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ABILITY OF PHENOLICS IN ISOLATION, COMPONENTS PRESENT IN SUPINA TURF GRASS TO REMEDIATE *CANDIDA ALBICANS* (A72 and SC5314) ADHESION AND BIOFILM FORMATION

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by

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ABILITY OF PHENOLICS IN ISOLATION, COMPONENTS PRESENT IN SUPINA TURF GRASS TO REMEDIATE *CANDIDA ALBICANS* (A72 and SC5314) ADHESION AND BIOFILM FORMATION

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Candida albicans inhabits the gastrointestinal tract dormant commensal member but can become an opportunistic pathogen when the host microflora or immune system is compromised. Adhesion to a biological or synthetic surface, followed by a morphological change from the yeast to hyphae phenotype. Biofilm formation is becoming a common occurrence on types of medical devices. Because *C. albicans* resistant to commonly available anti-fungal drugs is increasing, innovative treatments are critically needed. Phenolic compounds are promising anti-fungal synergists. Supina grass was used as the complex matrix as it is abundant and highly sustainable source of phytochemicals even though grass cuttings are typically disposed. *Therefore, the objective of this research is to determine the potential of phenolic compounds in isolation, as a combination and in present matrix, supina turf grass, to act synergistically in remediating C. albicans adhesion and biofilm formation from a synthetic surface*.

Chapter 1 of this study focused on the ability of 7 phenols (ferulic, gallic, sinapic, coumaric, epicatechin, catechin and quercetin) and 2 non-phenolic (farnesol and chlorophyll) common in natural systems to combat the cited virulent factors using different incubation times, 1, 3, 6 and 24 h, and treatment dosages (0.06-4.00 mM). The highest potency for occurred at 6 h post treatment but only chlorophyll, farnesol, and catechin were effective against adhesion while all the compounds were able to act

against biofilm formation. However, percent remediation ranged from < 0 to 40% with *C. albicans* (A72) being more stable and G, F being the most effective phenol. Interestingly, the lower dosages resulted in the greatest effectiveness.

Chapter 2 describes the ability of two phenols to act synergistically to remediate adhesion and biofilm formation. After screening combination of compounds described above, the same 6 sets were the most effective against adhesion and biofilms, and included F-G, S-Q, F-E, E-C, CAT-Q, and CAT-C. In combination, the non-phenolic compounds in combination were not as effective as in isolation. The efficacy of the phenol combination was 20-60%, which again occurred with the lower dosages, (0.03-0.25) while the higher concentration (up to 4 mM) resulted in limited or no inhibition. The phenols acted synergistically to detach bound cells as the factionary inhibitory concentrations (FIC) was less than 0.05. For the biofilm experiments, with the FIC was 0.5<FIC>1.0 indicating partial synergy.

Lastly, Chapter 3 demonstrated that a supina grass extract (at ng levels) was able to remediate *C. albicans* cellular adhesion and biofilm formation (50-70%). This study therefore generated information on the ability from a readily available agricultural stream, which then is expected to facilitate the development of efficacious anti-fungal treatments capable of remediating potentially life-threatening *C. albicans* infections.

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LITERATURE REVIEW

I. *Candida albicans* and Morphological Transitions

C. albicans inhabits the gastrointestinal tract (GI) as a typical dormant commensal member that resides in the human gut microflora, oral and mucosa surfaces. Although *C. albicans* is hosted by approximately 70% of healthy individuals without causing any problems (Gow, 2013), it can become an opportunistic pathogen when the immune system, GI enzymes or host microflora is compromised (Schulze and Sonnenborn, 2009). Such an event is typically accompanied by a deficient immune response (e.g., in HIV/AIDS patients), interventions used to treat chronic diseases (e.g., cancer and diabetes), injuries or surgeries or medical implants (Brown et al., 2014; Pfaller et al., 2007). In particular, candidaemia and disseminated candidiasis are serious medical infections caused by *C. albicans,* currently accounting for 30-40% of the mortality rate (Kullberg and Filler, 2002). It has been estimated that 40,000 cases of invasive candidiasis occur annually in the U.S alone (Centers for Disease (Control and Prevention and US Department of Health and Human Services, 2013). As such, virulence factors leading to opportunistic infections that include phenotype switching ability, hydrolytic enzyme secretion, adhesion to both endothial and epithelium cells and biofilm formation are a critical area of research (Wang and Helliwe, 2000).

C. albicans can colonize any site from the oral cavity to the rectum and peri-anal tissues in the gastrotestinal tract due, in part, to their moist surfaces that can sustain needed nutrients. However, viable cells can survive for 24 h on dry surfaces if the degree of contamination is sufficiently high (Cannon and Chaffin, 1999). Regarding its physiology, *C. albicans* is a diploid polymorphic yeast with eight pairs of chromosomes that can colonize under both aerobic and anaerobic conditions (Schulze and Sonnenborn, 2009). Additionally, *C. albicans* is able to switch to three distinct

morphological phenotypes consisting of budding yeast (round shaped cells), pseudohyphae (branched structures with lateral buds) and hyphae (presence of elongated filaments) (Fig. 1). All three forms can be induced in laboratory under select medium and incubation conditions (Biswas et al., 2007; Veses and Gow, 2009). For example, yeast is able to transition to the hyphae phenotype in the presence of serum, neutral pH, 5% CO_2 (the partial pressure of CO_2 in the bloodstream) and N-acetyl-d-glucosamine (GlcNAc) (Sudbery, 2011). In the host, however, *C. albicans* has adapted to grow and form hyphae in the host under a variety of conditions due to the microenvironment diversity present. For example, *C. albicans* morphological switching involves communicating not only with other *C. albicans* cells, but also the surrounding bacteria present in the environment. *C. albicans* is further equipped with a quorum sensing mechanism to sense its own population density in the environment, which is based on farnesol secretion into the environment to prevent hyphal formation (Sudbery, 2011). This microorganism can also form chlamydospores (a spore-like structure) and opaque phenotype, which is important for biofilm formation, as will be discussed further in the next section (Scaduto and Bennett, 2015; Mayer et al., 2013; Odds, 1985; Sonneborn et al., 1999).

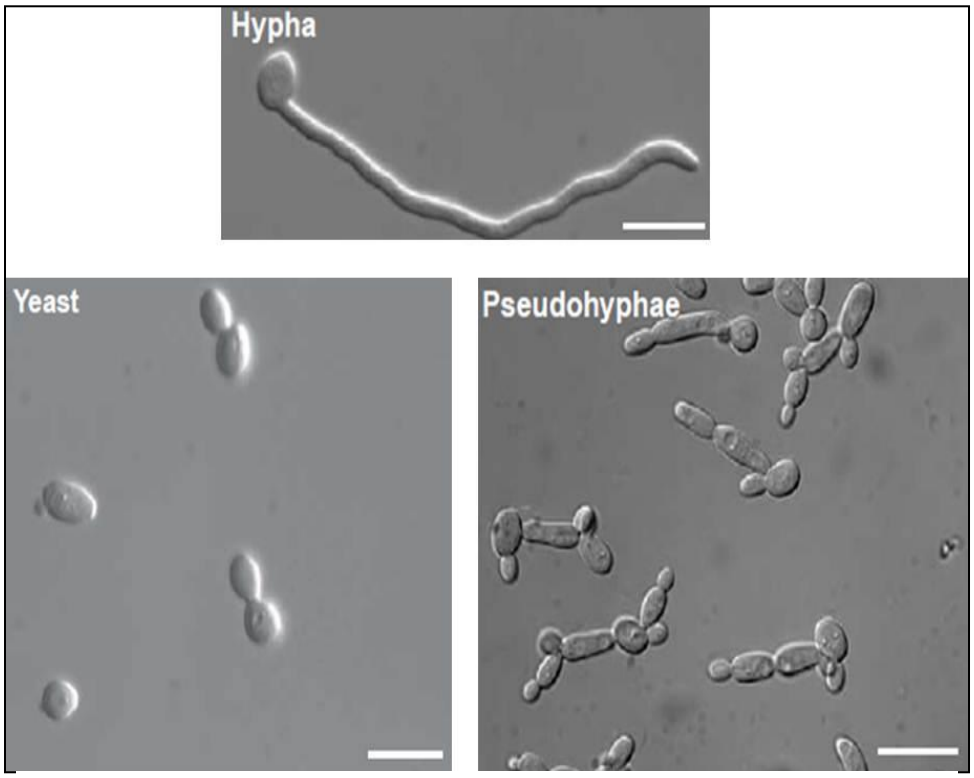


Figure 1: Different morphologies of *C. albicans*

Notably, the hyphal form of *C. albicans* facilitates adhesion and invasion and is considered the virulent phenotype that leads to systemic infections. Indeed, both the

yeast and hyphal forms are present at infection sites (Jacobsen et al., 2012; Pope et al., 1982; and Saville et al., 2003). However, hyphae invade the epithelial barriers or the interepithelial cells, and are considered the virulent phenotype that leads to systemic infections. *C. albicans* that has transitioned into the hyphae state is also the most resistant to anti-fungal agents (Gale et al., 1998; San-Blas, et al., 2000; Oh, et al., 2012.; and Sudbery, 2011). Therefore, information on the fundamental roles of yeast-hyphae morphological transition is critical to understanding the different infection stages of *C. albicans* bloodstream infections (Jacobsen et al., 2012).

The hyphae phenotype is fundamentally associated with elevated adhesiveness; therefore, will more readily colonize other cells and organs than will the yeast cells (Saville et al., 2003). Hyphal invasion into epithelial cells are mediated by two distinct mechanisms: induced endocytosis of host and active penetration. Induced endocytosis is defined as a host-driven process activated by interaction between the *C. albicans* invasin Als3 and host E-cadherin. (Almeida et al., 2008). Alternatively, active penetration is a fungal-driven process that is moderated by hyphal extension and other hyphal-related health risks. For example, the invasive hyphae are responsible for epithelial tissue damage (Jacobsen et al., 2012), and are crucial for escaping phagocytic cells, which are the first line of defense in combating infections. (Lorenz et al., 2004).

This mechanism is facilitated by the presence of *C. albicans* yeast cells that can be phagocytosed by macrophages, but once engulfed transition to hyphae form damaging the surrounding membrane and eventually killing the phagocyte (Lorenz et al., 2004; Jacobsen et al., 2012). It is clear that morphological transition from yeast to hyphae plays a fundamental role in *C. albicans* infection stages, involving escape from epithelium surfaces into the bloodstream, colonization of internal organs and disruption of the immune defense systems. As C*. albicans* that has transitioned into the hyphae

state is the most resistant to anti-fungal agents (Gale et al., 1998; San-Blas, et al., 2000; Oh, et al., 2012.; and Sudbery, 2011), inhibition or mitigation of the yeast-hyphal switch is a potential antifungal target to control *C. albicans* infections.

II. Adhesion and Biofilm Virulence Factors of *C. albicans*

Microbial virulence factors can be defined as "the capability of a micro-organism to cause infectious diseases via pathogenic (virulent) factors that directly interact with host cells, or a pathogenic component that can damage the host" (Yang, 2003; Casadevall and Pirofski, 2001). As stated previously, *Candida* spp. are naturally present in the human microbiota, thereby allowing the interaction with implanted biological and synthetic surfaces, which include dentures, shunts, prostheses, implants, endotracheal tubes, pacemakers and various types of catheters.

Adhesion of *C. albicans* to a surface, whether biological or synthetic, is the first step in its pathogenic phase followed by a morphological change from the yeast to hyphae phenotype (the virulent state) (Schulze and Sonnenborn, 2009; Yang, 2003; Han, et al., 2011). *C. albicans* cells' initial attachment to a surface is mediated by nonspecific and specific factors (Ramage et al., 2005). The nonspecific factors include cell surface hydrophobicity and electrostatic forces, as well as fungal surface specific adhesion stimulating ligands in the habituation films, such as serum proteins and salivary factors. The ligand receptors are fibrinogen and fibronectin (Ramage, et al., 2005; Chaffin, et al., 1998; Webb et al., 1998). Therefore, adhesion is a potential colonization strategy, and it is the connection point between the cell wall of *Candida* and host surfaces (Cannon and Chaffin, 1999).

C. albicans adhesion to host cells is highly associated with the strain and the host cells, as several studies have shown at least four candida-host cell recognition systems (Calderone, 1993; Calderone, 1994; Webb et al.,1997). First, *Candida* strains within the

oral cavity or that have been isolated including *C. glabrata, C. tropicalis, C. kefyr, C. krusei,* and *C. guilliermondii*, which produce blastoconidium mannoprotein with lectin-comparable properties that recognize the terminal fucose or n-acetylglucosamine-containing glycosides of host epithelial cells. Second, the CR2/CR3 complement receptors are present in select *C. albicans* strains which are thereby able to identify endothelial cells. In addition, two other recognition systems are enabled due to the mannan oligosaccharide and the Candida cell wall chitin content. It is expected that a clear understanding of these ligand-recognition systems will demonstrate sites susceptible to Candida invasion throughout the body and onto synthetic devices (Webb et al.,1997).

Moreover, *C. albicans* biofilm formation is a virulent factor, which if not treated, can lead to life threatening systemic infections (Ramage, *et al*., 2005; Nobile and Mitchell, 2006; Bauer et al., 2002). Biofilm formation is a common occurrence on both biological and synthetic surfaces (Ramage et al., 2005; Nobile and Mitchell, 2006; Bauer et al., 2002). Biofilms are three-dimensional assemblies of microorganisms surrounded by extracellular polymeric substances (Figure 2) that protect yeast cells from being attacked by the surrounding environment and the immune system. (Li et al., 2016). The biofilm complex structure facilitates nutrient influx, waste product disposal, and microniche establishment throughout the biofilm (Ramage et al., 2005).

Biofilm process is known as a stepwise process (shown in Figure 3) initiated by adhering to a given foreign substrate followed by yeast cell proliferation, hyphal transition and mature biofilm formation (Blankenship and Mitchell, 2006). Gene expression alteration begins after 30 min of adhesion to a surface. Recent studies in quorum sensing suggest that a dispersal stage might be an additional step in the *C. albicans* life cycle, which involves non-adherent yeast cells. Therefore, adhesion, yeast-

hyphae transition, and quorum sensing are a vital key process in biofilm development (Blankenship and Mitchell, 2006; Hornby et al., 2001; Ramage et al., 2002).

The hyphal form plays an important role in maintaining the structural integrity of the multilayered architecture of a fully developed biofilm (Ramage et al., 2005). Ramage et al. (2002) conducted a study on the molecular pathways that regulated filamentation to form biofilms. A series of genetically defined *C. albicans* mutant strains that are unable to form hyphae under different environmental conditions were used and tested for their ability to form biofilm. It was determined that the single *fg1* and double *cph1 efg1* deletion mutants were unable to form biofilms or even hyphae. The regulator protein Efg1 is the key element to form a fully mature biofilm on most biological and artificial surfaces (Ramage et al., 2005).

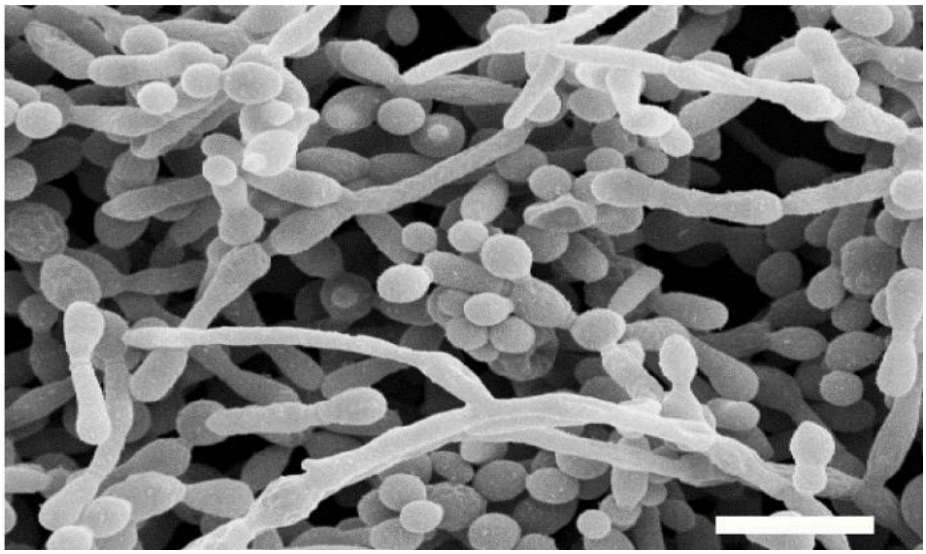


Figure 2: Biofilm formation

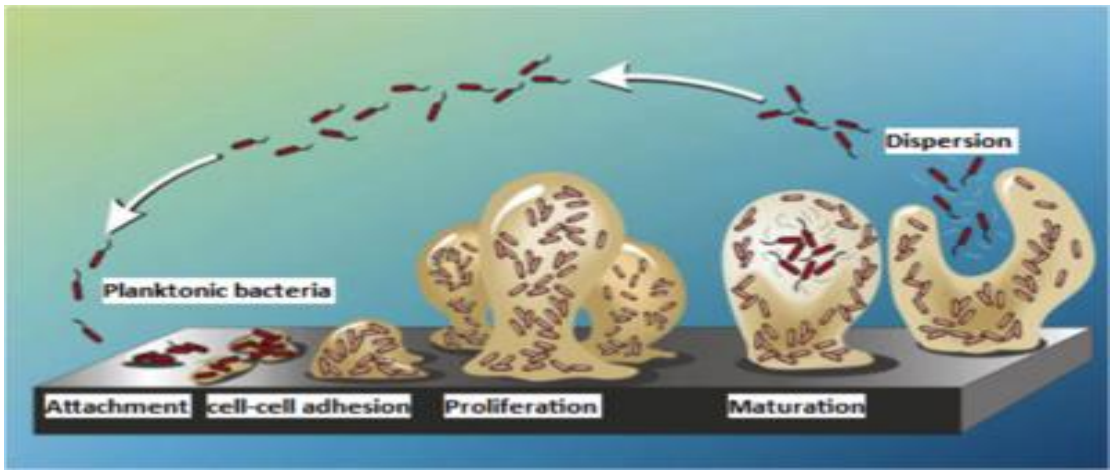


Figure 3: Biofilm formation process

In addition to filamentation, a quorum sensing strategy is facilitating a cell-cell communication process which is a fundamental to biofilm formation that involves an array of microbial activities, such as biosynthesis of extracellular enzymes, antibiotic, extracellular polymeric substances, and extracellular virulence factors (Simoes et al., 2010). Quorum sensing is beneficial for biofilm in terms of self-organization by preventing unwanted overpopulation and regulating nutrient competition. Microorganisms can detect and respond to their own population densities through the environmental sensing system producing an auto-inducer organic signal (AL). This organic signal accumulates during the growth in the surrounding environment resulting in either trigger gene expression or physiological responses (Simoes et al., 2010).

