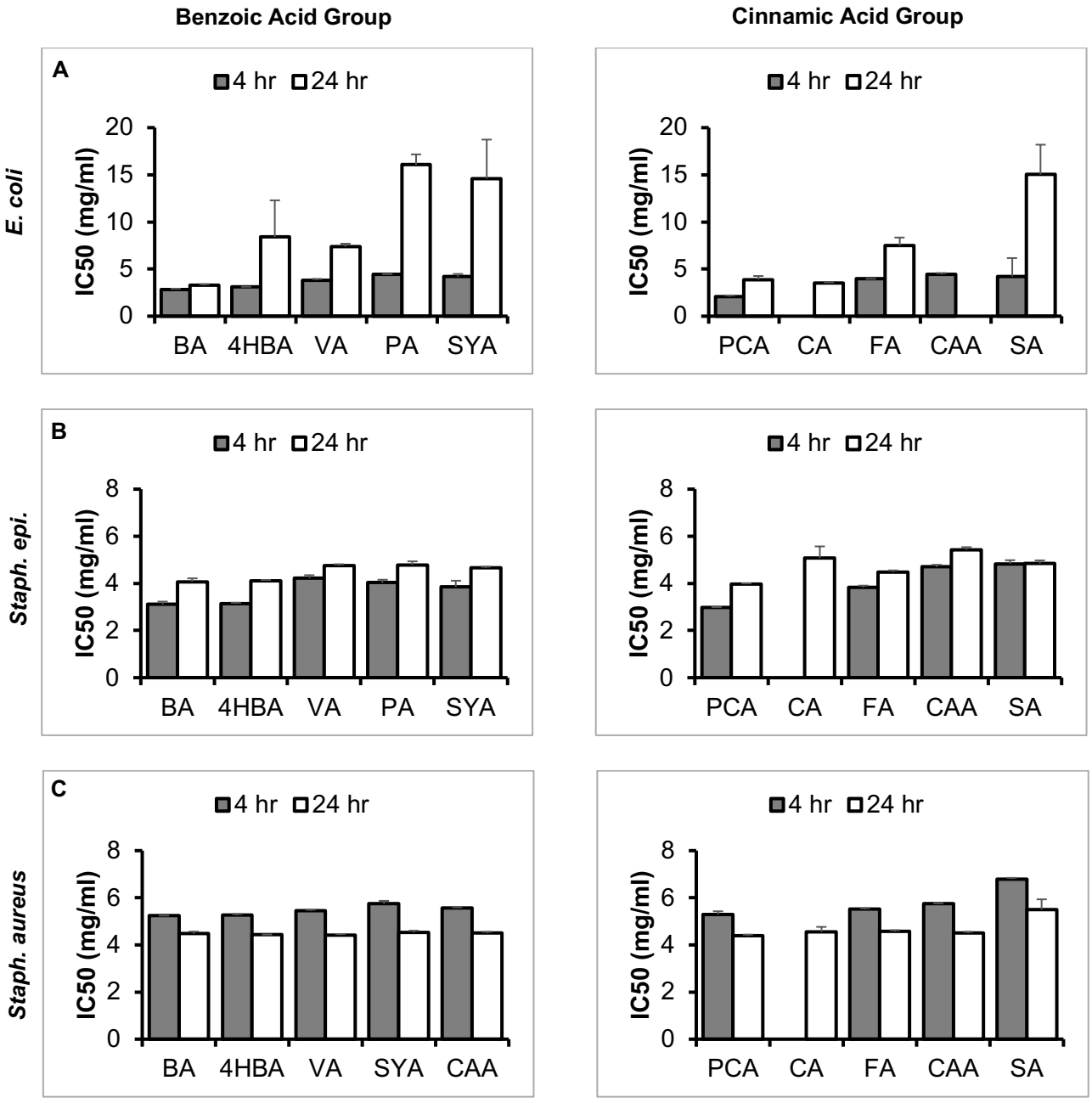


Figure 6. IC50 results of (left) Benzoic acid group and (right) Cinnamic acid group against native (A) *E. coli*, (B) *Staph. epi.*, and (C) *Staph. aureus*.

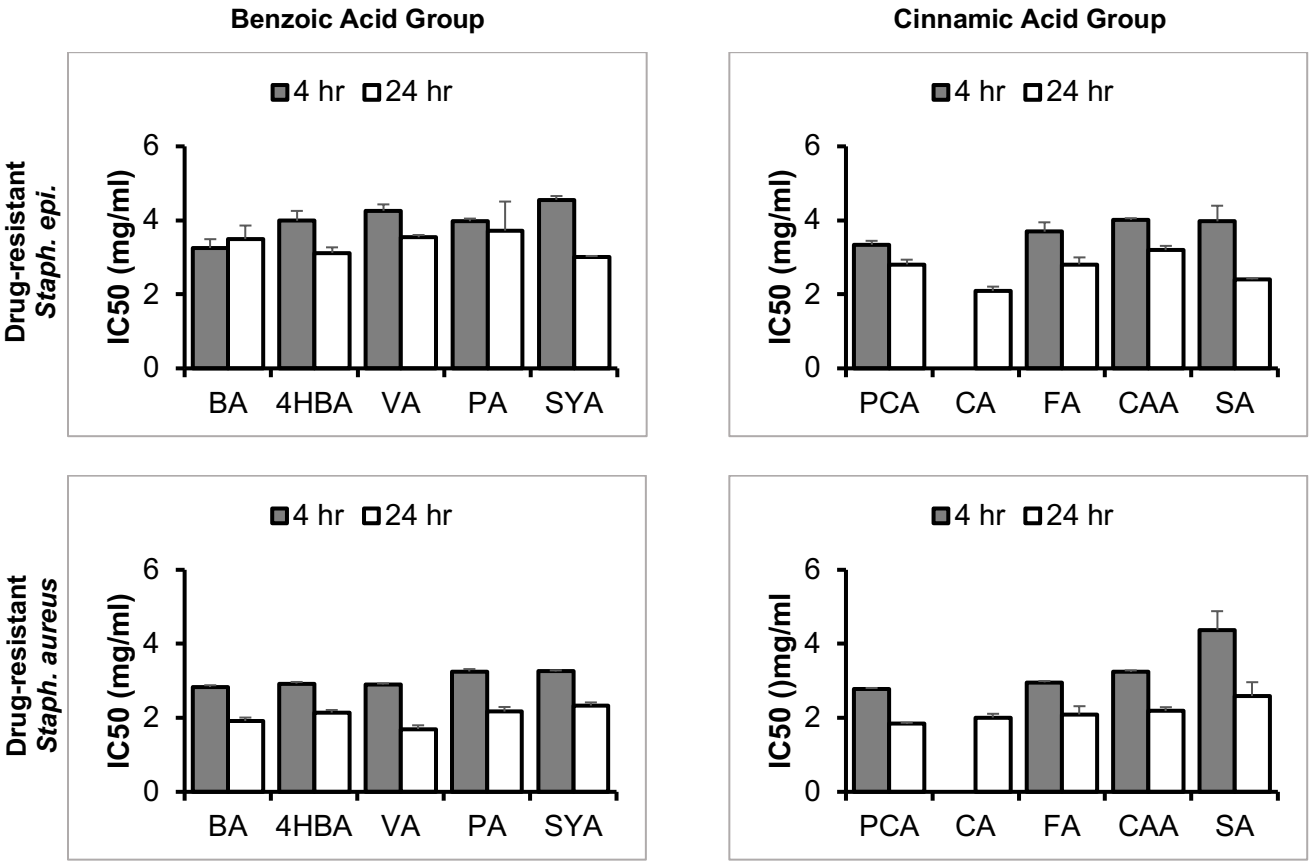


Figure 7. IC50 results of (left) Benzoic acid group and (right) Cinnamic acid group against drug-resistant (A) *Staph. epi.* and (B) *Staph. aureus*.

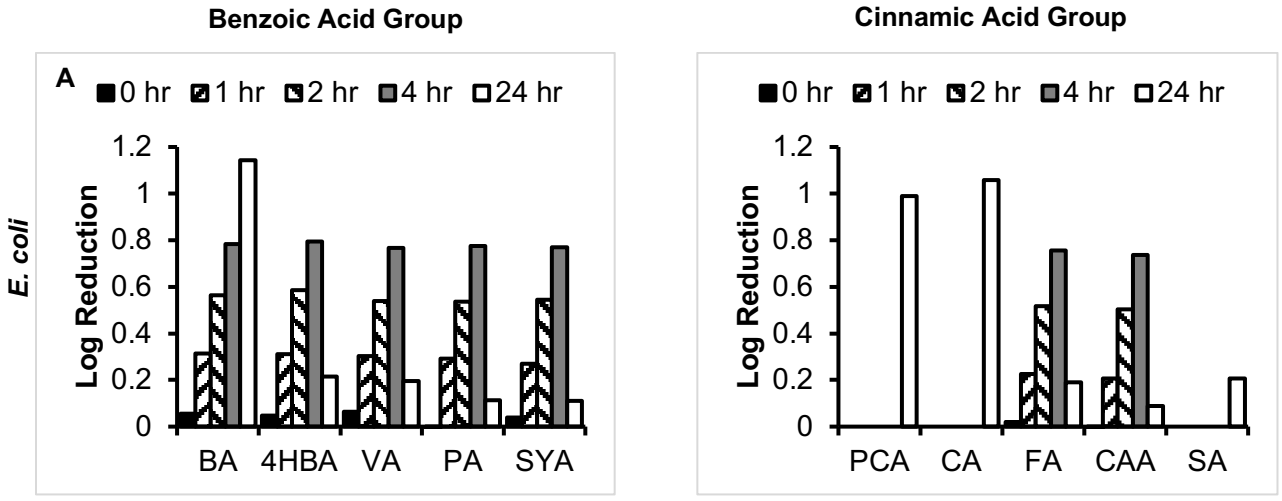
It could be observed from the experiment that CA solubility improved over time in the presence of bacteria strains, and CA solubility didn't change without the presence of bacteria. This finding could provide the basis of future studies, for example, an experiment could be designed to test if the solubility change is due to digestion by bacterial proteases, and a mutant strain that doesn't release protease could be applied in the test. Also, it could be tested if CA is taken up over time, spectroscopy techniques could be applied to analyze CA's concentration over time.

Three important results that can be gleaned from this data include: (1) All PAs show some efficacy against all tested bacteria strains, indicating their potential for use in wound infection prevention. (2) PA IC50 values are within the range of possible incorporation into SMP scaffolds, and (3) PAs are equally effective against native and drug-resistant strains of *Staph. epi.* and *Staph. aureus*. Beyond this project, this study provides essential information that could be utilized in PA use in other biomaterial systems to provide localized antimicrobial properties.

Except for the polymers with improved antimicrobial properties demonstrated above, phenolic acids could also incorporate with natural rubber (NR) forming SMPs with tunable triggering temperature. They can form a epoxidized natural rubber crosslinked network with excellent shape memory effect.[39]

3.2.3 Log Reduction

Bacterial log reductions in the presence of the selected PAs are shown in **Figures 8-9**.