However, it is notable that the physiological response is highly dependent on the achieved critical threshold signal molecule concentration. Therefore, quorum sensing systems are important in the infection process (Simoes et al., 2010). As an example, the farnesol molecule is produced during high cell density growth to inhibit *C. albicans* filamentation (Ramage et al., 2005; Hornby et al., 2002; Kruppa et al., 2004). Therefore, a study used various concentrations of farnesol to test the ability to inhibit *C. albicans* biofilm formation, and the results indicated that biofilms decreased for farnesol-treated samples compared to the untreated controls (Ramage et al., 2002). Yet, such infections (candidiasis) are increasing due to the biofilm formation on these surfaces as the anti-fungal resistance to commonly used anti-fungal agents is augmented (Schulze and Sonnenborn, 2009; Yang, 2003; Magee and Chibana, 2002). Although it is more logical to prevent biofilm formation it is still critical to mitigate this virulent effect when it does arise (Simoes et al., 2010). Therefore, the inhibition or remediation of both cell adhesion and biofilm formation can be a promising strategy to reduce *C. albicans* colonization of

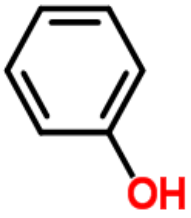
synthetic products used for human health purposes, such as catheters. (Wang et al., 2012).

III. Phenols as Anti-Fungal Agents

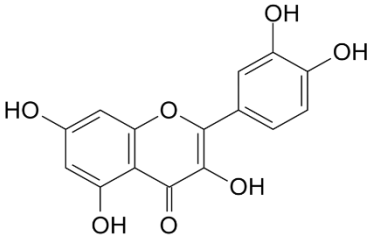
Phenolic compounds are highly diverse class of compounds with more than 8000 known structures (Bravo 1998), all of which are classified by the presence of at least one or more hydroxyl groups bonded to one or more phenol groups (Figure 4). Phenolic compounds are widely distributed throughout the plant kingdom and include phenolic acids, terpenoids, organosulfur compounds, isoquinoline alkaloids, flavonoids, lactone, and naphthoquinone. These compounds are synthesized through the pentose phosphate, shikimate and phenylpropanoid pathways, and, as secondary metabolites, act as defense chemicals against biotic (e.g., pathogens) and abiotic (e.g., UV light) stresses to protect the host (Treutter, 2005 and Bravo, 1998).

In terms of human health protection, studies have shown that phenols are able to protect against cellular stresses, such as oxidation and inflammation, as well as providing treatment for various conditions or diseases, including cancer, hypertension, vascular fragility, allergies, diabetes and hypercholesterolemia (Treutter, 2005; Bravo, 1998; Papadopoulou, et al., 2005).

Simple phenols

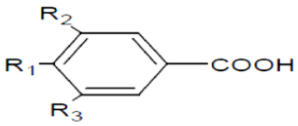


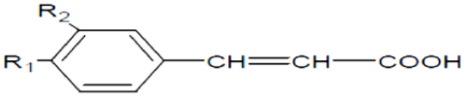
Basic structure

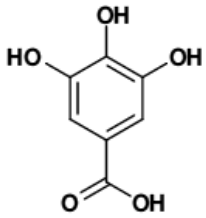


Flavonoids

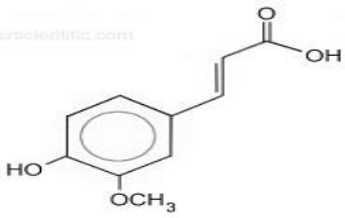
Phenolic acids



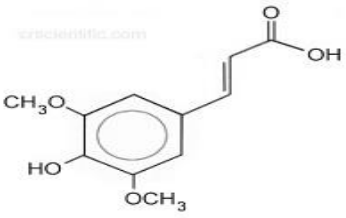




Gallic Acid
3,4,5-TriHydroxyBenzoic Acid



Ferulic acid



Sinapic acid

Figure 4: Common phenolic compounds chemical structure

However, the bioavailability and solubility of phenols can be affected by derivatization to other types of molecules, such as sugars, *in vivo* (Wesgner, 2011). Still, phenolic compounds have been reported to act as *C. albicans* anti-fungal agents through various mechanisms of action, including inhibition of fungal toxin, adhesion, secretory systems, virulent gene expression, and quorum sensing strategies (Ivanova et al., 2013). Phenolic compounds have thus been proposed to be promising anti-fungal agents due to their capability to inhibit the virulence factors (Ivanova et al., 2013).

For example, Vikrant et al. (2015) have shown that gallic acid, capric acid, carvacrol, and terpenen-4-ol were able to inhibit the growth of *C. albicans* by preventing the normal budding process due to destruction of membrane integrity (Vikrant et.al, 2015). Multiple studies have also shown the anti-fungal effect of various plant extracts against certain microorganisms, including *C. albicans*. One such study completed by Papadopoulou et al. (2005) demonstrated that non-alcoholic red wine prevented the growth of several pathogenic microorganisms that included *C. albicans, Staphylococcus aureus, and Escherichia coli.* These results were attributed to the presence of a broad range of flavonoids (flavonols, flavanols and anthocyanins) and non-flavonoids (mainly phenolic compounds) present in red wine. Moreover, seven phenolic compounds extracted from olive leaves (caffeic acid, verbascoside, oleuropein, luteolin 7-O-glucoside, rutin, apigenin 7-O-glucoside and luteolin 4'-O-glucosid) inhibited the growth of *C. albicans* and gram positive (*B. cereus, B. subtilis, S. aureus*) and gram-negative bacteria *(E. coli, P. aeruginosa, K. pneumoniae).*

Phenolic compounds have also been shown to act as anti-fungal agents against *Candida* virulence factors. For example, flavonoid-rich extracts from honey prevented hyphal formation of *C. albicans* (Teodoro et al.2015 and Candiracci et al. 2012). Studies

in our own lab have shown that phenols in combination work synergistically to prevent formation of the hyphal phenotype in vitro (Shi, 2016; Camara, 2015).

Yet, studies remain limited on the inhibitory/remediation effects of phenols on *C. albicans* adhesion to and biofilm formation on synthetic materials, albeit research has shown that other small molecules, such as filastatin (one of 50,000+ small molecules screened), are effective against both these virulence targets (Fazly et al., 2013; Wong et al., 2014). It must be noted that these studies did not focus on the synergistic potential of small molecules in combination to prevent or remediate these events of two different species.

Interestingly, it has been demonstrated that the green tea phenol, catechin epigallocatechin gallate (EGCG), acted synergistically with miconazole, fluconazole and amphotericin B (typical anti-fungal drugs used to treat *C. albicans*) resulting in reduced levels of anti-fungal agents needed to inhibit *C. albicans* biofilm formation (Ning et al., 2015). Another study by Sun et al., (2015) reported that the two neolignan compounds. Magnolol and honokiol, which were extracted from *M. officinalis,* exerted a synergistic antifungal effect against *C. albicans* inhibiting adhesion, yeast-hyphae transition and subsequently biofilm formation through the Ras1-cAMP-Efg1 pathway. Therefore, ability of phenols and polyphenols to act as synergists might be a promising and potent treatment strategy to prevent and/or remediate adhesion and biofilm formation as multi-target mechanisms of action are expected.

IV. Determination of Anti-Fungal Synergists

When two combined drugs produce a similar effect, a quantitative analysis is required to differentiate exaggerated or diminished effects from an additive versus from a synergistic action (i.e., compounds exert a greater response together than if the responses exerted by the individual components were added together) (Tallarida, 2001).

Methods to identify dietary synergists are not consistent among the reported studies (Tan et al., 2003; Barrera et al., 2005; Berenbaum, 1989) but construction of isobologram plots (first introduced by Berenbaum in 1989) may be the most reliable approach for this purpose (Wagner and Ulrich-Merzenich, 2009). (A typical isobologram is illustrated in Figure 5, where x and y axes represent the dosage of two or more substances [e.g., isolated phenolic compounds, or an extract] while the lines represent the type of interaction of the compounds that inducing a given response [e.g., inhibition or remediation of adhesion of *C. albicans* at a pre-established level].) When the specified response is obtained at the given dose combination, a point is plotted.

The combinations of the different compounds are tested again until a line can be plotted based on the position of the points. The compounds that produce a concave, linear, or convex correlation indicate that these agents act as synergists, additives or antagonists, respectively, with one another or within the complex matrix. This effect can be confirmed by determining the fractional inhibitory concentration index (FICI), as defined below.

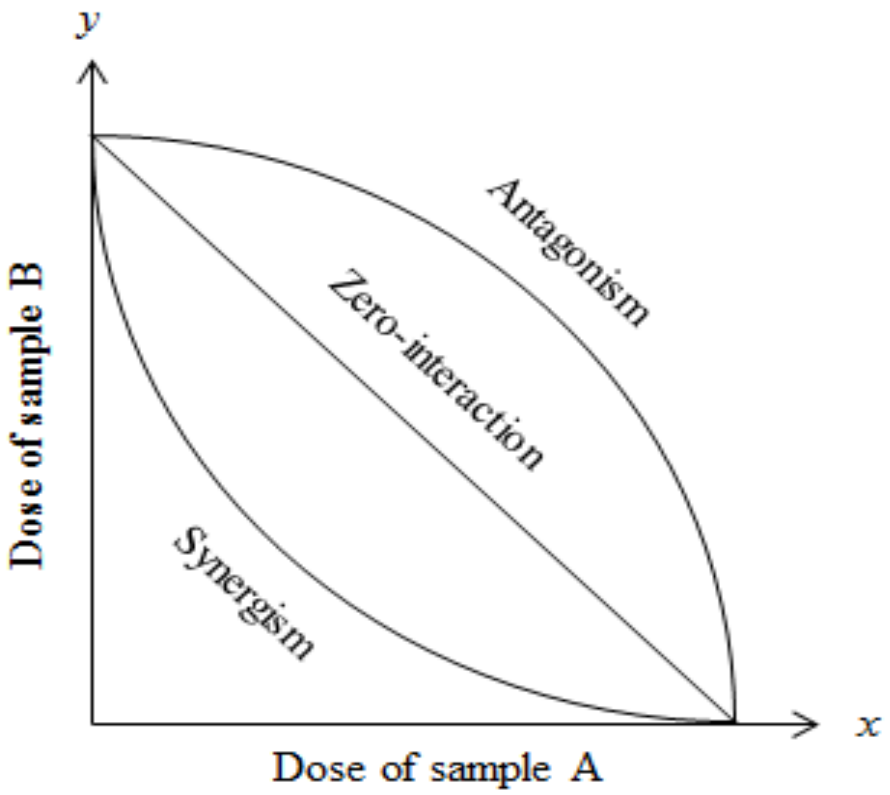


Figure 5: Illustration of an isobologram

The equation of FICI is:

I=Σi (xi / Xi)

where **I** is the FICI

i is the **ith** individual substance in the combination

xi is the dose of the individual substance in the combination

Xi is the dose of individual substance, which has same effect as the combination.

FICI <1 suggests synergism, whereas FICI >1 suggests antagonism. If FICI = 1, no interaction occurred between the two substrates and the effect of two is, rather, additive. This method was applied to a methanol rosemary extract alone and combined with butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) to determine its antimicrobial activity. The isobologram confirmed the extract acted synergistically with both of these compounds (Tallarida, 2001; Romano et al*.,* 2009; Moreno et al., 2008). Moreover, the efficacy of a combination of antimicrobial drugs (sulphadoxin and pyrimethamine) to treat human malaria caused by *P. falciparum* was analyzed by constructing an isobologram. A strong concave curve was generated, indicating strong synergism (Bell, 2005). It was farther reported that therapeutic efficacy of a combination of plant extracts (*Gingko biloba* and Echinacea) was due to a synergic interaction (Williamson, 2001).

In term of phenolics compounds, one study indicated that curcumin combined with five azoles and two polyene antifungal drugs provided increased inhibition of 21 clinical isolates of *C. albicans* as well as some sensitive laboratory strains (Sharma et al., 2010b). Moreover, theaflavin and epicatechin showed a synergistic effect to inhibit *C. albicans* growth in a plate (Betts et al., 2013). However, extensive studies are still required to identify the individual, synergistic or additive effects of phenolic compounds

on the different virulence factors of *C. albicans,* with an emphasis on adhesion and biofilm formation as no known studies have been reported to date.

V. Supina Turf Grass as a Source for Phenolic Anti-Fungal Agents

The efficacy and safety of any drug, including anti-fungal agents, depends on their consistent production and delivery. Although phenols are available in all plants, they are typically consumed as part of a larger matrix of dietary compounds and thus are difficult to monitor for targeted drug-related purposes. Isolation of the phenolic synergists from a single source that is highly sustainable is thus optimal in development of drugs. The *Poaceae* or grass family is among the most abundant and renewable plant families on the planet that may offer a novel source of phenols (Margorie, 1999; Thompson and Thompson, 2010; and Odey et al., 2012).

The cereal species in particular (corn, rice, and wheat) are staple foods that are widely consumed on a global basis, and these species have been recognized as the primary nutraceutical sources within the *Poaceae* family (Thompson and Thompson, 2010). *Poa supine,* shown in Figure 5, is an interesting species among the grass family due to its turf characteristics and as a native species to the European Alps (Leinauer, et al., 1997). This grass is easy to cultivate in short time periods.

Figure 6: Supina Grass

VI. Objectives and Specific Aims

The objective of this study was to determine the synergistic potential of selected phenolic compounds in isolation (Chapter 1) and combination followed by phenolic-rich turf grass extracts in inhibiting or remediating *C. albicans* adhesion to and biofilm formation on substrates simulating medical devices. This research is *significant* as it is the first to: a) to screen the ability of several selected isolated phenolic compounds to inhibit or remediate the adhesion and biofilm virulence factors of two strains of *C. albicans* (A72 and SC5314), using synthetic surface, b) to study the synergistic effect of a combination of phenolic compounds targeting *C. albicans* adhesion and biofilm virulence factors and c) determine the potential synergistic interplay of the phenols within a complex matrix, using extracts from Supina turf grass to inhibit *C. albicans* virulence factors.

The rationale for this study is that information will be generated to provide a foundation to determine the feasibility of obtaining synergistic *C. albicans* anti-fungal agents from a readily available agricultural stream (in the short term), which then is expected to facilitate the development of efficacious anti-fungal treatments capable of preventing potentially life-threatening *C. albicans* infections (in the long term). Therefore, the central hypothesis for this project is that the richly diverse phenolic compounds present in grasses will impart a great anti-fungal effect in terms of inhibiting or remediating adhesion and biofilm formation by *C. albicans.* This hypothesis will be tested, and the study objective satisfied by completing the following specific aims.

Specific Aim 1 (chapter 1): **To screen the ability of several select phenolic compounds to remediate the adhesion and/or biofilm formation of *C. albicans* (A72 and SC5314).** *The working hypothesis for this specific aim is that certain phenols will be more effective than others in remediating adhesion and biofilm formation but will*

do so in a dose dependent manner with varying percent inhibition concentration values (%IC).

Specific Aim 2 (Chapter 2)**: To determine the potential synergistic interplay of the most effective phenols established in Specific Aim 1 to prevent and/or remediate the adhesion and biofilm formation of *C. albicans* (A72 and SC5314).** *The working hypothesis for this specific aim is that the phenols will act synergistically to prevent or remediate the adhesion/biofilm formation to a greater degree than can be elicited by the isolated components alone but will be dependent on the type of phenolic and concentration.*

Specific Aim 3 (Chapter 3): **To determine the potential synergistic interplay of the phenols within a complex matrix, using extracts from Supina turf grass, to prevent and/or remediate the adhesion and biofilm formation of *C. albicans* (A72 and SC5314).** *The working hypothesis for this specific aim is that the phenols will act synergistically with Supina turf grass extracts to prevent or remediate the adhesion to a greater extent than can be elicited by the isolated component alone.*

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Chapter 1

Natural compounds, with an emphasis on the phenols, are able to reduce biofilm formation and cell adhesion established by *C. albicans* (A72 and SC5314) but the degree of mitigation is based on yeast strain, compound and concentration

1.1 Abstract

C. albicans is an endogenous and an opportunistic member in the human microbiota. *C. albicans* can switch from budding yeast form to hyphal form leading to life-threating infections. Moreover, *C. albicans* not only can adhere to epithelial and endothelial cells of the host, but also can colonize internal and implanted devices by forming biofilms. Recently, *C. albicans* has evolved and developed resistance to commonly available antifungal agents for these virulence factors. Therefore, there is a high necessity to develop innovative antifungal agents to increase the treatment efficacy against *C. albicans* infections. The objective of this study was to determine if 8 compounds, with an emphasis on the phenols, were able to remediate *C. albicans* (A72 and SC5314) infection by reducing existing adhered cells and/or biofilms. After exposing *C. albicans* (A72 and SC5314) to the isolated compounds at 9 different concentrations ranged from 0.06-4.00 mM) at four time points (1, 3, 6, and 24h), it was determined that for most of the compounds were most effective at the 6 h point. Among the compounds, catechin, chlorophyll, and farnesol were able to reduce adhesion of *C. albicans* (A72 and SC5314), while gallic acid, ferulic acid, sinapic acid, quercetin, catechin, epicatechin, and coumaric acid, as well as chlorophyll and farnesol were all able to reduce biofilm formation at all concentrations by 50-60%. However, cellular adhesion varied from 0-50% due to the high variability of the test.