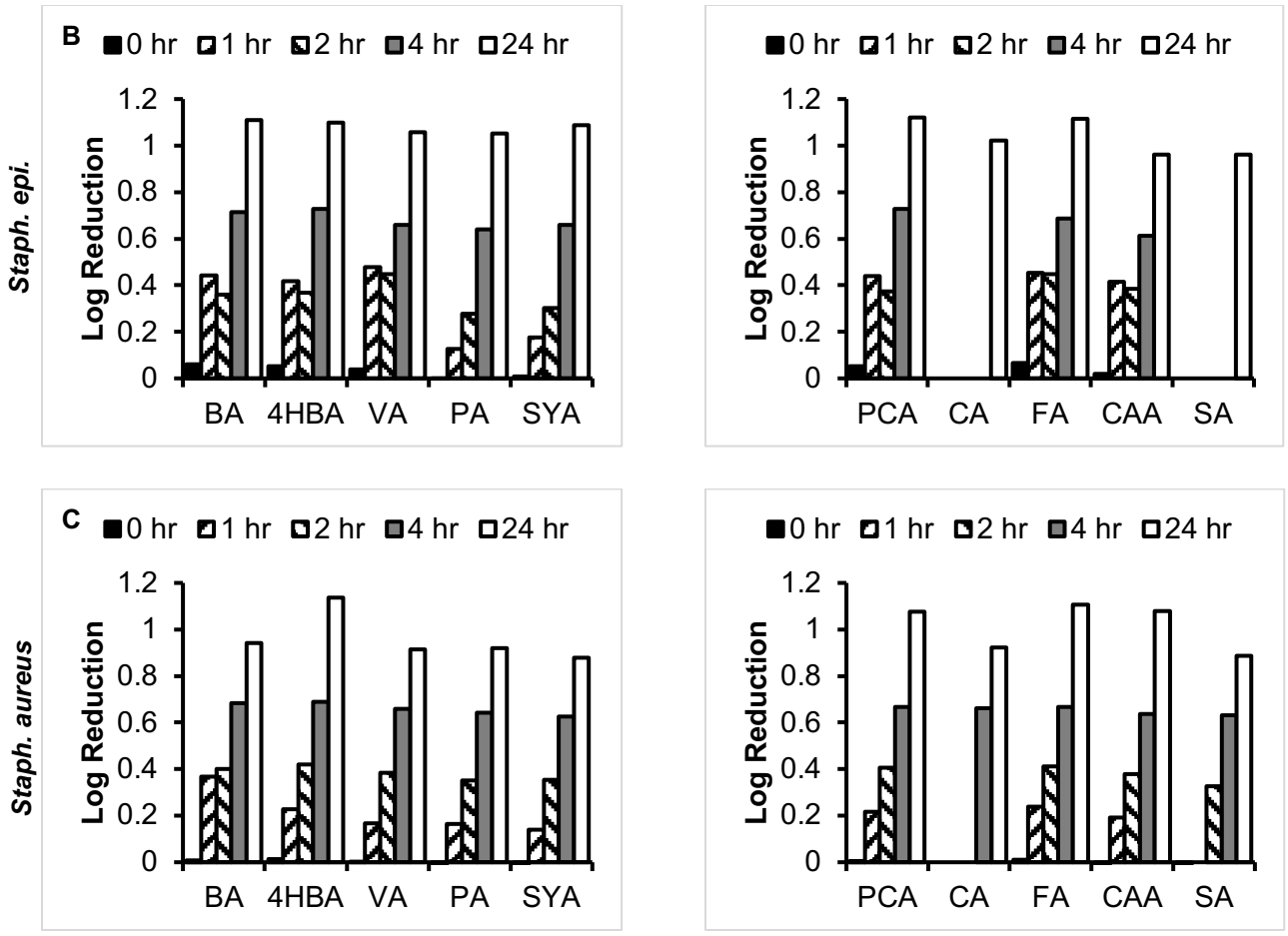
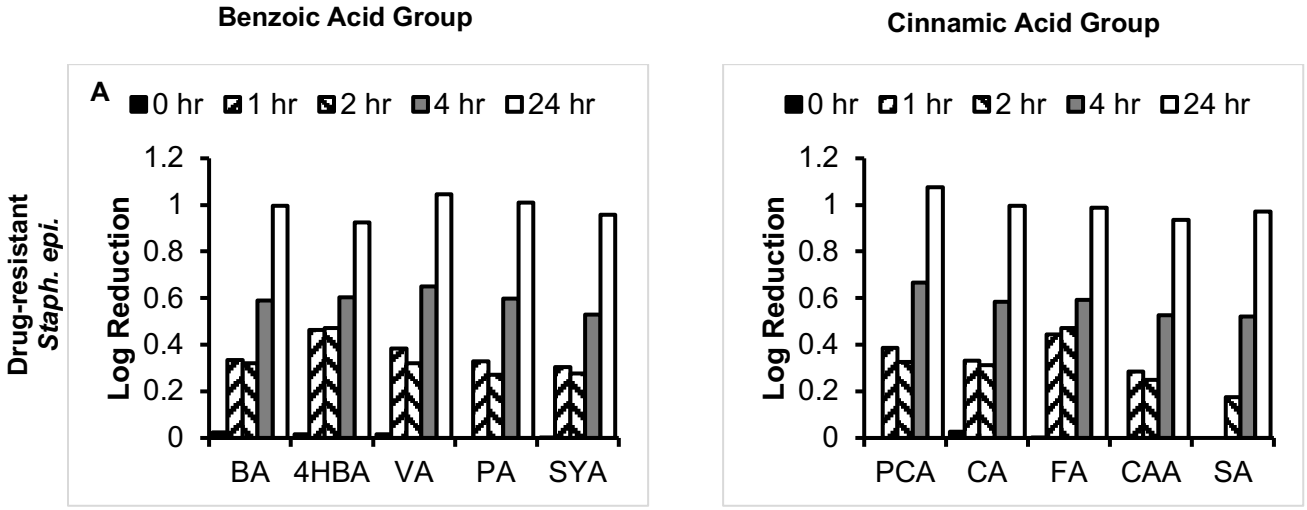