Keywords: Candida, phenols, biofilm formation, cellular adhesion

1.2. Introduction

Candida albicans is a dormant commensal member of the human microbiota, which inhabits the gastrointestinal (GI) tract and oral cavity but can become an opportunistic pathogen when the host microflora is compromised (Jacobsen et al., 2012; Sudbery et al., 2004). Although 70% of individuals carry this yeast without showing health problems (Gow, 2013), *C. albicans* infections (candidiasis) can be life threatening, particularly in individuals who are critically ill, (immunodeficiency syndrome, hematological malignancy). As such, mortality rates are often more than 30-40% when these infections become septic (Kullberg and Filler, 2002; Garcia et al. 2014). It also has been reported that multiple chronic conditions are also associated with *C. albicans,* such as depression, denture stomatitis and more recently obesity (Edwards DA, 1985; Guggenheimer et al., 2000; Salerno et al., 2011; Srebrnik and Segal, 1990).

C. albicans is metabolically flexible, which contributes to itis ability to switch morphology (yeast-hyphae), adhere to host surfaces and form biofilms (da Silva Dantas et al., 2016). Adhesion of *C. albicans* to a surface, whether biological or synthetic, is the first step in its pathogenic phase followed by a morphological change from the yeast to hyphae phenotype (Schulze and Sonnenborn, 2009; Yang, 2003; Han, et al., 2011. As such, cellular adhesion is an important feature of pathogenicity (Wang et al., 2012, Sundstrom, 2002). Moreover, biofilm formation is another critical virulence factor of *C. albicans* as it forms on both biotic and abiotic surfaces (Simoes et al., 2009) with catheters and other types of intravenous devices being primary targets. If such colonized devices are not surgically replaced, *C. albicans* biofilms can lead to life-threatening systemic infections (Nobile et al., 2006; Bauter et al., 2002; Mayer et al., 2013) that have accounted for costs exceeding a billion dollars a year in the United States alone (Miller et al., 2001).

Biofilm formation occurs in sequential steps with initial adhesion of yeast cells to a surface, yeast cell proliferation, and hyphal cells forming the top part of the biofilm and creating extracellular matrix material. Lastly, the yeast cells start to disperse or scatter from the biofilm complex (Mayer et al., 2013). Indeed, mature biofilms are far more resistant to commonly used anti-fungal agents and becoming even more so compared to the planktonic form counterpart due to the high complexity of biofilm architecture, matrix of biofilms, enhanced drug efflux pump expression and *Candida* metabolic plasticity (Mayer et al., 2013).

Azoles and polyenes are currently the most commonly prescribed anti-Candida drugs, while other treatments are in various phases of clinical development, including amorolfine, natifine, terbinafine, tolaftate, rilopirox, cilofungin, pradimycin, and benanomicin A (Ellepola, 2000). Still, fungal infections are more difficult to treat than their bacterial counterparts as multiple infection areas are possible making development of anti-fungal treatments even more complicated (Ellepola, 2000). Additionally, the anti-fungal agents azoles and polyenes, stimulate adverse side effects (Miceli et al., 2011; Ellepola, 2000), one of which is killing other benign microbiota consortium, thereby providing potential niches that other pathogens may colonize (Candiracci et al., 2011). Thus, development of innovative and multiple targeted anti-fungal agents that act on biofilms formed by *C. albicans* is critical.

As such, natural compounds are currently being studied extensively as alternates for current treatment approaches to protect from or mitigate already existing microbial infections (Gallucci et al., 2014; Nguyen et al., 2013; Palaniappan and Holley, 2010; Saleem et al., 2010). In particular, phenolic compounds are steadily growing as anti-fungal agents for human usages because, as secondary metabolites in plants, it is their function to protect against multiple threats, including fungal based diseases that

attack the host (Papadopoulou et al., 2005; Hirasawa et al., 2004). Phenols are widely distributed throughout the plant kingdom and include terpenoids, organosulfur compounds, isoquinoline alkaloids, flavonoids, lactone and naphthoquinone (Treutter, 2005 & Bravo, 1998). Studies have also shown that phenolic compounds possess multiple human health promoting properties due to their ability to protect against cellular stresses, such as oxidation and inflammation, as well as to treat conditions or diseases, includin, cancer, hypertension, vascular fragility, allergies, diabetes and hypercholesterolemia (Treutter, 2005; Bravo, 1998; Papadopoulou, *et al.,* 2005).

Despite such benefits, a critical gap of knowledge exists on the ability of phenolic compounds to target *C. albicans* adhesion and biofilms already established on synthetic devices used for human health purposes. Therefore, the objective of this work was to screen the ability of several phenolic compounds ubiquitous throughout nature, including ferulic acid, gallic acid, sinapic acid, quercetin, catechin, epicatechin, and coumaric, as well as chlorophyll and farnesol to remediate biofilm formation and cellular adhesion. The expected outcome of this study was to identify phenolics and their degree of efficacy to remediate the cited virulence factors, as isolated compounds, at different concentrations and against two strains of *C. albicans*, A72 and SC5314.

1.3 Materials and Methods

1.3.1 *Preparation of C. albicans yeast stock culture*

Two *C. albicans* strains (SC5314 and A72) were obtained from Kenneth Nickerson, University of Nebraska-Lincoln. A stock culture was grown to the stationary phase in 500 ml of yeast extract 5 g, peptone 2.5 g, and dextrose 10 g medium (YPD). The medium (25 ml) was then added to 125 ml Erlenmeyer flasks and a ½ loopful of *C. albicans* (A72 and SC5314), which was maintained on YPD agar for not more than 1 month, was incubated in a shaking water bath at 30°C for 22 h (or until cells achieved

stationary phase, i.e., no budding observed microscopically). The cells were then washed three times with potassium phosphate buffer (pH 6.5) followed by centrifugation until a clear supernatant was obtained. The ensuing pellet was then resuspended in 7.5 ml of phosphate buffered saline and maintained at 8-10 ^{o}C until use.

1.3.2 *Treatment preparation.*

Serum media (Atlanta Biological) was thawed at room temperature for 5 min, and then 5 ml was dissolved in 45 ml of potassium phosphate buffer (pH 6.5) prior to use. Stock solutions of gallic acid (G), ferulic acid (F), sinapic acid (S), epecatchin (E), farnesol (FA), and þ-coumaric acid (C) were freshly prepared at 200 mM in 100% ethanol until the solids had completely dissolved. Alternatively, quercetin, catechin (CAT), and chlorophyll (CH) were diluted in 50:50 ethanol: water solution. The concentrations of the natural compounds used for this study were prepared by diluting the stock solution such that the final delivery system was consistently 2% ethanol while the natural products ranged from 4.0-0.06 mM in eight 2-fold increments. Ethanol (2%) was selected as the delivery system as the chosen solvent had to completely dissolve the natural products while at a concentration that did not inhibit biofilm formation or cellular adhesion preliminary experiments had shown that 2% ethanol met these requirements. Methanol and dimethyl sulfoxide were screened as other potential delivery candidates. Methanol at any concentration inhibited adhesion and biofilm formation assays by inhibiting a control with only the cells + the media, whereas dimethyl sulfoxide quickly oxidized the phenols (data not shown).

Chlorophyll was also used for these studies due to the high abundance of this compound in grasses and other natural systems (Şükran, et al., 1998), whereas farnesol was selected as it is a quorum sensing compound produced by *C. albicans* to inhibit the yeast from switching to the hyphae state (Shirtliff et al., 2009). Farnesol is also present

in many dietary compounds so is not expected to be lethal to *C. albicans* at low quantities, but also could be an important compound for inhibiting other *C. albicans* virulence factors, such as biofilm formation and cellular adhesion (Ramage, et al., 2002).

1.3.3 *Biofilm/adhesion remediation treatments*

To determine the effects of the natural compounds on established adhered cells and biofilm formation, *C. albicans* strains (A72 and SC5314) were added at 5 x 10^6 cell per ml to Immunol 2HB 96 well plates with each well containing 140 μl serum. The plates were covered with aluminum and incubated at 37 °C. After the 24 h incubation, 60 μl of a given phenol at various concentration were added to each well. Another set of wells that contained and did not contain the cells were prepared with only 60 μl of 2% ethanol to serve as the negative and positive control, respectively. The treatments were monitored for adhered cells at 1, 3, 6 and 24 h against the negative control.

1.3.5 *Adhesion assay*

The adhesion assay was performed according to Pierce *et al.* (2008). Briefly, the medium from each well was carefully removed and 50 μl of crystal violet was added to the wells. The plates were covered again and incubated at room temperature for 45 min. After incubation, each plate was rinsed gently with 400 μl of ice cold water 5-10 times. The plates were inverted onto a paper towel to remove any non-adherent cells and water. The plates were then incubated for another 30 min at room temperature after adding 200 μl of 75% methanol to each well. The absorbance was determined at 590 nm using a microtiter plate reader (Fazly et al*.,* 2013).

1.3.5 *Biofilm assay*

Biofilm formation was determined by using the (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) XTT kit according to the manufacturer's direction (Sigma-Aldrich). (The XTT kit consists of XTT labeling reagent

and electron coupling reagent. This assay relies on yellow tetrazolium XTT salt cleavage to an orange formazan dye by the active metabolic cells, which indicates the viable cells, and is based on procedures cited by Pierce *et al.,* 2008 & Sudjana, *et al.,* 2012). The XTT labeling reagent and electron coupling reagent were thawed in a water bath set at 37 ^{o}C, and then 0.1 ml of electron coupling reagent was added to 5 ml of XTT labeling reagent to be activated prior to use. The XTT mixture 100 μl was added to each well and incubated for 2 h. The absorbance was determined at 450 nm using a microtiter plate reader.

1.3.6 *Percent remediation calculations*

Remediation of biofilm formation or *C. albicans* adhesion in already established films were defined as % remediation for both cases, which was determined by the following equation (Romano *et al.,* 2009):

$$\% \text{ Remediation} = (A_{control} - A_{sample})/ (A_{control})*100$$

Where: $A_{control}$ is the absorbance of untreated cells

A_{sample} is the cells with the treatment.

1.3.7 *Statistical analysis*

The biofilm/adhesion experiments were completed on 3-9 replicates for each treatment/concentration used and time point monitored. After data outliers were removed by the Grubs test at a 5% confidence interval, the final results were reported as the mean +/- standard deviation. One-way ANOVA was used to determine whether various treatments differed in terms of % remediation at 95% confidence interval ($p < 0.05$) using Tukey's honest significant difference. The statistical analyses were obtained with Stats Graphic Centurion XVI.1.

1.4 Results and discussion

The ability of *C. albicans* to develop resistance against the currently available antifungals is a contributing factor for increases of mortality rate (up to 30-40%) for patients who are critically ill (González de Molina et al., 2012; and Fazely et al., 2013). For this reason, efforts to discover new strategies to treat virulence factors of *C. albicans,* such as biofilm formation and cellular adhesion, are intensifying.

Many researchers have investigated the potential of phenolic compounds for such antimicrobial properties (Borges et al., 2013; Candiracci et al., 2012; Faria et al., 2011; Gallucci et al., 2014; Kazuko et al., 2010; Nguyen et al., 2013; Palaniappan and Holley, 2010; Saito et al., 2013; Wang et al., 2009). For example, resveratrol ranging from 200 µM to 900 µM prevented yeast-to hyphae transition of *C. albicans* (SC5134) (Kazuko et al., 2010). Another study reported that hyphal formation of *C. albicans* was impaired by catechin at a concentration of 2.8 mM (Saito et al., 2013). However, there is a lack of notable studies using naturally isolated phenolic compounds to remediate *C. albicans* adhered cells and formed biofilms.

In this study, several isolated phenolic compounds were screened for their ability to remediate *C. albicans* (SC5134 and A72) adhesion. As stated previously, the remediation effect was determined at four-time points (1, 3, 6 and 24 h) for each natural compound at eight different concentrations. However, the 6 h time point showed the most potent and significant effects for the majority of the compounds at the various concentrations. For example, at 3 h, the compounds aided in cellular adhesion (data not shown), while the 24 h data showed a decrease in the remediation, again for most of the treatments (data not shown). Given this initial screening process, only the 6 h treatments were analyzed further by statistically comparing the various compounds that showed remediation at a given concentration (Figure 1, 3; Table 1, 3) and a given

compound across concentrations (Figure 1.2, 1.4, and Table 1.2, 1.4) for *C. albicans* strains A72 and SC5314.

1.4.1 *Remediating* C. albicans *(A72) cellular adhesion*

In the case of *C. albicans*, A72, only one phenol, catechin (CAT), and the two non-phenols, chlorophyll (CH) and farnesol (FA), were able to reduce cellular adhesion using concentrations soluble at 2% ethanol (Figure 1.1). However, percent remediation was not significantly different among these compounds at the low concentrations ($p > 0.05$) (Table 1.1), which ranged from a low of -18% (CAT at 0.06 mM) to a high of ~ 30% (CAT at 0.06, 0.5 mm; FA 0.06) (Figure 1.1) indicating that the three compounds could be used at these low concentrations but the efficacy of each would not be consistent. Despite these results, at 2, 3 and 4 mM, the compounds showed significant differences from each other at each level while exerting different responses at each level (Table 1). For example, CAT was significantly different relative to CH and FA at 2.00 mM, which in turn were statically different from one another. Indeed, CAT exhibited a % remediation of > 50%, while the effect was substantially lower for CH and FA at 0 and 20%, respectively (Figure 1.1). Yet, when the level increased to 3.00 mM, CAT affected cellular remediation similar to both CH and FA, while the latter compounds were statistically different. Interestingly, at this higher concentration, the reduction of cellular adhesion exhibited by both CAT and FA decreased compared to 2.00 mM concentration, i.e., by 28 percentage points for CAT and 11 percentage points for FA. On the other hand, cellular adhesion remained at approximately 0 to 10% when treated with CH at 3.00 mM, but at a concentration of 4.00 mM the response increased to 44%. This value was statistically different compared to FA and CAT, which were similar in that both negatively affected remediation at this concentration.

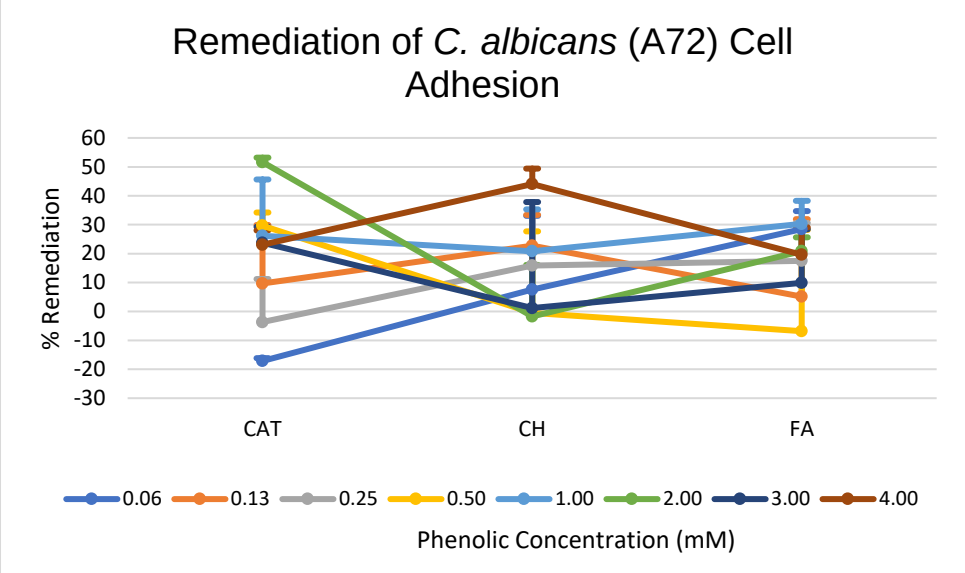

Figure 1.1: Effect of catechin (Cat), chlorophyll (CH), and farnesol (FA) on remediating *C. albicans* (A72) cellular adhesion 6 h post exposure of a given treatment concentration. Each point and vertical bar represent the mean ± standards deviation of three replicates.

Table 1.1. Rows with different letters show significant difference ($p < 0.05$) in remediation of *C. albicans* (A72) cellular adhesion across the different compounds but at the same treatment concentration.

mM	CAT	CH	FA
0.06	-17.12	7.54	28.47
0.13	9.68	22.73	5.11
0.25	-3.66	15.91	17.48
0.5	29.56	-0.51	-6.82
1.00	26.17	20.79	30.28
2.00	51.65[a]	-1.72 [b]	20.88 [c]
3.00	23.76[ab]	1.17 [b]	9.93 [a]
4.00	23.08 [a]	44.04 [b]	19.5 [ab]

mM - millimolar concentration of compound
CAT – Catechin, CH – Chlorophyll, FA – Farnesol

Figure 1.2 and Table 1.2 respectively show the reduction of cellular adhesion plots of each compound across concentration and the corresponding statistical analysis. Clearly, a typical log dose response curve was not generated in these studies. When the cells were exposed to either CH and FA, the different concentrations were not statistically different, which was unexpected given a low concentration of 0.06 mM and the high of 4.00 mM (Table 1.2). These results indicate that unreliability of developing either of these compounds as a means to mitigate cellular adhesion of *C. albicans*, A72, given that the % remediation exerted by CH and FA was approximately 0 to 45% and -10 to 30% for CH and FA, respectively, regardless of concentration (Table 1.2). The values obtained from the various concentrations most likely were due to the high variability caused by the interaction of these compounds with *C. albicans*, A72, as evidenced by the large error bars shown in Figure 1.2.

In contrast, decreased variability occurred when CAT was used at most concentrations, which cannot be explained at this point in our research. As shown in Table 1.2, the responses exerted by both the 0.13 and 0.25 mM CAT treatments were statistically similar to 0.06 mM, while 0.13 mM treatment also trended with all the other concentrations except 2.00 mM. Notably, the treatments that ranged from 0.50 to 4.00 mM were all statistically similar resulting in % remediation of approximately 25-50%. Although this range is fairly large for developing a consistently efficacious anti-virulence treatment mitigating *C. albicans* (A72) adherence to cells, the significance of this data is that it shows the potential of CAT to remediate cellular adhesion. In another study, CAT was able to inhibit hyphal formation of *C. albicans* (NUD-202) at 2.8 mM with 10% fetal calf serum as the inducing agent, i.e., the same concentration and medium used herein (Saito 2013).

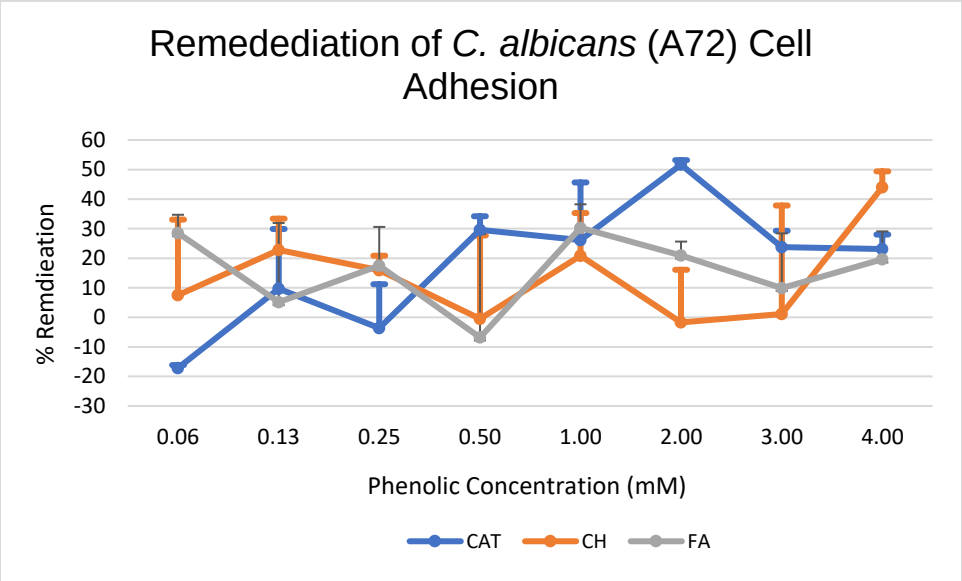

Figure 1.2: Effect of different concentrations of catechin (Cat), chlorophyll (CH), and farnesol (FA) on remediating *C. albicans* (A72) cellular adhesion 6 h post treatment exposure. Each point and vertical bar represent the mean ± standards deviation of three replicates.

Table 1.2. Rows with different letters show significant difference ($p < 0.05$) in percent adhesion remediation across different treatment concentrations using a given compound.

mM	**0.06**	**0.13**	**0.25**	**0.5**	**1.00**	**2.00**	**3.00**	**4.00**
CAT	-17.12^{a}	9.68 ^{abc}	-3.66^{ab}	29.65^{bc}	26.17	51.65 ^{c}	23.7 ^{abc}	23.0 ^{abc}
CH	7.54	22.73	15.91	-0.51	20.79	-1.72	1.17	44.04
FA	28.47	5.11	17.48	-6.82	30.28	20.88	9.93	19.58

mM - millimolar concentration of compound
CAT – Catechin, CH – Chlorophyll, FA – Farnesol

As such, development of CAT as a consistently efficacious treatment for reducing cellular adhesion may be possible with more research to understand the interaction of this compound with *C. albicans.* Khan et al., (2012) further showed that the cell suspension concentration and the medium used affects the properties of an anti-fungal, which also could translate into reducing established film formation.

Plant extracts that derived *from Krameria, Aesculus hippocastanum*, and *Chelidonium majus* demonstrated potential remediation activity against *Staphylococcus aureus and Staphylococcus* epidermidis strain adhesion after 24 h of exposure (Artini et al., 2012). Although the plant extracts were not characterized, this study shows the potential of using natural products rich in micronutrients, including the phenols, as are all plant products, of remediating *C. albicans* A72 cellular adhesion.

1.4.2 *Remediating* C. albicans *(*SC5314*) cellular adhesion*

Similar to *C. albicans* (A72), only CAT, CH and FA showed remediation properties against *C. albicans* (SC5314) (Figure 1.3). Still, differences occurred in the effectiveness across concentrations between the strains, as shown by the difference between the trend lines vs concentration for each strain (Figure 1.1 and 1.3). In particular, the percent remediation of *C. albicans* (SC5314) cellular adhesion was not significantly different among these compounds at the low concentrations 0.06-0.25 mM; CAT did vary at 0.05 and 1.00 mM compared to CH and FA at these concentrations; and all three compounds were similar at the high concentrations of 2.00-4.00 mM, unlike *C. albicans* A72 (Table 1.1).

At 1.00 mM treatment, the percent remediation was significantly different between CAT and the other two compounds (CH and FA), which a low of 0% for CAT, but increased to 40% and 45 % for to CA and FA, the latter showing the most potent

responses (Figure 1.2) among the treatment combinations. For the other significantly different response caused by a concentration (2.00 mM), the effective values were much closer in their degree in potency that CAT, CH and FA, the percent remediation produced was 20, 35 and 25%, respectively.

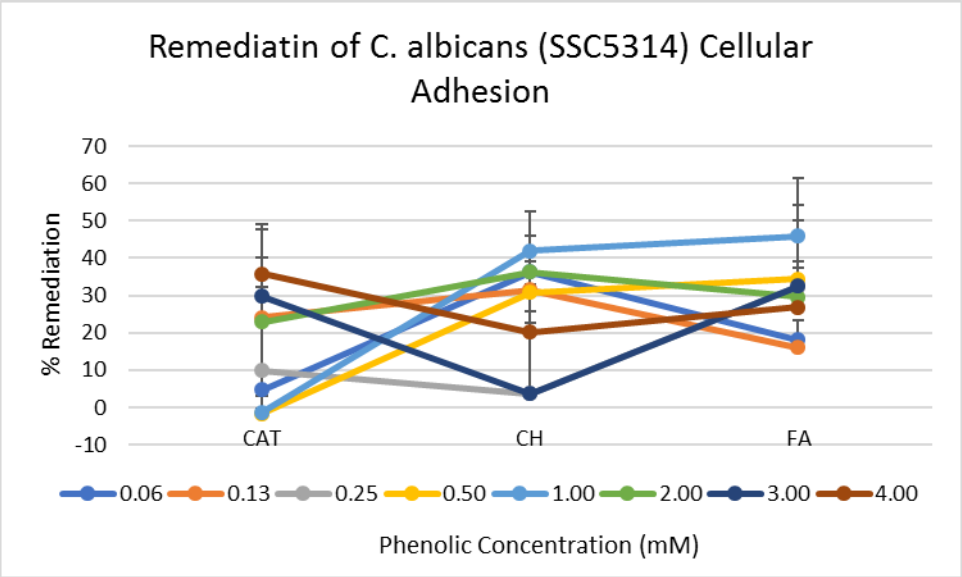

Figure1. 3 Effect of different concentrations of catechin (CAT), chlorophyll (CH), and farnesol (FA) on remediating *C. albicans* (SC5314) cellular adhesion 6 h post treatment exposure. Each point and vertical bar represent the mean ± standards deviation of three replicates.

Table 1.3. Rows with different letters show significant difference ($p < 0.05$) in remediation of *C. albicans* (SC5314) cellular adhesion across the different compounds but at the same treatment concentration.

mM	CAT	CH	FA
0.06	4.74	36.31	18.16
0.13	24.19	31.52	16.13
0.25	9.92	3.73	32.3
0.5	-1.58 [a]	30.78 [b]	34.36 [b]
1.00	-1.25 [a]	42.03 [b]	45.91 [b]
2.00	22.87	36.32	29.62
3.00	29.72	3.62	32.47
4.00	35.77	20.12	26.9

mM - millimolar concentration of compound
CAT – Catechin, CH – Chlorophyll, FA – Farnesol

Clearly, the compounds act upon the two strains differently (Figures 1.1 and 1.3). The mid-range concentrations (1.00 and 2.00 mM) were more effective against the *C. albicans* SC5314 strain while the higher concentrations were the most efficacious in reducing the virulent factor produced by *C. albicans* A72 (Table 1.2). Although the reduction in adhesion differed across compounds at the same concentration in some cases (Table 3), the adhesion responses were statistically similar for each compound across concentrations regardless of dosage (Table 1.4), similar to that shown for *C. albicans* (A72) (Table 2). The adhesion reduction for CAT, CH, and FA ranged respectively from 0-35% (at 0.5 and 1.00 mM for the low and 4.00 mM for the high), 2-40% (0.25 and 3.00 mM for the low and 1.00 mM for the high), and 20-40% (0.05 and 0.13 mM for the low and 1.00 for the high).

Again, similar to *C. albicans* (A72), the relatively broad dosage ranges indicate that unreliability of developing any of these compounds at any concentration to alleviate cellular adhesion of *C. albicans* (SC5314), albeit FA may be acceptable given that a low dosage of 0.06 mM could still elicit quite high reductions. This is especially important as farnesol is a well-characterized quorum signal molecule, which blocks the transition from yeast to hyphae (Derengowski et al., 2009). Moreover, this data showed the ability of a relatively large molecule, chlorophyll, to mitigate *C. albicans* cellular adhesion, as most studies used small molecules (Fazely et al., 2013; Wong, et al*.,* 2014). Nonetheless, inhibition or reduction of the initial adherence could be an ideal strategy to prevent mature biofilm formation (Cerca et al., 2005) given their resistance to anti-fungal agents. Thus, more studies are needed on the remediation of adhered cells and it must be applied to different strains and species of *Candida* because the treatment may act differently on each as indicated here.

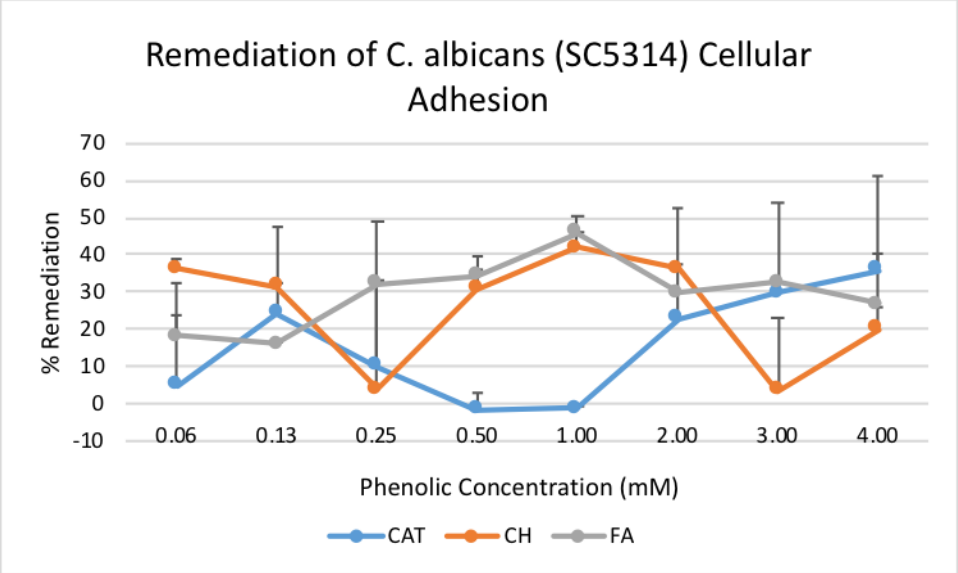

Figure 1.4: Effect of catechin (Cat), chlorophyll (CH), and farnesol (FA) on remediating *C. albicans* (SC5314) adhesion after 6 h exposed to the specified treatment concentration.
Each point and vertical bar represent the mean ± standards deviation of nine replicates.

Table 1.4. Rows with different letters show significant difference ($p < 0.05$) in percent adhesion remediation exerted by the different concentrations but at the same treatment compounds.

Mm	0.06	0.13	0.25	0.50	1.00	2.00	3.00	4.00
CAT	4.74	24.19	9.92	-1.58	-1.25	22.87	29.72	35.77
CH	36.31	31.52	3.73	30.78	42.03	36.32	3.62	20.12
FA	18.16	16.13	32.3	34.36	45.91	29.62	32.47	26.9

mM - millimolar concentration of compound
CAT – Catechin, CH – Chlorophyll, FA – Farnesol

1.4.3 *Remediating* C. albicans *(A72) biofilm formation*

Yeast to hyphae transition of *C. albicans* is a virulence factor of critical concern, as it participates in host tissue penetration, enabling invasive growth followed by biofilm formation (Raut et al., 2013). The construction of biofilms can be divided into 2 phases: initial adherence at 3 to 4 h followed by biofilm formation at 12 to 24 h (Ceonye et al., 2011) resulting in a heterogeneous structure that consists of yeast, hyphae, and pseudohyphea, and is coated with extracellular polymeric materials. The yeast to filaments transition strengthens and support the biofilm structure (Raut et al., 2013), which makes biofilms the most resistant virulence factor to available antifungal agents (Fazely et al., 2013).

To this end, the natural compounds used in the cell adhesion assays were screened at the 8 concentrations cited previously (Sections 1.4.1- 1.4.2) for their ability to migrate biofilms produced by *C. albicans* SC5134 and A72. Similar responses to the treatments at the different concentrations occurred for the various time points (1, 3 and 24 h) as described for the adhesion work (Section 1.4.1-Section 1.4.2) with the most potent effect occurring at the 6 h time point (data not shown). Therefore, results obtained from reduction of biofilm formation in the presence of several natural compounds at this time point will be the focus for the next two sections.

For *C. albicans* A72, several compounds (G, F, S, E, Q, C, CAT, CH and FA) were able to attenuate biofilm formation (Figure 1.5) compared to those that promoted the reduction of cellular adhesion, namely CAT (Figure 1.1). At the concentrations ranging from 0.06-1.00, the compounds did not differ significantly in terms of their ability to reduce established biofilms with the exception of FA (at 0.05, 0.25, and 0.05 mM), C / CH (0.25 mM), and Q (0.06, 0.13 and 0.25 mM) (Table 5). For the phenol (Q), the remediation results were statistically different from G, F, S and E at

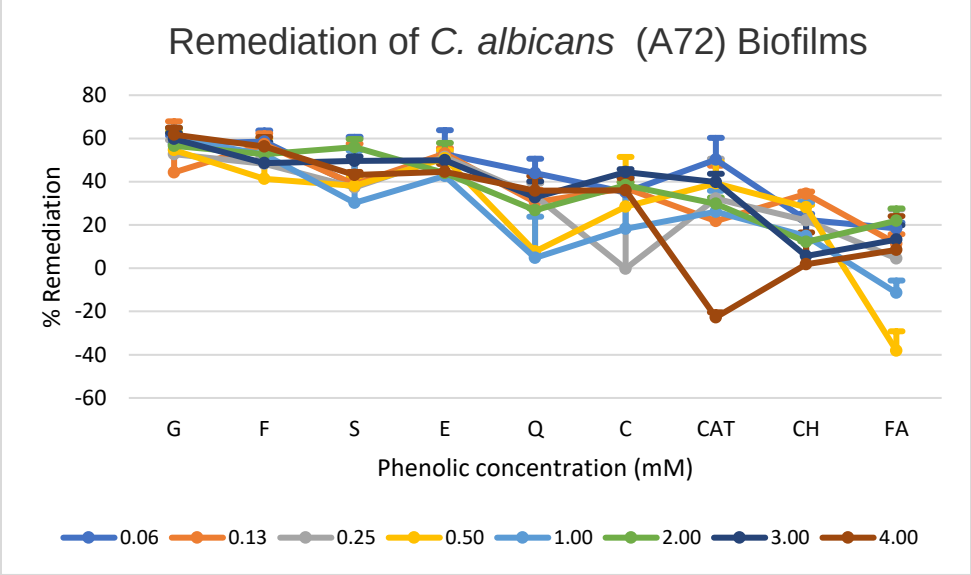

Figure 1.5: Effect of gallic acid (G), ferulic acid (F), sinapic acid (S), epicatechin (E), quercitin (Q), p-coumaric acid (C), catechin (CAT), chlorophyll (CH), and farnesol (FA) on remediating *C. albicans* (A72) biofilm formation 6 h post exposure of a given treatment concentration.
Each point and vertical bar represent the mean ± standards deviation of three replicates.

Table1.5. Rows with different letters show significant different ($p < 0.05$) in percent remediation of *C. albicans* (A72) biofilm formation exerted by compounds 6 h post exposure of a given treatment concentration.

mM	G	F	S	E	Q	C	CAT	CH	FA
0.06	57.49	58.55	38.38	52.75	44.05 ^{ab}	35.06 ^{ab}	50.16 ^{ab}	22.43^{b}	18.4 ^{b}
0.13	44.25	57.24	38.81	53.08	30.49 ^{ab}	36.97 ^{ab}	21.74 ^{ab}	34.41^{ab}	11.32 ^{b}
0.25	52.72	48.27	37.3	51.43	33.52 ^{ab}	-0.19 ^{bc}	32.22 ^{bc}	22.14^{bc}	4.42 ^{c}
0.5	54.61	41.33	38.11	50.83	7.72 ^{b}	28.46 ^{a}	39.3 ^{a}	28.16^{ab}	- 38.2 ^{b}
1.00	60.05	52.57	30.1	42.59	4.84 ^{bc}	18.27^{ab}	26.04 ^{ab}	14.9 ^{bc}	-11.42^{abc}
2.00	56.57^{a}	52.68 ^{a}	56.04 ^{a}	43.9	26.78 ^{bc}	38.42^{ab}	29.83 ^{bc}	12.17 ^{b}	21.89 ^{bc}
3.00	59.78	48.6	49.5 ab	49.8	32.81 ^{c}	44.44^{abc}	39.8 ^{bc}	5.6 ^{c}	13.17 ^{bc}
4.00	61.17 ^{a}	56.26 ^{a}	43.1 ^{abc}	44.5	35.48^{bcd}	36 ^{abcd}	- 22 ^{d}	1.86 ^{cd}	8.49^{abcd}

mM - millimolar concentration of compound G – Gallic acid, F- Ferulic acid, S- Sinapic acid, E- Epicatechin, Q- Quercetin,C- þ-coumaric acid, CAT – Catechin, CH – Chlorophyll, FA – Farnesol

concentrations > 1.0 mM. In fact, Q, CAT, CH and FA started to trend differently from the other molecules in most cases with increasing treatment levels exhibiting lower remediation effect < 40 %, i.e., those greater than 1.0 mM (Figure 1.5 and Table 1.5), except for the FA at 4.00, which was statistically similar to multiple compounds.