Figure 8. Native (A) *E. coli*, (B) *Staph. epi.*, and (C) *Staph. aureus* log reduction in presence of (left) Benzoic acid group and (right) Cinnamic acid group at 5 mg PA/ml.



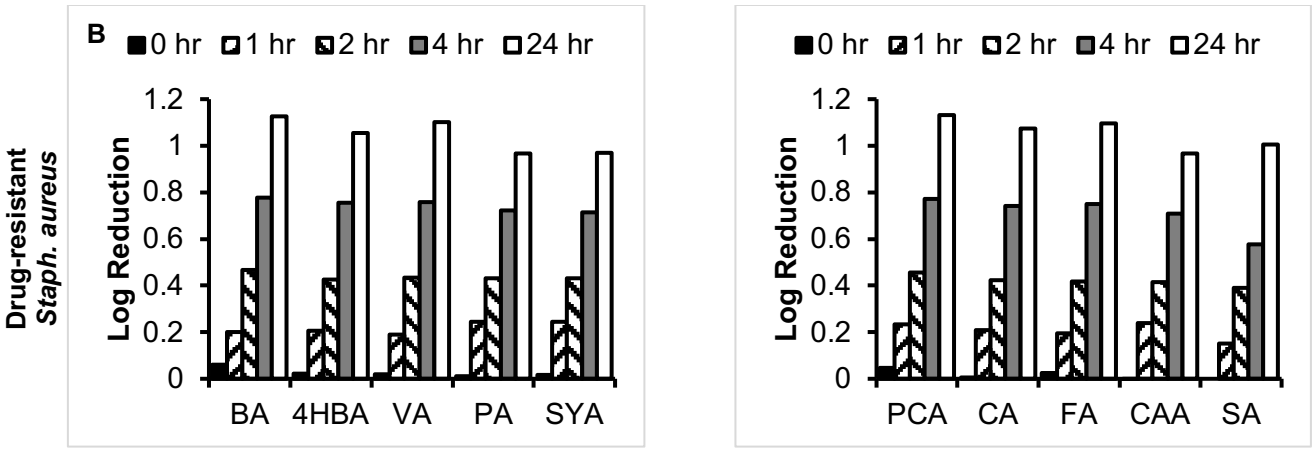


Figure 9. Drug-resistant (A) *Staph. epi.* and (B) *Staph. aureus* log reduction in presence of (left) Benzoic acid group and (right) Cinnamic acid group at 5 mg PA/ml.

The data collected in **Figure 8** is based on the O.D. value of 5 mg/ml PA solutions collected in the 96 well plate, the lack of some data is due to solubility issues that affected absorbance measurements. In general, a larger log reduction corresponds with improved antimicrobial capacity. From **Figure 8A**, except for the PAs without log reduction data, only BA shows consistently increased antimicrobial capability over the full 24 hours for *E. coli.* However, all PAs with available data show a log reduction of ~0.8 at 4 hours, which is comparable to that of the drug control, indicating initial antimicrobial properties at clinically effective levels. In contrast, **Figure 8B** and **8C** show that the log reduction value and antimicrobial capability of all tested PAs increased with time for *Staph. epi.* and *Staph. aureus*. These differences are likely due to differences in bacterial growth rates. Additionally, the uptake of PAs into gram positive bacteria (*Staph. epi.* and *Staph. aureus*) may be more effective than that into gram negative *E. coli* due to differences in the cell walls. Based on the potential mechanisms of PA's antimicrobial properties, the differences in bacterial cell cytoplasmic membranes can indeed affect the antibacterial