Yet, at a concentration of 0.50 mM FA (-38%) was the least effective in remediating biofilm formation followed by 4.00 mM CAT and CH resulting in a % remediation of -22% and 1.86 respectively. It must be noted that 4.00 mM CAT was also statistically similar to 4.00 mM CH. Notably, these three compounds were the most effective in reducing cellular adhesion. Moreover, a 0% reduction occurred with 1.00 mM Q treatment, but this result was statistically comparable with several of the remaining compounds, which ranged in a high of 60% for F, G, and E. Additionally, Q was statistically similar in % biofilm reduction with that of FA at 0.50 mM, (-38%) and CH (1.86%).

These results most likely are due to the structure-function effect of the compounds and thereby their mode of interaction with the *C. albicans* biofilm formation. The phenols, Q and CAT, are aglycone flavonoids and farnesol is a sesquiterpene; each are fairly small compounds consisting of 15 carbons whereas chlorophyll is a much larger compound that is essential for photosynthesis (Esten and Dannin, 1950), Although different structurally, all of these compounds are highly insoluble in water due mainly to the lack of hydroxyl groups, soluble derivatization groups and/or size of the molecule (Esten and Dannin, 1964; Mishra et al., 2011). These structures thereby promote greater solubility in more non-polar delivery systems, which also may be limiting their ability to directly interact with the films. As phenolic acids, G, F, S, and C are small molecular weight molecules containing an acid group and hydroxyl groups located at different position on the phenol; whereas epicatechin is a glycosylated flavonoid.

Additionally, the structural characteristics could also be affecting their degree of potency due to differences in their antioxidative capacities, especially at the higher concentrations. This hypothesis is supported by Bores et al. (2013) who attributed the structure of the antimicrobial phenolics to the phenolic antioxidant capacity and ultimately increasing toxicity to microorganisms. Still, it is unclear whether the phenols are actively destroying existing biofilms or affecting the ability of *C. albicans* to promote the growth of biofilms via their antioxidative properties.

These results further demonstrate that the behavior of Q coincides with the phenolic acids and epicatechin phenols when the concentrations are low (< 2.00 mm) but is more similar to the non-phenols, CH and FA, at concentration of 3 and 4 mM (Table 1.5). The chemical substructure of quercetin is such that it is able to stop oxidative processes by acting both as a scavenger of free radicals and by chelating reactive metals involved in oxidation (Spencer et al. (2004). In fact, quercetin has been cited in various reports as exerting higher antioxidative capacities than any of the other phenolic compounds used herein (Rice-Evans, 1997). Yet, terpenes, such as FA, and chlorophyll are able to scavenge free radicals, but a direct correlation to phenols have not been reported to our knowledge. Again, many of the test compounds in reducing the biofilms were similar to FA, CAT, Q and CH and each other at one concentration but different at another dosage indicating that treatment levels may promote a different mode of action for each compound.

The compounds FA, CH, CAT, and Q also showed relatively high variability across treatment concentration similar to that discussed in the cellular adhesion sections for both *C. albicans* A72 and SC5314, which may account, in part, for their comparable results across compounds (Figure 1.5 and Table 1.5). The trend lines representing these FA, CH, CAT and Q (Figure 1.5), show more randomness between concentration points

and thus ranged from 0 to 30%, 42 to -22%, 0-20% and -40 -22% for Q, CAT, CH and FA, respectively. On the other hand, G, F, S, E and S produced less variability, and the highest remediation values. More specifically, gallic acid (G) was able to reduce biofilm formation by 55-60% regardless of dosage (Figure 1.6), which was not statistically different (Table 1.6). Moreover, G was the only phenol that did not trend concurrently with another compound (Table 1.5) or across concentration (Table 1.6). Although biofilm reduction was not affected by a given compound-based dosage levels for any of the treatments (Table 1.6), G, F, S, E and C showed the most consistent trend lines across concentrations with a remediation range of 40-60%. These results indicate that one or more of the phenolic acids are potential candidates as anti-fungals for remediation of biofilms produced on synthetic devices.

1.4.4 *Remediating* C. albicans *(SC5314) biofilm formation*

In the case of *C. albicans,* strain SC5314, the compounds G, F, S, E, Q, C, CAT, CH and FA were again able to reduce the formation of the corresponding biofilm formation (Figure 1.7). At the lower concentrations ranging from, 0.06-0.50 mM, the compounds were not significantly different in terms of their ability to reduce established *C. albicans* biofilms with the exception of FA (at 0.06 0.13, 0.25, 0.5 mM), CH and Q (0.13 and 0.50 mM), CAT (0.25 and 0.50 mM) (Table 1.7). The compounds, Q, CAT, CH, and FA, again started to trend differently from the other molecules with increasing concentrations, i.e., those greater than 1.0 mM. The flavonoid, Q, was significantly different in % remediation compared to S and E at 1.00 and 2.00 mM; CH, FA at 2.00 mM, and G, F, E, and S at 3.00 and 4.00 mM. It has been proposed by Nitiema et al. (2012) that antimicrobial activities increase when lower groups of hydroxyls are bonded to phenols or flavonoids, which results in high chemical affinity to the pathogenic lipid membrane. Yet, this research indicates again that the least soluble compounds in

aqueous solution due in part to the presence of hydroxyl groups was the least effective in negatively impacting biofilm formation. For example, at concentration 0.05 mM FA was the least effective, resulting in % remediation of ~ 4% and ~ 6%, respectively.

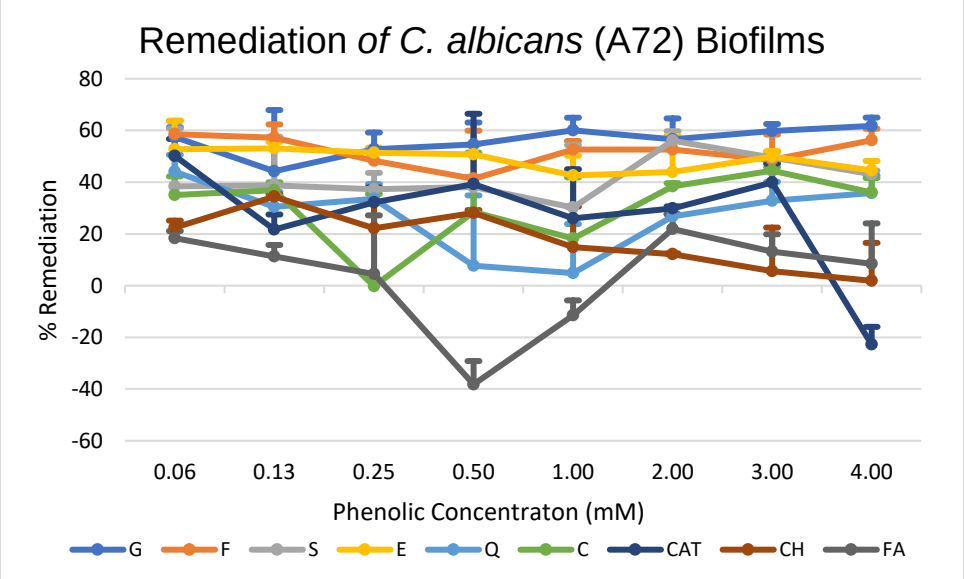

Figure 1.6: Effect of different concentrations of gallic acid (G), ferulic acid (F), sinapic acid (S), epicatechin (E), quercetin (Q), catechin (CAT), p-coumaric acid (C), chlorophyll (CH), and farnesol (FA) on remediating *C. albicans* (A72) biofilm formation 6 h post treatment exposure. Each point and vertical bar represent the mean ± standards deviation of three replicates.

Table 1.6. Rows with different letters show significant difference ($p < 0.05$) in percent biofilm formation across different treatment concentrations using a given compound.

Mm	**0.06**	**0.13**	**0.25**	**0.50**	**1.00**	**2.00**	**3.00**	**4.00**
G	57.49	44.25	52.72	54.61	60.05	56.57	59.78	61.17
F	58.55	57.24	48.27	41.33	52.57	52.68	48.6	56.26
S	38.38	38.81	37.3	38.11	30.1	56.04	49.5	43.1
E	52.75	53.08	51.43	50.83	42.59	43.9	49.8	44.5
Q	44.05	30.49	33.52	7.72	4.84	26.78	32.81	35.48
C	35.06	36.97	-0.19	28.46	18.27	38.42	44.44	36
CAT	50.16	21.74	32.22	39.3	26.04	29.83	39.8	-22 b
CH	22.43	34.41	22.14	28.16	14.9	12.17	5.6	1.86
FA	18.4	11.32	4.42	-38.2	-11.42	21.89	13.17	8.49

mM - millimolar of compound concentration G – Gallic acid, F- Ferulic acid, S- Sinapic acid, E- Epicatechin, Q- Quercetin, C- þ-coumaric acid, CAT – Catechin, CH – Chorophyll, FA – Farnesol

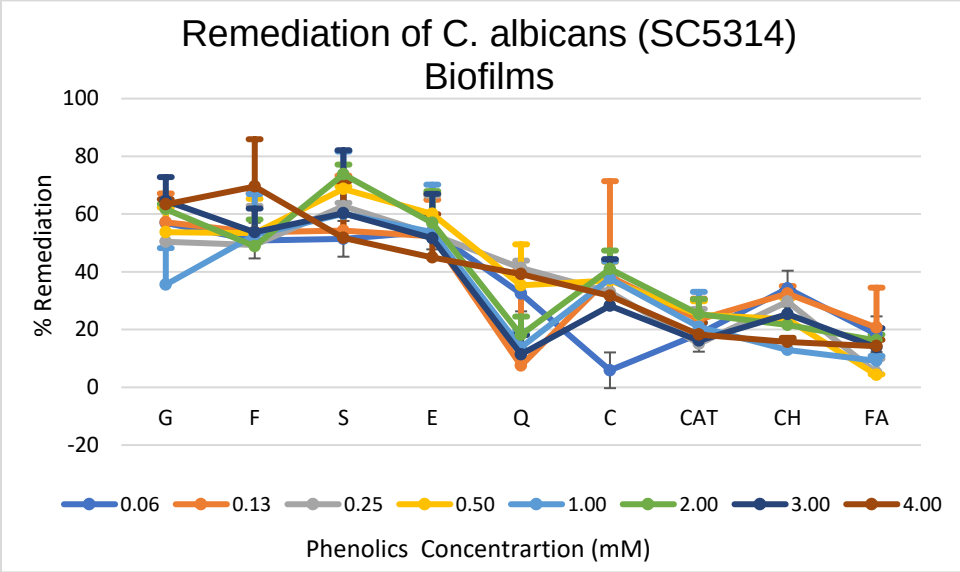

Figure 1.7: Effect of gallic acid (G), ferulic acid (F), sinapic acid (S), epicatechin (E), quercetin (Q), p-coumaric acid (C), catechin (CAT), chlorophyll (CH), and farnesol (FA) on remediating *C. albicans* (SC5314) biofilm formation 6 h post exposure of a given treatment concentration. Each point and vertical bar represent the
mean ± standards deviation of three replicates.

Table 1.7. Rows with different letters show significant difference ($p < 0.05$) in percent 6 h remediation of *C. albicans* (SC5314) biofilm formation exerted across compounds 6 h post exposure of a given treatment concentration.

mM	G	F	S	E	Q	C	CAT	CH	FA
0.06	56.9	50.83	51.43	53.97	32.51	5.9	18.53	34.21	18.39
0.13	57.16	53.69	54.24	57.16	7.5 ^{b}	38.38	23.47^{bcd}	32.5^{abcd}	20.68 ^{d}
0.25	50.38	49.25	62.75	52.99	41.3^{abc}	33.03	15.05 ^{cd}	29.66^{abc}	5.01 ^{d}
0.5	53.76	53.43	68.72	60.06	35.32 ^{bc}	37.04	25.09 ^{cd}	23.59 ^{cd}	4.29 ^{c}
1.00	35.55	52.75	60.09	53.7	13.83 ^{c}	37.66	20.96 ^{bc}	12.92 ^{c}	9.14 ^{c}
2.00	61.69	48.85	73.77	56.95	18.14 ^{bc}	41	25.37 ^{cd}	21.61 d	16.23 ^{d}
3.00	64.6	53.69	60.26	51.63	11.44 ^{b}	28.25	16.13 ^{b}	25.36 ^{ab}	13.87 ^{b}
4.00	63.43	69.54	51.73	44.97	39.2 ^{abc}	31.65	18.25 ^{c}	15.76 ^{c}	14.24 ^{c}

mM - millimolar concentration of compound G – Gallic acid, F- Ferulic acid, S- Sinapic acid, E- Epicatechin, Q- Quercetin C- þ-coumaric acid, CAT – Catechin, CH – Chlorophyll, FA – Farnesol

G, F, S, and E were able to reduce biofilm formation by equivalent amounts at all concentrations, with relatively high biofilm reduction of 50-60% (Figure 1.8), (Table 1.8). It has also been reported that phenolic compounds cause reduction of pathogenic infections by the accumulating at the site of infection (Nicholson,1992). The results presented in this manuscript might be attributed to the smaller molecular weight of G, F and S, in comparison to flavonoids i.e., Q and E or non-phenolic compounds FA and CH. Due to their size, the phenolic acids could accumulate at the site and thereby reduce biofilm formation through altering the hydrophobicity causing cytoplasmic content leakage. Another possible mode of action is by acting on reducing the ergosterol biosynthesis, which can damage the cell membranes and eventually reduce the growth of *C. albicans* and thus biofilm formation (Teodoro et al. 2015). At any rate, these results are supported by Wang et al. (2009), who demonstrated that gallic acid prevented *C. albicans* biofilm formation at an MIC50 of 5.9 mM. The compounds, CH and Q, were significantly different in terms of remediation biofilm across treatment concentrations (Figure 1.8) further supporting that their structure may be impacting biofilm formation for *C. albicans* (SC5314) differently as both are uniquely different from the remaining compounds. Despite the ability of Q to remediate biofilm formation, variability was high among the various concentrations, which ranged from a low of 8% to a high of 42%. Yet for the phenolic acid, C, the high variability caused a % remediation between the low

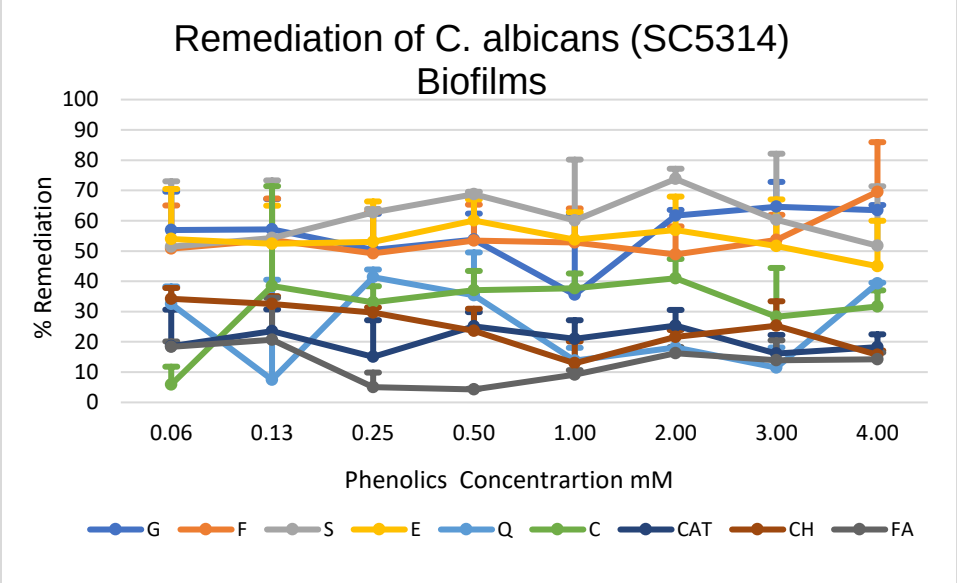

Figure 1.8: Effect of different concentrations of gallic acid (G), ferulic acid (F), sinapic aicd (S), epicatechin (E), catechin (CAT), p-coumaric acid (C), chlorophyll (CH), and farnesol (FA) on remediating *C. albicans* (SC5314) biofilm formation 6 h post treatment exposure. Each point and vertical bar represent the mean ± standards deviation of three replicates.

Table 1.8. Rows with different letters show significant difference ($p < 0.05$) in percent adhesion remediation across different treatment concentrations using the given compound.
mM - millimolar concentration of compound

mM	**0.06**	**0.13**	**0.25**	**0.50**	**1.00**	**2.00**	**3.00**	**4.00**
G	56.9	57.16	50.38	53.76	35.55	61.69	64.6	63.43
F	50.83	53.69	49.25	53.43	52.75	48.85	53.69	69.54
S	51.43	54.24	62.75	68.72	60.09	73.77	60.26	51.73
E	53.97	52.32	52.99	60.06	53.7	56.95	51.63	44.97
Q	32.51^{bcd}	7.5 ^{a}	41.3 ^{d}	35.32 ^{cd}	13.83 ^{ab}	18.14 ^{d}	11.44 ^{a}	39.2 ^{bcd}
C	5.9	38.38	33.03	37	37.66	41	28.25	31.65
CAT	18.53	23.47	15.05	25.09	20.96	25.37	16.13	18.25
CH	34.21 ^{a}	32.5 ^{a}	29.66^{ab}	23.59^{abc}	12.92 ^{c}	21.61^{abc}	25.36^{abc}	15.76^{bc}
FA	18.39	20.68	5.01	4.29	9.14	16.23	13.87	14.24

G – Gallic acid, F- Ferulic acid, S- Sinapic acid, E- Epicatechin, Q- Quercetin, C- þ-coumaric acid, CAT – Catechin, CH – Chorophyll, FA – Farnesol

concentration 0.06 mM of ~6% and high concentration 2.00 mM of 41% (Figure 1.8), which were not significantly different (Table 1.8).

In conclusion, biofilm formation was reliably and substantially reduced when phenolic acids and derivative flavonoids, particularly gallic acid, ferulic acid, sinapic acid and epicatechin, were used as the treatments. Moreover, the effects of the doses were not significantly different across concentrations indicating that even at low doses, biofilm formation by either *C. albicans* strain (A72 or SC5314) could be inhibited. Despite the efficacy of these compounds, they were unable to inhibit cellular adhesion. In fact, the opposite occurred, in that the compounds able to reduce cellular adhesion, again for both strains, were least effective in reducing biofilm formation. Nonetheless, these studies show that phenols and other compounds ubiquitous in plants (CH) or *C. albicans* (FA) may be potent anti-fungal agents that act on established cellular adhesion and/or biofilm formation.

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Chapter 2

Natural compounds, with an emphasis on phenols, act synergistically to remediate cellular adhesion and biofilm formation produced by *C. albicans* (A72 and SC5314) on synthetic devices

2.1 Abstract

C. albicans is a commensal member of the microbiome that inhabits the gastrointestinal and oral mucosa. However, it can be an opportunistic pathogen and cause superficial infections when the environment is compromised, and it can also cause life threating systematic infections. *C. albicans* strains have become resistant to available antifungal agents, which leads to growing interest toward inventing novel strategies to remediate adhesion and biofilm formation. The objective of this study was to determine the potential synergistic interplay of the 7 phenols and 2 non-phenolic compounds to remediate cell adhesion and biofilm formation of *C. albicans* (A72 and SC5314)**.** After exposing *C. albicans* (A72 and SC5314) to the different treatment combinations at 5 different concentrations 0.03-0.5 mM to *C. albicans* for (6 h) most of the treatment combinations (G-F: Gallic Acid-Ferulic Acid, S-Q: Sinapic Acid-Quercetin, F-E: Ferulic Acid-Epicatechin, E-C: Epicatechin-Coumaric Acid, CAT-Q: Catechin-Quercetin, CAT-CH: Catechin-Chlorophyll) were effective on mitigating cellular adhesion of *C. albicans* (A72 and SC5314) by > 50 % with FIC > 0.5. Further, the same combinations with the addition of CAT-C: Catechin- Coumaric Acid were effective on remediating ~ > 30 % of biofilm with 0.5< FIC <1.

Keywords: Candida, phenols, synergism

2.2 Introduction

Candida albicans inhabits the gastrointestinal tract (GI) as a typical commensal member but can become an opportunistic pathogen when the immune system, GI enzymes, or host microflora is compromised (Schulze & Sonnenborn, 2009). As such, mortality have been reached 30-40% due to Candida infections of the mucosal membranes (candidiasis) or of the bloodstream (candidemia) especially in individuals who are critically ill (González de Molina et al., 2012; Shareck and Belhumeur, 2011; Underhill and Iliev, 2014; Miceli et al., 2011; Pfaller and Diekema, 2007; Pfaller et al., 2012). For hospitalized patients, *C. albicans* is considered the most prevalent cause of nosocomial bloodstream infections (Magill et al., 2014). Expenditures of $6,000–$29,000 have been estimated by the Centers for Disease Control for each *C. albicans* case of infection, which have resulted in increasing costs to U.S health care of millions of dollars annually (CDC (Centers for Disease Control and Prevention), 2013).

The ability of *C. albicans* to transition from yeast to hyphal growth is the predominant virulence factor that has been linked to its pathogenicity (Hazan et al., 2002; Sudbery et al., 2004). The filamentous form is more invasive than the yeast form as it is able to penetrate and colonize the other body organs (Dalle et al., 2010; Phan et al., 2007; Sudbery et al., 2004; Weide and Ernst, 1999; Zhu and Filler, 2010). Moreover, the hyphal form is highly resistant to host defenses (Clark and Hajjeh, 2002; Yan et al., 2013). *C. albicans* infections are also capable of forming biofilms on the surfaces of synthetic devices used for human health purposes, such as an intravascular or urinary catheter, and endotracheal tubes, as well as devices implanted in their entirety into the body, including, but not limited to, subprosthetic heart valves, cardiac pacemakers and

joint replacements, all of which are susceptible to *C. albicans* cellular adhesion and biofilm formation. (Douglas, 2003).

Notably, *C. albicans* biofilm is 10–1000 times more resistant to antifungal drugs in comparison to other *C. albicans* morphologies (Douglas, 2003). Amphotericin B, fluconazole, flucytosine, itraconazole and ketoconazole are important antifungal drugs clinically investigated for their efficacy in treating *C. albicans* biofilm formation. However, these drugs were less effective in treating biofilms compared to the other *C. albicans* virulence factors (Hawser et al., 1995). Moreover, only a limited number of antifungal drugs are currently available to address *C. albicans* biofilm production. For example, lipid formulations of amphotericin B and two echinocandins (caspofungin and micafungin) were reported as antibiofilm to *C. albicans* (Kuhn et al., 2002). Also, caspofungin was approved as an antifungal drug that suppresses biofilm formation by inhibiting the major *C. albicans* cell wall component β1,3-glucan synthesis (Bachmann et al., 2002; Ramage et al., 2002). In this context, specific mechanisms of action of *C. albicans* reaction to drugs have been identified to contribute to their resistance, such as restricted drug penetration into the biofilm matrix, decreased growth rate or lack of nutrients due to phenotypic changes, and an increase in resistance gene expression induced by surface contact (Douglas, 2003).

Recently, researchers have proposed an approach to *C. albicans* resistance to commonly used drugs by developing agents that maintain the organism in its non-virulent phenotype (Shareck and Belhumeur, 2011). As *C. albicans* is a benign member of microbiota when in its yeast form, targeting the non-lethal morphology maybe will ensure that a more lethal pathogen will not colonize the vacant niche (Lewis and Kontoyiannis, 2001).

Natural compounds, such as phenolic compounds, have been extensively investigated as promising antifungal agents (Gallucci et al., 2014; Nguyen et al., 2013; Palaniappan and Holley, 2010; Saleem et al., 2010). Phenolic compounds are a highly diverse class of compounds widely distributed throughout the plant kingdom and include terpenoids, organosulfur compounds, isoquinoline alkaloids, flavonoids, lactone, and naphthoquinone (Bravo, 1998). Phenolic compounds have been reported to act as *C. albicans* anti-fungal agents through various mechanisms of action. For example, Vikrant *et al.* (2015) has reported that gallic acid, capric acid, carvacrol, and terpene-4-ol were able to disrupt membrane integrity, prevent the normal budding process, and potentially inhibit the growth of *C. albicans*.

Yet, studies remain limited on the inhibitory/remediation effects of phenols on *C. albicans* adhesion to and biofilm formation on synthetic materials. Additionally, the previously cited studies did not focus on the synergistic potential of phenolic compounds in combination to prevent or remediate these events. Instead, these studies have focused on isolated phenols despite emerging evidence showing that complex phenolic-rich extracts impart greater benefits than the sum of the individual components (synergism) (Lewis and Kontoyiannis, 2001, Junio et al., 2011).

As such, a significant gap of knowledge exists on the ability of phenolic compounds to act as synergists to remediate *C. albicans* cell adhesion and biofilm formation. Therefore, the main objective of this work is to investigate the ability of the phenols (gallic acid, ferulic acid, sinapic acid, quercetin, epicatechin, cumeric, catechin) and the other natural compounds (chlorophyll, and farnesol) to act synergistically inhibiting adhesion and biofilm formation. These phenols were selected because they

were present in multiple food systems and/or are ubiquitous throughout the plant kingdom thereby sustaining their availability pending their degree of efficacy. Moreover, these compounds were used for this research based on another study that showed these phenols were the most effective in remediating cellular adhesion or biofilm formation (Chapter 1). They were thus combined with other phenols with the expectation that they would provide highly potent synergists or additives to reduce adhesion and/or biofilm formation. The significance of this research is that antifungal resistance by *C. albicans* species will diminish due to the multi-targeted effect provided by several natural compounds acting synergistically or additively. Additionally, potential synergists/additives that specifically protect against cellular adhesion or biofilm formation will be identified.

2.3 Materials and Methods

2.3.1 *Preparation of* C. albicans *yeast stock culture*

Two *C. albicans* strains (SC5314 and A72) were obtained from Kenneth Nickerson, University of Nebraska-Lincoln. A stock culture was grown to the stationary phase in 500 ml of 5 g of yeast extract, 2.5 g of peptone, and 10 g of dextrose (YPD). The medium (25 ml) was then added to 125 ml Erlenmeyer flasks and a ½ loopful of *C. albicans* (A72 and SC5314), which was maintained on YPD agar for not more than 1 month, was incubated in a shaking water bath at 30°C for 22 h (or until cells achieved stationary phase, i.e., no budding observed microscopically). The cells were then washed three times with potassium phosphate buffer (pH 6.5) followed by centrifugation until a clear supernatant was obtained. The ensuing pellet was then re-suspended in 7.5 ml of phosphate buffered saline and maintained at 8-10 ^{o}C until use.

2.3.2 *Treatment preparation.*

Serum media (Atlanta Biological) was thawed at room temperature for 5 min, and then 5 ml was dissolved in 45 ml of potassium phosphate buffer (pH 6.5) prior to use. Stock solutions of gallic acid (G), ferulic acid (F), sinapic acid (S), epicatechin (E), farnesol (FA), and þ-coumaric acid (C) were freshly prepared at 200 mM in 100% of ethanol until the solids had completely dissolved. Alternatively, quercetin, catechin (CAT), and chlorophyll (CH) were diluted in 50:50 ethanol: water solution. The natural compounds were prepared by diluting a freshly prepared stock solution such that the final delivery system was consistently 2% ethanol while the phenols and non-phenols ranged from 4.0-0.06 mM in eight 2-fold increments. Ethanol (2%) was selected as the delivery system as preliminary experiments completed with this solvent were able to dissolve the phenols and non-phenolic compounds at the various concentrations utilized that did not prevent biofilm formation or cellular adhesion. Methanol and dimethyl sulfoxide were also screened as potential delivery candidates. However, methanol at most of the concentrations screened prevented adhesion and biofilm formation assays, whereas dimethyl sulfide quickly oxidized the phenols (data not shown).

2.3.3 *Biofilm/adhesion remediation treatments*

C. albicans strains (A72 and SC5314) were added at (5×10^6 cell per ml) to Immunol 2HB 96 well plates with each well containing 140 μl serum. The plates were covered with aluminum and incubated at 37 ^{o}C for 24 h. The two different compounds that served as the treatments were added at final concentrations of 0.03 to 0.5 mM to determine the effects of the natural compounds on established adhered cells and biofilm formation, After the 24 h incubation, 60 μl of a phenolic combination at various concentrations were added to each well. Again, another set of wells that contained and did not contain the cells were delivered but only 60 μl of 2% ethanol was added to serve

as the negative and positive control, respectively. The plates were covered again with aluminum and incubated at 37 ^{o}C for 6 h.

2.3.4 *Adhesion assay*

The adhesion assay was performed according to Pierce *et al.* (2008). Briefly, the medium from each well was carefully removed and 50 μl of crystal violet was added to the wells. The plates were covered again and incubated at room temperature for 45 min. After incubation, each plate was rinsed gently with 400 μl of ice cold water 5-10 times. The plates were inverted onto a paper towel to remove any non-adherent cells and water. The plates were then incubated for another 30 min at room temperature after adding 200 μl of 75% methanol to each well. The absorbance was determined at 590 nm using a microtiter plate reader (Fazly et al., 2013).

2.3.5 *Biofilm assay*

Biofilm formation was determined by using the (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) XTT kit according to the manufacturer's direction (Sigma-Aldrich). (The XTT kit consists of XTT labeling reagent and electron coupling reagent. This assay relies on yellow tetrazoluim XTT salt cleavage to an orange formazon dye by the active metabolic cells, which indicates the viable cells and is based on procedures cited by Pierce *et al.,* 2008 & Sudjana, *et al.,* 2012). The XTT labeling reagent and electron coupling reagent were thawed in a water bath set at 37 ^{o}C, and then 0.1 ml of electron coupling reagent was added to 5 ml of XTT labeling reagent to be activated prior to use. One hundred μl of the XTT mixture was added to each well and incubated for 2 h. The absorbance was determined at 450 nm using a microtiter plate reader.

2.3.6 *Percent Remediation calculations*

Remediation of biofilm formation or *C. albicans* adhesion of already established films were defined as % remediation for both cases, which was determined by the following equation (Romano *et al.,* 2009):

$$\% \text{ Remediation} = (A_{control} - A_{sample})/ (A_{control})*100$$

Where: $A_{control}$ is the absorbance of cells without a treatment

A_{sample} is the cells with the treatment.

2.3.7 *Synergistic Interaction*

Synergism was determined by calculating the fractional inhibitory concentration. FIC_A is the activity of phenolic compound A in the presence of B/activity of phenolic compound B alone. FIC_B is the activity of phenolic compound B in the present of A/activity of phenolic compound A alone. The equation $FIC_{indix} = FIC_A+FIC_B$ was used to determine if the compounds acted as synergists, additives or antagonists. The synergist values were considered in the range of FIC_{indix}, whereas compounds with values <1, 0, or > 1 acted as synergists, additives or antagonists, respectively (Romano et al., 2009).

2.3.8 *Statistical analysis*

The biofilm/adhesion experiments were completed on 4 replicates for each compound/concentration used and the time point monitored. After data outliers were removed by the Grubs test at a 5% confidence interval, the final results were reported as the mean +/- standard deviation. One-way ANOVA was used to determine whether the various treatments differed in terms of % remediation at 95% confidence interval ($p < 0.05$) using Tukey's honest significant difference. The statistical analyses were obtained with Stats Graphic Centurion XVI.1.

2.4 Results and discussion

2.4.1 *Remediating* C. albicans *(A72 and SC5314) cellular adhesion in response to natural compounds*:

C. albicans adhesion to a surface is the first in sequential steps to colonization and biofilm formation (Mayer et al., 2013) and thus must be considered the first line of attack when developing anti-candida drugs. Yet, reports remain non-existent to our knowledge on discovering drugs that specifically remove *Candida* cells bound to a given surface, whether it be biological or synthetic, with the following exception. After screening 30,000 small molecules for their protective properties against several *Candida* virulence factors, Fazly et al. (2013) showed the number of bound cells established on a polystyrene surface for 4 h was reduced by 35-40% after 8 h of exposure to 50 μM filastatin, but the results were not as effective as when the molecule was initially co-incubated with unbound *Candida* cells (% Inhibition = ~90%).

The adhesive method for screening compounds used herein was administered to intact *C. albicans* cells. As such, the compounds were able to directly interact with the cells, and thus act on various potential targets that include secreted cellular adhesives, the cell membrane, (either indirectly by transporting the compound into the cells and affecting internal pathways that cause adhesion, or directly by affecting the membrane wall), or as a signal transduction agent. As secondary molecules in plants, phenols are expected to be potent ant-adhesive *Candida* agents due to their ability to complex proteins, disrupt microbial membranes, act as cell signaling agents, and provide anti-oxidative protection properties (directly and indirectly) (Papadopulou et al., 2005; Kanwala et al. 2010; Candiracci et al 2012; Brovo and Lazo; 1997; Hirasawa and Takada, 2004).

Yet, in our own lab (Chapter 1), 7 different phenols, which are ubiquitous throughout nature (gallic acid (G), ferulic acid (F), sinapic acid (S), p-coumaric acid (C) epicatechin gallate, (E) quercetin (Q) and catechin (CAT)), provided minimal or no benefits in reducing the quantity of *C. albicans* cells bound to a synthetic surface regardless of concentration, with the exception of CAT. In fact, CAT was the most effective anti-fungal adhesive when applied to both of the two stains of *C. albicans*, A72 and SC5314, used in this study compared even to *C. albicans*'s own quorum sensing molecule farnesol (FA), and chlorophyll (CH). Chlorophyll is another molecule consumed throughout the world due to the intake of green plants that has also been shown to exert antimicrobial activities against *C. albicans* but has been studied for its cell growth prevention properties (Maedawa et al. 2007). Yet, % remediation effects in response to CH and FA were highly variable (ranging from 0% to 40%) making an efficacious treatment for reducing bound *C. albicans* cells on a synthetic surface highly improbable. Although catechin showed promise as an active anti-cell adhesive for *C. albicans*, it too would require further development due to it is wide range in providing protection (% remediation =10-50% (Chapter 1)).

Limited studies have shown that complex phenolic-rich extracts impart greater health properties (synergism) than the sum of the individual components, as reviewed by Wagner and Ulrich-Merzenich (2009) and Mukherjee et al. (2011), especially when applied to remediation of *Candida* spp. cellular adhesion. As a result, critical gaps in research remain on the synergistic effects of phenolics on *Candida*-based infections as well as other health related benefits. However, one such study was conducted by Ackland et al. (2005), who reported an antiproliferative effect caused by flavanols, i.e., quercetin and kaempferol, that acted synergistically to reduce the proliferation of human gut cancer cell lines (HUTI-80 and Caco-2) and a breast cancer cell line (PmC42). The

combinations of the two flavanols were more effective compared to the additive effect of each compound.

In the context of our work, a synergistic effect between anti-*Candida* drugs (i.e., amphotericin and fluconazole) and phenolic compounds (caffeic acid, cinnamic and benzoic acids, thymol, and 2,3- and 2,5-dihydroxybenzaldehydes) has been reported (Faria, et al. (2011), Dai et al. 1987) to result in decreased cell growth. Another study targeted the suppression of the yeast-to-hyphae switch by using a flavonoid-rich honey extract (Canonicom 2014), which resulted in the inhibition of *Candida albicans* morphogenesis by modulating DNA and mitochondrial function. The authors proposed that the agents worked synergistically, considering that the standard phenolics identified in the extracts showed lower yeast-to-hyphae inhibition activities when tested as isolated compounds. However, these studies did not follow up on the synergistic hypothesis by confirming with isobolograms and/or calculating the FIC or focusing on the virulence factors cellular adhesion or biofilm formation. For this study, the same 7 phenols (G, F, S, E, C, Q, CAT) and 2 non-phenols (CH and FA) used in isolation (Chapter 1) were screened for their potential as synergists to prevent *C. albicans* (SC5314 and A72) cellular adhesion and biofilm formation. This section focuses on the former virulence factor.