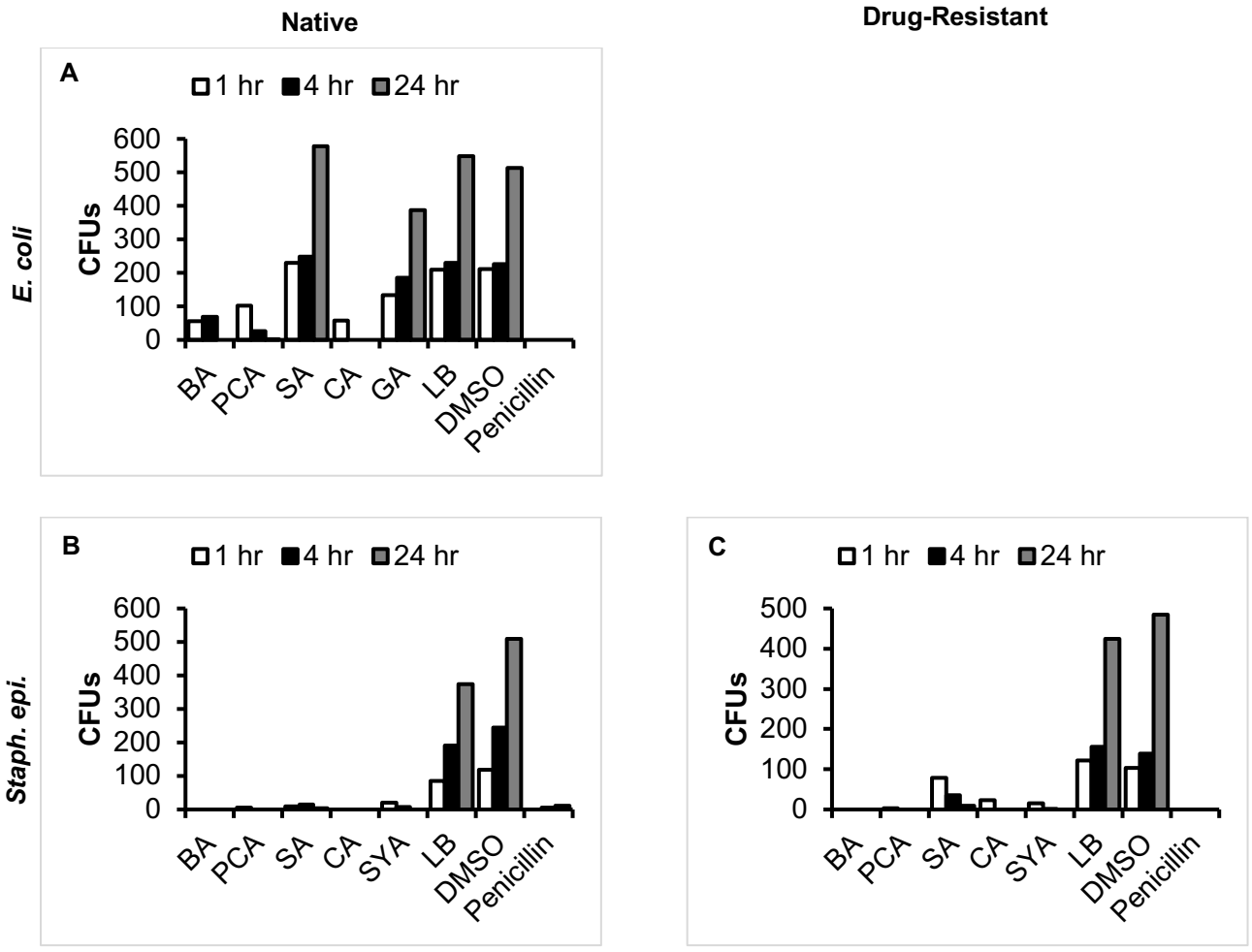
properties of PA. Because PAs could alter bacteria's surface tension components, they can also change the hydrophobicity and electron receptors (receptor components increase on gram-positive, decrease on gram-negative) on the bacterial cell surface. Therefore, gram negative *E. coli* shows different results compared with other bacteria strains. In **Figure 9A and 9B**, it can be seen that the antimicrobial capability of each PA is increased over time for drug-resistant *Staph. epi.* and *Staph. aureus* to comparable levels to those seen in the native strains. These results confirm the IC50 results and show that only 5 mg PA/ml is required for effective antimicrobial properties at levels that are comparable to drugs. Previously synthesized SMP foams with cinnamic acid contained between ~5 and 20 mg CA/ml (cm^3) of foam.

Compared with those previously presented in the literature, the antimicrobial results of CA in this paper show the same outstanding antimicrobial properties on *E. coli* and *Staph. epi.*.[27] However, there are also some differences, some results in previous literature show that protocatechuic acid demonstrates high antimicrobial capacity on gram-positive bacteria strains,[40] which could not be seen in the results of this paper. According to PA's antimicrobial mechanisms, one possible explanation for this difference could be the different proportions of lipids and phospholipids contained in the cell walls of the bacteria strain (*Bacillus cereus*) used in the previous literature.[41]

3.2.4 Colony Forming Units

The antimicrobial properties of PAs with limited solubility (cinnamic acid (CA), sinapic acid (SA), p-coumaric acid (PCA), and syringic acid (SYA)) and the synthesized MPAs (modified benzoic acid (BA), SYA, CA, and PCA) could not be measured directly using

the multi-well plate assay. The sediment that was present with these PAs and MPAs blocked the travel of light through the sample, affecting measurements of bacteria density via absorbance. Therefore, a Petri dish assay is utilized to quantify the antimicrobial properties of PAs and MPAs with limited solubility in terms of colony forming units (CFUs) over time, as shown in **Figures 10-12**.



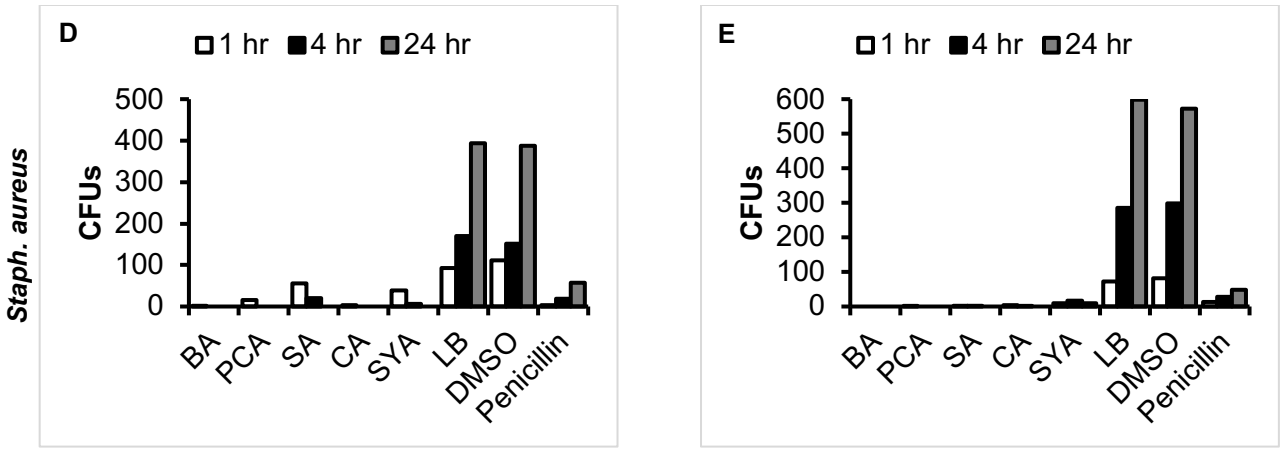
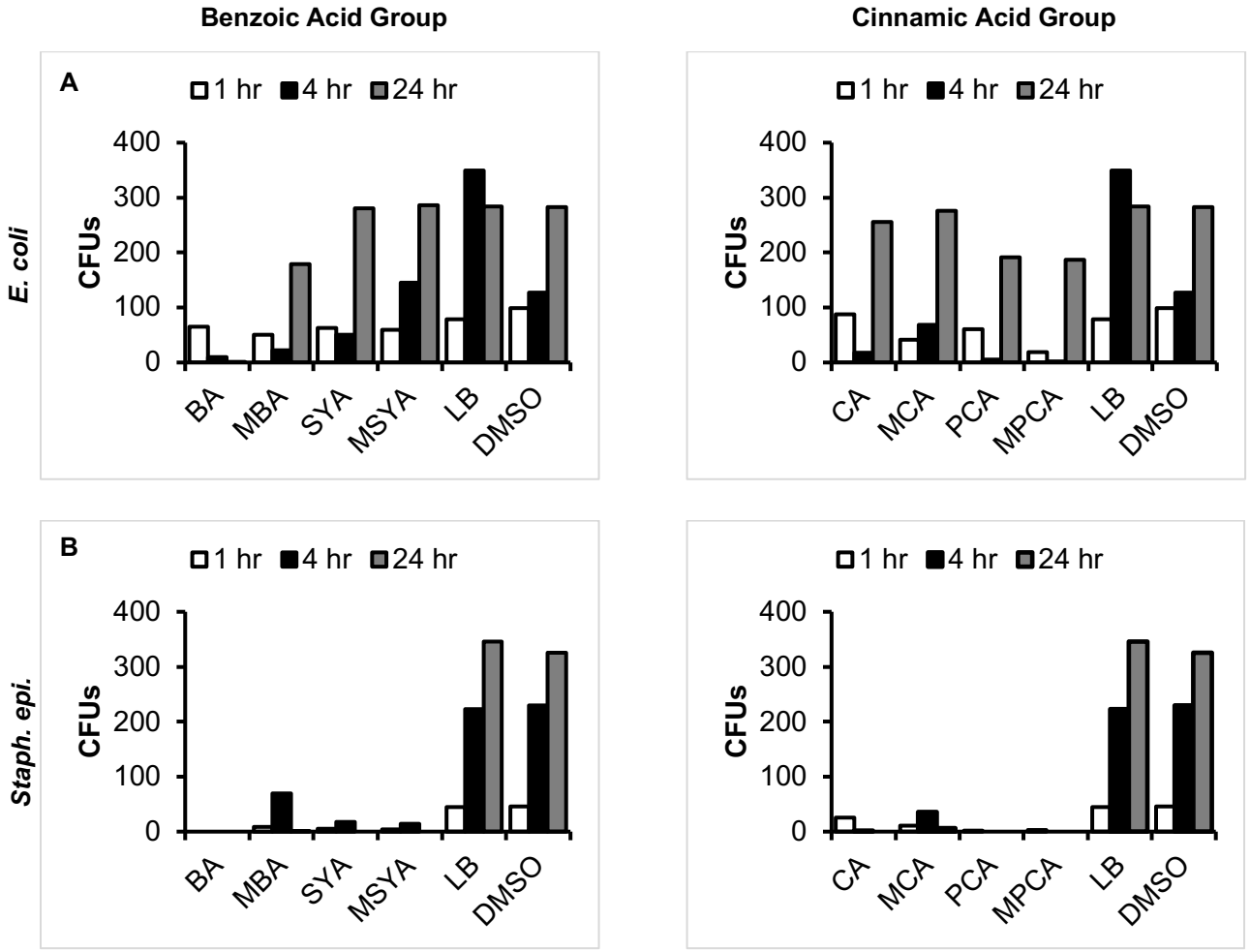


Figure 10. CFU counts of (A) *E. coli*, (B) native *Staph. epi.*, (C) native *Staph. aureus*, (D) drug-resistant *Staph. epi.*, and (E) Drug-resistant *Staph. aureus* after exposure to PAs with limited solubility.

The CFU results were quantified based off of ¼ the plate area using ImageJ software. Compared with LB and DMSO, it could be seen that all PAs with limited solubility are effective for 5 selected bacteria strains to levels that are comparable to the penicillin control, with the exception of SA and *E. coli*, **Figure 10**. In general, CA shows the best antimicrobial property among all the PAs with limited solubility, as there were no visible CFUs after 24 hours of exposure to CA. Again, comparable efficacy was observed with native and drug-resistant strains, indicating PA efficacy against drug-resistant bacteria, even when not fully solubilized.