2.4.1a *Remediating* C. albicans *(A72) bound cells exposed to 2 natural compounds:* For these studies, the compounds previously cited were prepared in different combinations by preparing a given concentration using a 1:1 ratio for each natural product. These combinations ranged from 0.03 to 4.00 mM and were then tested for their ability to remediate *C. albicans* (A72) cells adhered to a synthetic surface when incubated with the phenolic combinations for 6 h. This time duration was selected so as to remain consistent with the studies performed with the isolated components (Chapter

1). Only those combinations that, for the most part, showed cellular reduction greater than 50% at any concentration used are reported herein (Figure 2.1), albeit it must be noted that other phenolic sets were able to remediate cellular adhesion at percentages less than 50%.

As the combinations did not produce statistically different remediation results at the cited concentrations, each were equally capable of detaching the bound cells despite compound structural differences and the compound combination, with the exception of the 0.5 mM treatments (Figure 2.1). Only two of the seven phenols were a subset of 1 combination, while the other phenols played a role in two of the treatment sets. For example, CAT positively impacted remediation when combined with Q and C, while E altered adhesion when combined with either C or F These data may indicate that a given compound plays a different role within a combination compared to its counterpart, but the same when combined with another compound. However, more studies are needed to test this hypothesis. Interestingly, the non-phenolic compounds did not influence adhesion when combined with the phenolic compounds. As both FA and CH

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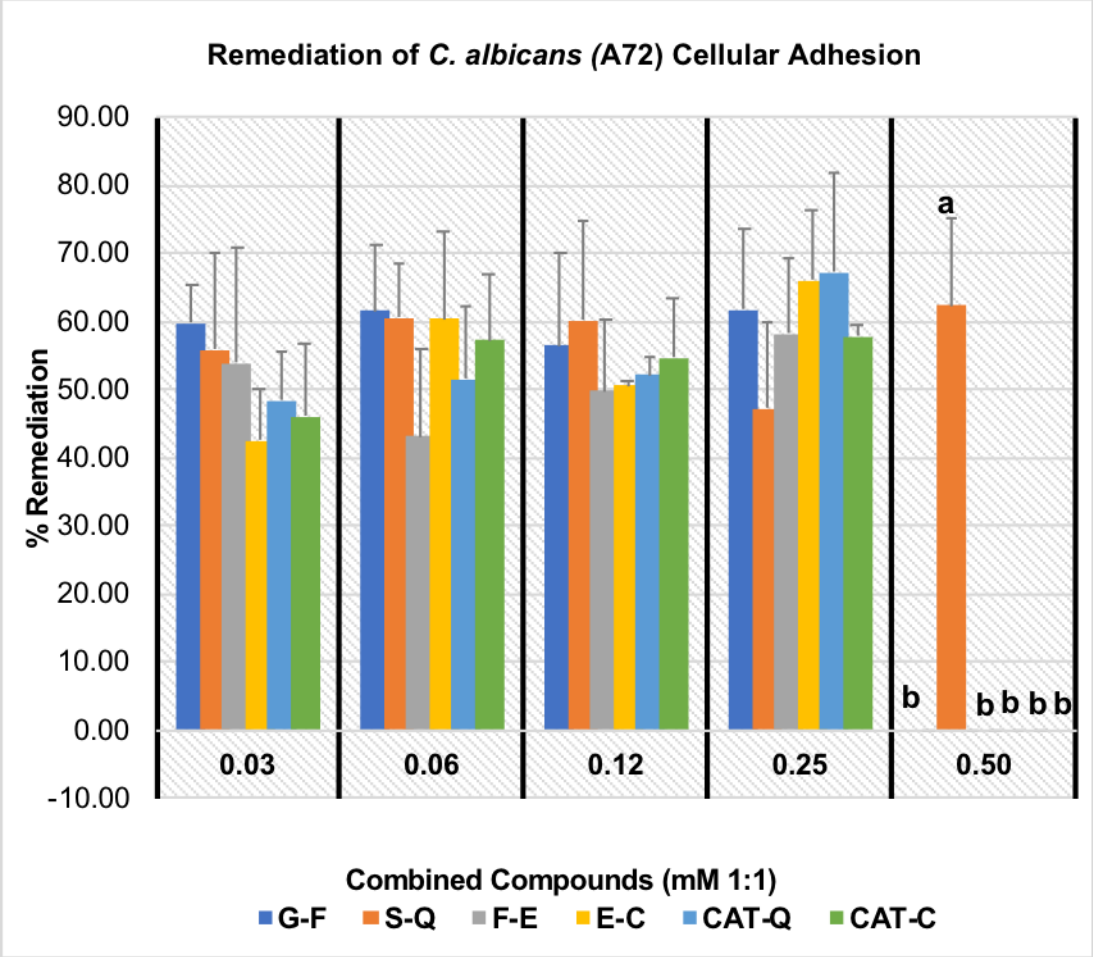

Figure 2.1: Percent remediation of *C. albicans* (A72) treated with different sets of dual compounds. Each set of bars represents remediation results for cited compounds at the concentration specified for each set. G-F: Gallic Acid -Ferulic Acid, S-Q; Sinapic Acid-Quercetin, E-C; Epicatechin- Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-C: Catechin-Coumaric Acid. (Different letters shown for a given compound combination for each treatment level indicate statistical difference ($p > 0.05$) in cellular adhesion). Bars represent the mean % remediation +/- standard deviation (n=4).

reduced adhesion when used as isolated compounds, these results were not expected and cannot be explained at this time.

For concentrations 0.03 – 0.25 mM, % remediation ranged from a high of ~80% (CAT-Q at 0.25) to a low of 30% (E-C at 0.03 mM) accounting for variability (Figure 2.1). At a concentration of 0.50 mM, only the S-Q treatment resulted in a reduction of bound cells, while the other combinations showed no inhibition. Percent remediation decreased for the majority of depicted treatment sets as well as the combinations that are not shown when the increasing concentration were used with several treatments even producing antagonistic effects (data not shown). These results could be due to the fact that the additional carbon contributed to cell growth when serum is used as the transition media, as was determined by other unrelated experiments completed in our lab. Alternatively, researchers have reported similar but atypical responses of cells exposed to low doses of pure natural agents as applied to number of responses (Kampa et al. 2004*;* Fimongnari, 2004; Yang et al. 2001*,* Daron and Casagrande, 2001, Zbasnik et al. 2010). Generally, the lower dosage produced a positive response, while increasing dosages either worsen the response for select dosages or improved or worsened the response depending on the condition being tested. For example, Zbasnik, et al. (2010) showed that low doses (< 100 μg/mL) of grain sorghum dry distiller's grain GS-DDG, a co-product of ethanol production with high levels of lipids, was able to lower the proliferation of Caco 2 cells. Yet, at higher levels of GS-DDG (200-400 μg/mL) proliferation actually increased, but then growth decreased and then plateaued with GS-DGG concentrations of 500-1000 μg/ml. Darbon and Casagrande (2001) attributed this low dose phenomenon to the ability of biological systems to compensate to lower levels of toxicity of a bioactive agent

but are unable to overcome this effect at higher treatment doses. More studies are critically needed to understand this low-dose response as these results may have important clinical implications.

As a result, the dual treatments that produced the most potent results (Figure 2.1) were further analyzed to see whether significant differences occurred across treatment levels. As shown by Figure 2.1 the only significant difference in terms of phenolic treatment levels occurred at 0.5 mM for all the combinations with the exception of S-Q. Again, this could be due to the phenols acting on different targets for this combination compared to the other dual treatments. That being said, the lower levels (0.03-0.25 mM) were not statistically different across these dosages for any of the combinations (Figure 2.2), which may be caused by saturation of the active sites at concentration lower than 0.03 mM.

However, as no significant difference occurred across combinations (Figure 2.1), the range of reduction of bound cells was 30-82%, which is high variability for treating cellular adhesion indicating that higher sample sizes may be needed to increase the signal to noise ratio so as to determine the optimal treatment option. Also, use of phenols (whether singly or in combination) appears to yield inconsistent results in reducing cellular adhesion of *C. albicans* A72 from a bound surface, as using only 1 phenol was also highly inconsistent for mitigating adhesion (Chapter 1). Yet, no combinations used at the ranges 0.03-0.25 mM resulted in negative remediation values, potentially making this approach the first line of attack in preventing biofilm formation.

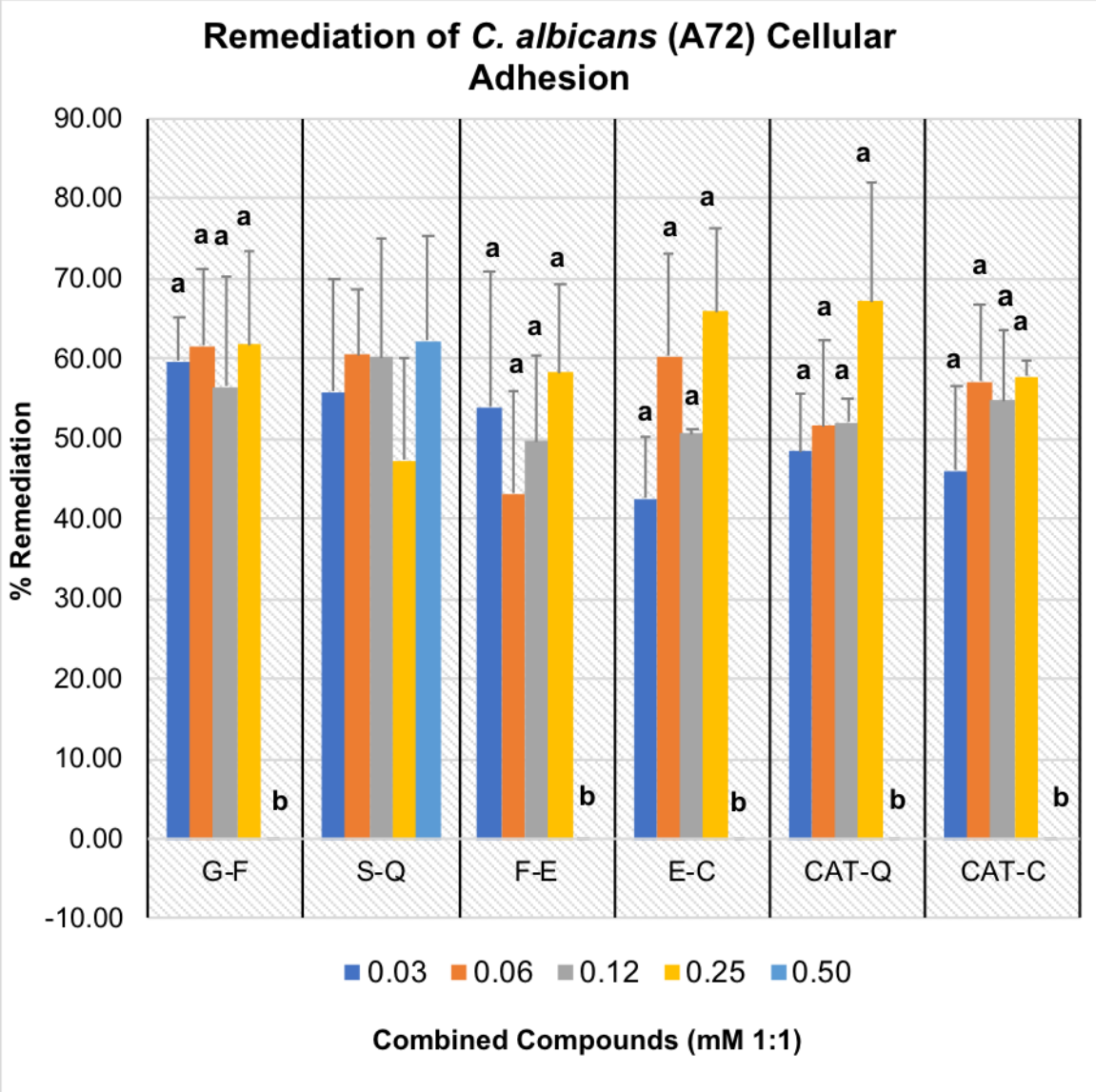

Figure 2.2: Percent remediation of C. albicans (A72) treated with different sets of dual compounds. Each set of bars represents remediation results for cited compound ranging at specified concentration provided for each set. G-F: Gallic Acid -Ferulic Acid, S-Q; Sinapic Acid-Quercetin, E-C; Epicatechin- Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-C: Catechin-Coumaric Acid. (Different letters shown for a given compound combination for each treatment level indicate statistical difference ($p > 0.05$) in cellular adhesion). Bars represent the mean % remediation +/- standard deviation (n=4).

Table 2.1: Results of FIC index show the results of the two phenolic treatments to remediate *C. albicans* (A2) cellular adhesion where FIC < 0.5 (synergistic), 0.5> FIC < 1 (partial synergy), and FIC >1.0 (antagonistic).

	G-F	S-Q	F-E	CAT-Q	CAT-C
FIC	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5

G-F: Gallic Acid -Ferulic Acid, S-Q; Sinapic Acid-Quercetin, E-C; Epicatechin- Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-C: Catechin-Coumaric Ac

This study further showed that that multiple combinations of dual phenols at low concentrations may be used sequentially to reduce established bound *C. albicans* for reasons that are not understood at this time. However, Salamatullah (2018) and Aldawsari (2018) both showed that lower concentrations of phenols combined with the amylase and glucosidase inhibitor of acrobose acted synergistically to inhibit the breakdown of starch and disaccharides, thus protecting against risk factors for diabetics, but the responses were not linear in terms of dosages. Another study completed in our laboratory by Columbanus (2018) further demonstrated the synergistic effect of phenols in modulating the macrophage phenotype with the lower doses again being the most effective but not in a dose dependent manner. Of course, more studies are needed to test this hypothesis. Still, these compounds acted synergistically as expected given that they were unable to inhibit adhesion as isolated compounds, even at high concentrations of 4.0 mM (Chapter 1). Calculation of the FIC confirmed these results as shown in Table 2.2.

As stated previously, most of the compounds that reduced cellular adhesion in combination were unable to do so as isolated compounds (F, G, Q, C, and S). Moreover, C and CAT that did impact adherence as single compounds (Chapter 1) showed relatively large variability making it difficult to determine their efficacy. Therefore, it is clear that a synergistic interaction occurred to remediate this virulence factor for most of the combined compounds. Still, as isoblograms would be difficult if not impossible to construct considering that the single concentrations were unable minimally to reduce adhesion at any

concentration (Chapter 1), FIC values were calculated to provide additional evidence that the compounds interacted as synergists. As shown in Table 2.1 all the FIC values were below 0.5 providing additional evidence that the each of the two compounds that were able to remediate adhesion interacted as synergists.

2.4.1b *Remediating* C. albicans *(SC5314) bound cells when concurrently exposed to 2 natural compounds:* Percent remediation of cellular adhesion in response to treatment with two phenols was also evaluated for *C. albicans* strain SC5314, to determine if the responses to the compounds differed with strain. Therefore, screening was again completed using different phenolic combinations and concentrations to ascertain the most potent phenol dual treatments and concentrations when using the 7 phenols and 2 non-phenolic compounds, as was done for *C. albicans* (A72). As stated previously (Chapter 1) only three of the 9 compounds tested in insolation were able to detach bound *C. albicans* (SC5314), which included the non-phenols, FA and CH, and the flavonoid, CAT, at concentrations ranging from 0.06-0.5 mM that was accompanied with high variability.

In this study, 6 treatment groups were able to reduce cellular adhesion at levels greater than 50% when exposed at the lower concentrations tested (0.03 to 0.25 mM). Again, higher levels were unable to remediate adhesion, similar to that reported for *C. albicans* (A2) (data not shown). At a concentration of 0.5 mM, only S-Q exerted a % remediation of 52 +/- 18 %, while the other compounds were not effective at this concentration (Figure 2.3). Moreover, the same phenolic combinations that also positively impacted

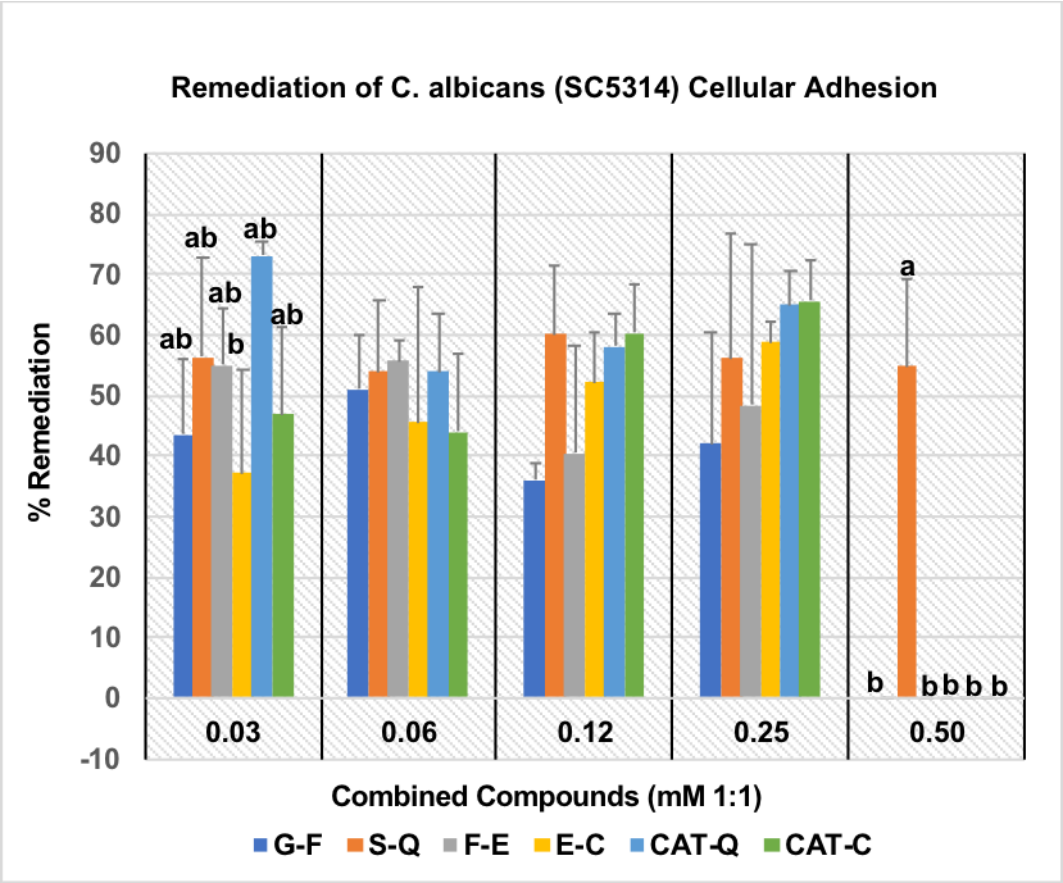

Figure 2.3: Percent remediation of C. albicans (SC5314) treated with different sets of dual compounds. Each set of bars represents remediation results for cited compound ranging at specified concentration provided for each set. G-F: Gallic Acid -Ferulic Acid, S-Q; Sinapic Acid-Quercetin, E-C; Epicatechin- Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-C: Catechin-Coumaric Acid. (Different letters shown for a given compound combination for each treatment level indicate statistical difference ($p > 0.05$) in cellular adhesion). Bars represent the mean % remediation +/- standard deviation (n=4).