The CFU results of MPAs in comparison with their PA controls are shown on **Figures 11** (native strains) and **12** (drug-resistant strains). All MPAs showed comparable results to their corresponding PAs for all bacteria strains. In general, PAs and MPAs were highly effective and reducing bacteria CFUs in the gram-positive strains (native and drug-resistant). Except for *E. coli*, PAs were clearly effective at reducing bacterial CFUs in

comparison with LB and DMSO. It should be noted that the unmodified PA CFU results are different from those observed in **Figure 10**. Thus, these studies should be repeated to verify PA efficacy against *E. coli*. However, the comparable CFU trends between PAs and corresponding MPAs over 24 hours of exposure indicates that PA modification at the carboxylic acid group, which is required for incorporation into SMPs, does not alter antimicrobial characteristics.



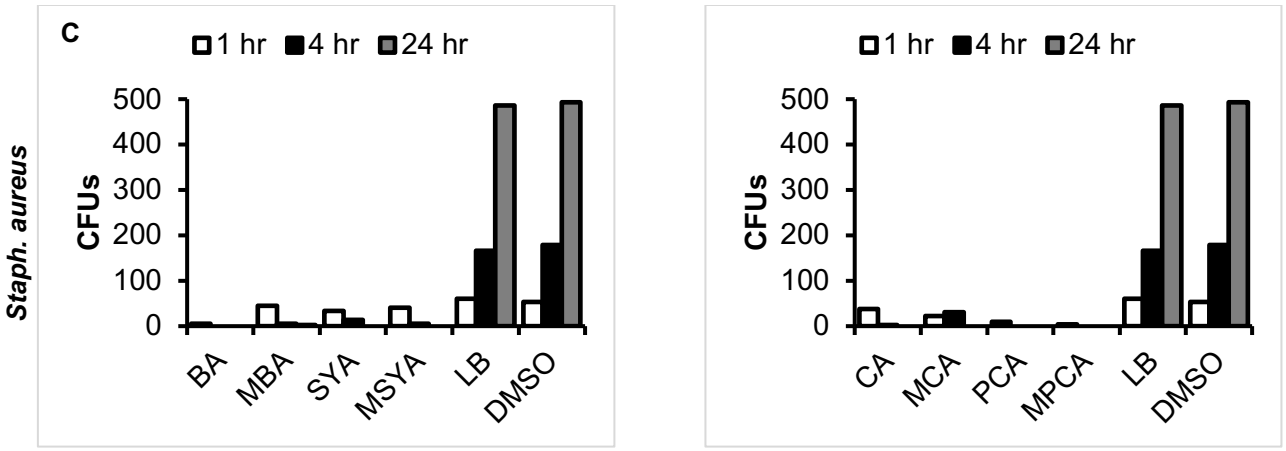


Figure 11. Native (A) *E. coli*, (B) *Staph. epi.*, and (C) *Staph. aureus* CFU counts in presence of (left) modified Benzoic Acid group and (right) modified Cinnamic Acid group.

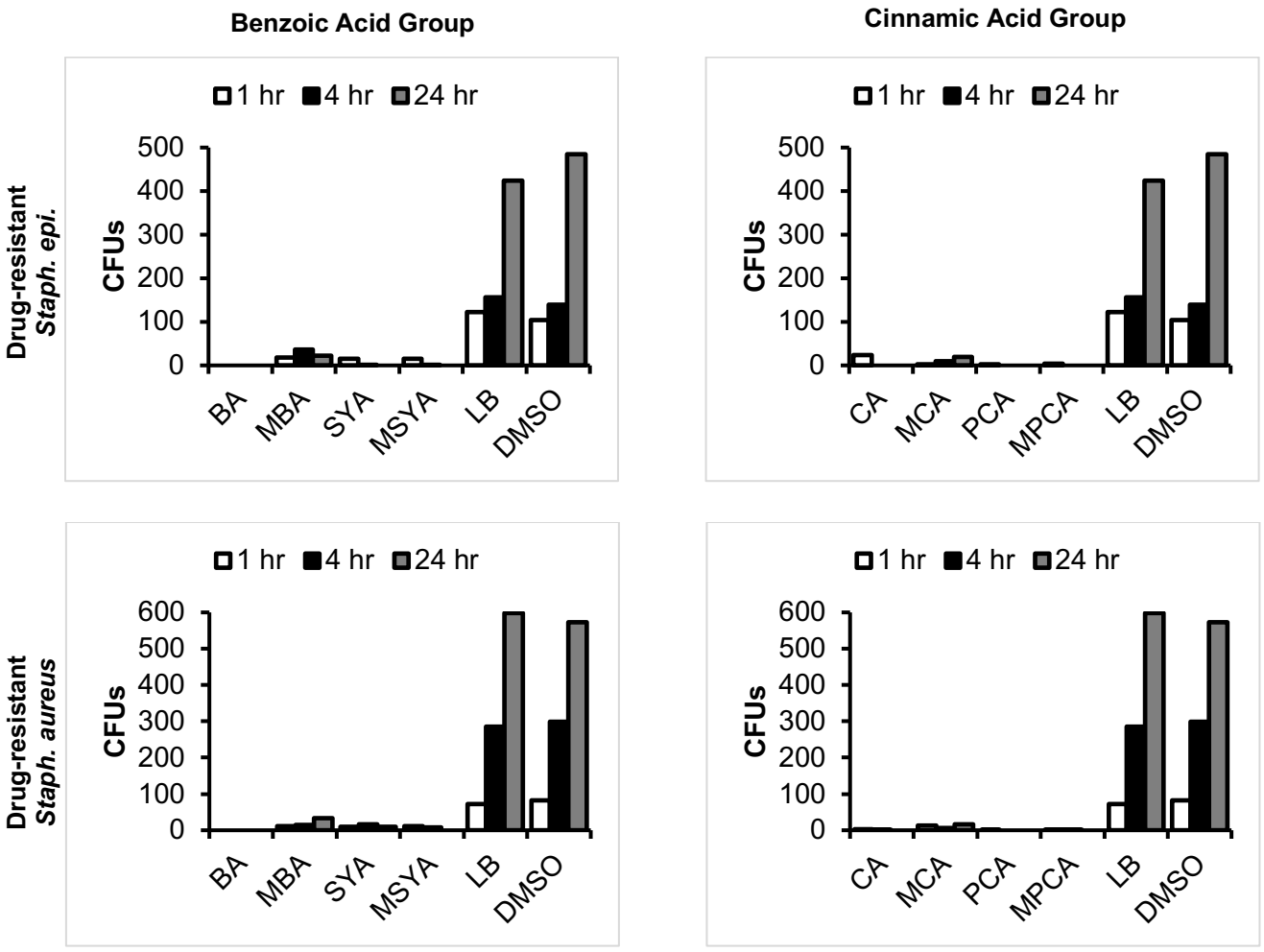


Figure 12. Drug-resistant (A) *Staph. epi.* and (B) *Staph. aureus* CFU counts in presence of (left) modified Benzoic Acid group and (right) modified Cinnamic Acid group.

4. Conclusion

These studies provide the first systematic characterization of PAs in terms of structure and antioxidant and antimicrobial properties with an emphasis on antimicrobial efficacy against common wound pathogens. The data collected thus far on modified PAs indicates that the carboxylic acid groups on PAs can be reacted with SMP components without loss in antioxidant or antimicrobial functionality. In general, hydrogen peroxide scavenging capabilities of phenolic acids increase with increasing radical scavenging pendant groups, eliminating cinnamic, benzoic, and 4-hydroxy benzoic acids as potential antioxidants. No consistent trends were observed between antimicrobial capacity and PA structure. While all PAs showed efficacy against all tested strains, there were variations in antimicrobial properties. PAs that had IC50 values greater than 10 mg/ml, maximum log reduction values below 0.7, and/or CFU counts above 100 include sinapic acid, syringic acid, and protocatachuic acid. Thus, these PAs will not be pursued in future studies required for antimicrobial SMPs. While solubility did not show a direct effect on functionality, limited solubility can hinder effective reactions between PAs and SMP components. Those PAs which have limited solubility include cinnamic acid, sinapic acid, p-coumaric acid, and syringic acid. Thus, these PAs will be given lower priority in future studies. Based on these trends, ferulic, caffeic, and vanillic acids will be pursued in future studies of synthesis of antioxidant and antimicrobial SMPs for use in hemorrhage control, **Figure 13**. This thesis provides a foundation for rational design of PA-containing biomaterial scaffolds with desired functionality that could be used in a range of applications where antioxidant and/or antimicrobial properties are required for improved clinical outcomes.

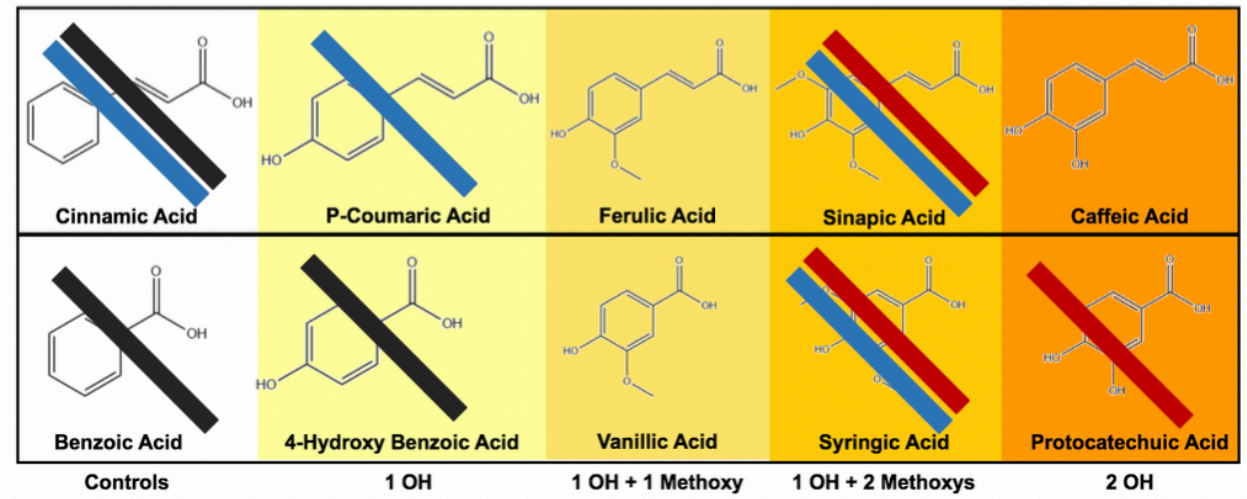


Figure 13. PA library down-selection for future incorporation into SMPs. Grey bars: No antioxidant efficacy. Red bars: Reduced antimicrobial efficacy. Blue bars: Limited solubility.

This research will guide future research on the rational synthesis of function foams with antioxidants, more studies could be on the synthesizing processes of improving PA content into the SMPs, which will enable SMPs to have better desired charasteristics. With the secreeing of 10 selected PAs, the PA library has been narrowed, which will help the future incorporating selection. Also, this research provides new fundamental information on PA structure/prooerty relationships, especially the relationship between PA structures and antioxidant properties. The future direction of this research is to test multiple bacteria strains together and test with biofilms. Because the battlefiled is a complicated enviroment, multiple bacteria strains may form a complex system.

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