C. albicans (A2) provided therapeutic responses (Figure 2.1 and 2.2). This data suggest that the phenols are acting on similar targets for both strains. The effect of the phenols was not statistically different across compounds for 0.03-0.25 mM, with the exception of S-Q as discussed above. In addition, E-C showed a statistical difference at 0.03 mM in comparison to the other treatments with a low of ~20%, even though the high was ~50%. The other phenolic combinations were statistically comparable resulting in % remediation that ranged from 35-75% (0.03 mM), accounting for variability. At 0.06, 0.12 and 0.25 mM, the % remediation respectively ranged from 30-70%, 35-70% and 40-75%. These values are again similar to that obtained when the phenols were used to treat *C. albicans* (A72).

As expected from the previous data, there was no statistical difference for a given phenol set across concentrations of 0.03-0.25 mM except for CAT-Q (Figure 2.4). In this case, the 0.03 mM concentration provided the optimal effect (70 +/- 2 %) but was not significantly different from the 0.12 mM (58 +/- 14 %) and 0.25 mM (65 +/- 5 %) treatments. Alternatively, the 0.06 mM concentration (52 +/- 10 %) produced statistically different % remediation results than the 0.03 mM treatment for synergistic effect of phenols in modulating the macrophage phenotype with the lower doses again being the most effective but not in a dose dependent manner. Still, these compounds acted synergistically as expected given that they were unable to inhibit adhesion as isolated compounds, even at high concentrations of 4.0 mM (Chapter 1). Calculation of the FIC confirmed these results as shown in Table 2.2.

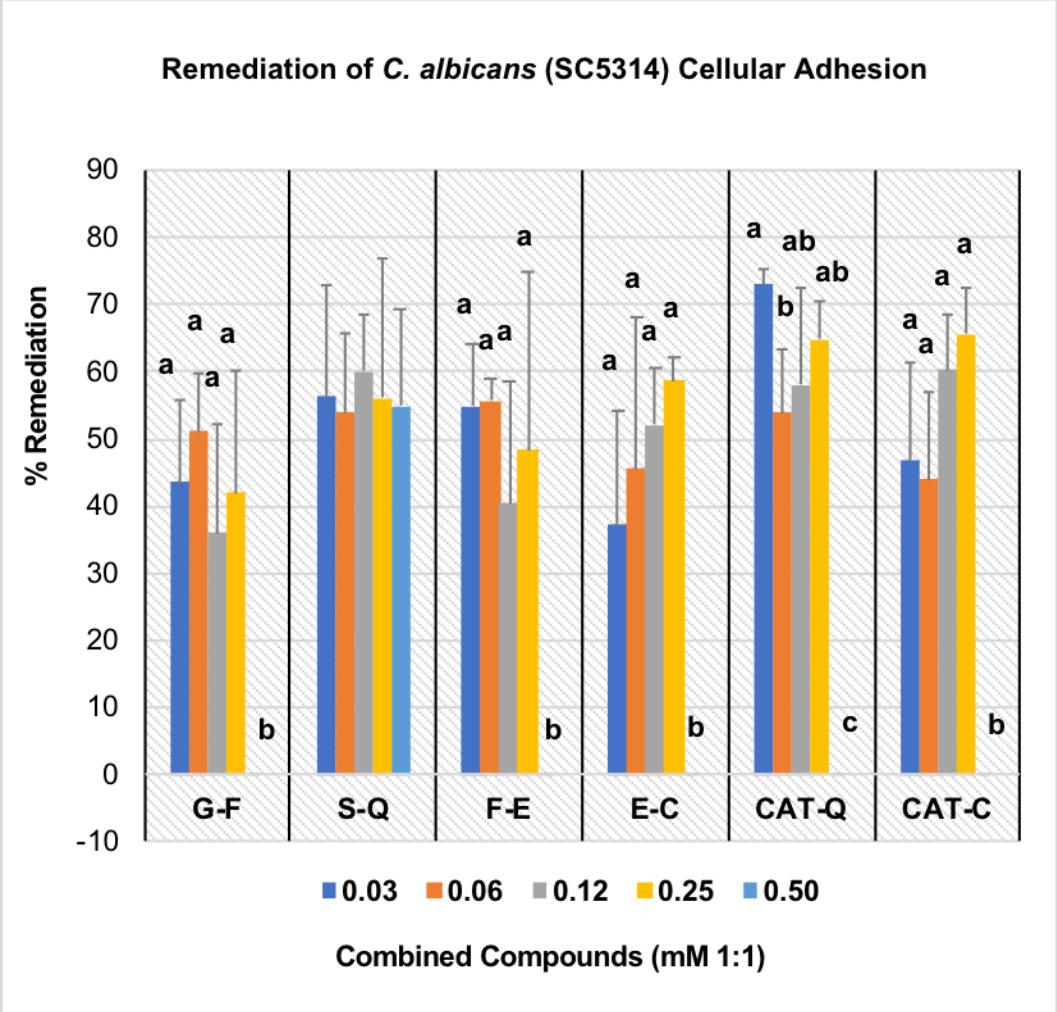

Figure 2.4: Percent remediation of C. albicans (SC5314) treated with differ sets of dual compounds. Each set of bars represents % remediation results for cited treatment levels ranging specified at for each compound dual. G-F: Gallic Acid -Ferulic Acid, S-Q; Sinapic Acid-Quercetin, E-C; Epicatechin- Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-C: Catechin-Coumaric Acid. (Different letters shown for \ a given concentration indicates statistical difference (p >0.05) in cellular adhesion). Bars show the mean % remediation +/- standard deviation (n=4).

Table 2.2: Results of FIC index show the results of the two phenolic treatments to remediate *C. albicans* (SC5314) where FIC < 0.5 (synergistic), 0.5> FIC < 1 (partial synergy), and FIC >1.0 (antagonistic).

	G-F	S-Q	F-E	CAT-Q	CAT-C
FIC	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5

G-F: Gallic Acid -Ferulic Acid, S-Q; Sinapic Acid-Quercetin, E-C; Epicatechin-Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-C: Catechin-Coumaric Acid

To our knowledge, only our group has studied the effects of phenols across multiple concentrations, thus more work is needed to understand the mechanism behind this phenomenon. Nonetheless, various phenolic combinations were able to reduce *C. albicans* (SC5314) adhesion (data not shown). Although variability ranged from 30%-80%, this information is significant for developing products that combat this virulence factor as a means to prevent the formation of biofilms (Figure 2.3 and 2.4).

2.4.2 *Remediating* C. albicans *(A72 and SC5314) biofilm formation when concurrently exposed to 2 natural compounds:*

C. albicans infections have become a serious clinical problem due to the ability of *C. albicans* to produce biofilms on synthetic and biological surfaces. Biofilms are not only more resistant to antifungal agents compared to the yeast form, but the antibiotic concentrations required to remediate biofilm formation are relatively high (Cerca et al,. 2005). In fact, *Candida* biofilm formation is a leading case of denture stomatitis development, which affects 65% of edentulous individuals (Brooun et al., 2000). C*. albicans* biofilms are extremely resistant to available antimicrobial agents, such as amphotericin B, chlorhexidine, nystatin, and fluconazole. Several mechanisms of action that may lead to the biofilm antimicrobial resistance have been investigated, including changes of cytoplasmic membrane, growth rate, efflux pump of cell wall, and dispersion of drugs (Ultee et al., 2002; Walsh et al., 2003; Puupponen-Pimiä et al., 2005; Campos et al., 2009). Nett et al., (2007) demonstrated that the changes on cell wall or matrix β 1,3-glucans were associated with the development of *C. albicans* biofilm antifungal resistance because the β 1,3-glucans of biofilm cells were 2-fold higher in comparison to the planktonic cells.

Despite this resistance, several studies demonstrated the synergistic/additive increased potency of combining phenols combined with currently *C. albicans* resistant antibiotics (Chandra et al., 2001; Chandra et al., 2001; Kuhn et al, 2002; Kuhn et al, 2002). For example, thymol, a monoterpenoid phenol derivative, combined with fluconazole antibiotic was able to reduce *C. albicans* (MTCC 227) biofilm formation after 24 h of exposure to the treatments, with the minimum inhibition concentration (MIC) for reducing 90% of the film increasing from 0.2 mg /ml to 0.5 mg /ml (Pemmaraju et al., 2013). Yet, reports remain limited on the efficacy of phenolic compounds acting as synergists on remediating *C. albicans* biofilm formation. In another study completed by Answari et al. (2019) the authors show that phenolic rich honey was able to disrupt the structure of established *C. albicans* biofilms (MIC = 40% w/v) by 70-75 % after an incubation period of 24 hr. Yet these studies did not identify the phenols responsible for this effect or if the compounds acted synergistically or additively.

However, in a study completed in our laboratory on the effects of isolated phenols on remediating biofilm formation, 4 of the 7 different phenols (G, F, S, and E used in the adhesion studies (Section 2.4.1) were able to remediate biofilm formation by *C. albicans* A72 and SC5314, ranging from 40-60% in comparison to flavonoids i.e., Q and E or non-phenolic compounds FA and CH, which exhibited low effect ~ 30 %. Therefore, to understand if these compounds were more potent in reducing biofilm formation when combined as couplets, the 7 phenolic compounds, G, F, S, E, C, Q, CAT, and 2 non-phenolic compounds, FA and CH, were investigated as potential synergistic agents for remediating *C. albicans* (SC5314 and A72) biofilm formation.

2.4.2a *Remediating* C. albicans *(A72) biofilm formation when concurrently exposed to 2 natural compounds:* Different concentrations of the previously cited compounds were prepared using a 1:1 ratio of each natural product with the different

combinations again ranging from 0.03 mM to 4.00 mM to screen for their ability to remediate matured biofilm formation of *C. albicans* A72 upon the exposure to the natural products for 6 h. Again, the 6 h incubation time was selected so as to be consistent with isolated compounds study (Chapter 1). However, this incubation time also provided the most efficacious results based on a time course study using 1, 3, 6 and 24 h time points. The combinations that reduced biofilm remediation ~ > 30% are reported for this study rather than ~ 50% used for the adhesion studies, mainly as most of the combinations exhibited values at approximately this value (Figure 2.5). Nonetheless, most of the tested combinations were able to decrease *C. albicans* biofilm formation at a given concentration, although less than 30%. For most the treatments, there was no statistical difference across compounds regardless of concentration. However, the highest reduction occurred for G-F at 0.03 mM, which was statistically different from the other compounds, exhibiting % remediation of 43% +/- 2 %, while the other combinations S-Q, F-E, E-C, CAT-Q, and CAT-CH ranged from 30-40%. For concentrations 0.06 and 0.12 mM, biofilm formation was reduced by 25-37%, and 25-50%, respectively. In terms of the 0.25 mM concentration, the highest inhibition occurred among the treatment for CAT-CH, which was 37 +/- 7%. This combination was statistically similar to all of the other compounds except for S-Q, which ranged in remediation from 23-33%. Interestingly, S-Q was again the only combination to reduce biofilm formation at 0.5 mM (30-40%), at which the other treatments showed no inhibition. Notably, all the combinations that reduced biofilm formation were the same as those cited for the adhesion studies for both strains of *C. albicans* (A72 and SC5314) across concentrations. This may be due to the fact that these compounds were able to detach the cells from the surface of the synthetic material thereby allowing lower biofilm formation and greater synergism in the similar compounds.

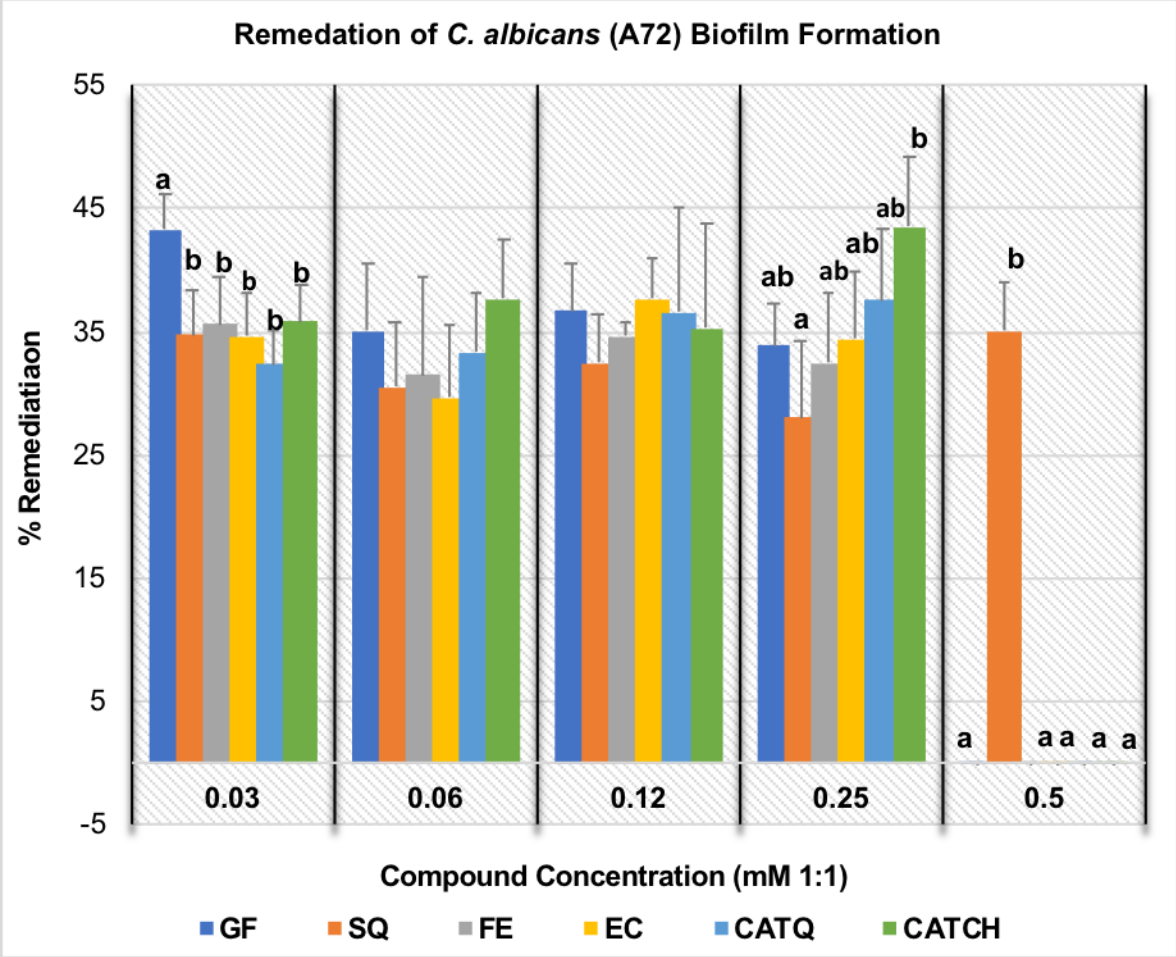

Figure 2.5: Percent remediation of *C. albicans* (A72) treated with different sets of dual compounds. Each set of bars represents remediation results for cited compound ranging at specified concentration provided for each set. G-F: Gallic Acid -Ferulic Acid, S-Q; Sinapic Acid-Quercetin, F-E; Ferulic Acid-Epicatechin E-C; Epicatechin- Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-CH: Catechin-Chlorophyll. (Different letters shown for a given compound combination for each treatment level indicate statistical difference ($p > 0.05$) in cellular adhesion). Bars represent the mean % remediation +/- standard deviation (n=4).

Again, the most potent duel treatments (Figure 2.5) were further analyzed to demonstrate if there were any significant differences across treatment levels (Figure 2.6). Most of the phenolic treatments were able to mitigate *C. albicans* A72 biofilm formation at levels that showed no statistically significant difference despite different concentrations. However, G-F acted significantly different at different concentration levels as stated previously. The effect decreased to < ~ 40 % upon increasing the concentration to 0.06, 0.12, or 0.25 mM. The phenol ferulic acid (F) played a positive role on remediating biofilm formation when it was combined with G, while F was less effective when combined with E, but E become more effective when combined with C. These results indicated that the compounds can perform distinct roles within a combination in comparison to its counterpart. It was obvious that at these concentrations 0.06, 0.12, 0.25 mM, the response was not statistically different among phenolic combinations, which might be attributed again to the saturation of the active sites. The combinations and levels cited could be used for treating *C. albicans* A72 biofilm formation as the percent ranged ~ 28- 43 %. As shown in Chapter 1, using single phenols or compounds was not as effective in remediating biofilm formation. Therefore, the significance of these results is that the phenolics in a combination can ideally target *C. albicans* A72 biofilm formation on synthetic surfaces. The FIC value (Table 2.3) supports that the phenols act at least partially synergistically in exerting this response. (Isobolobograms were again not constructed for this experiment as the lower doses were more potent than the higher doses (0.5 mM – 4.00 mM) (data not shown).)

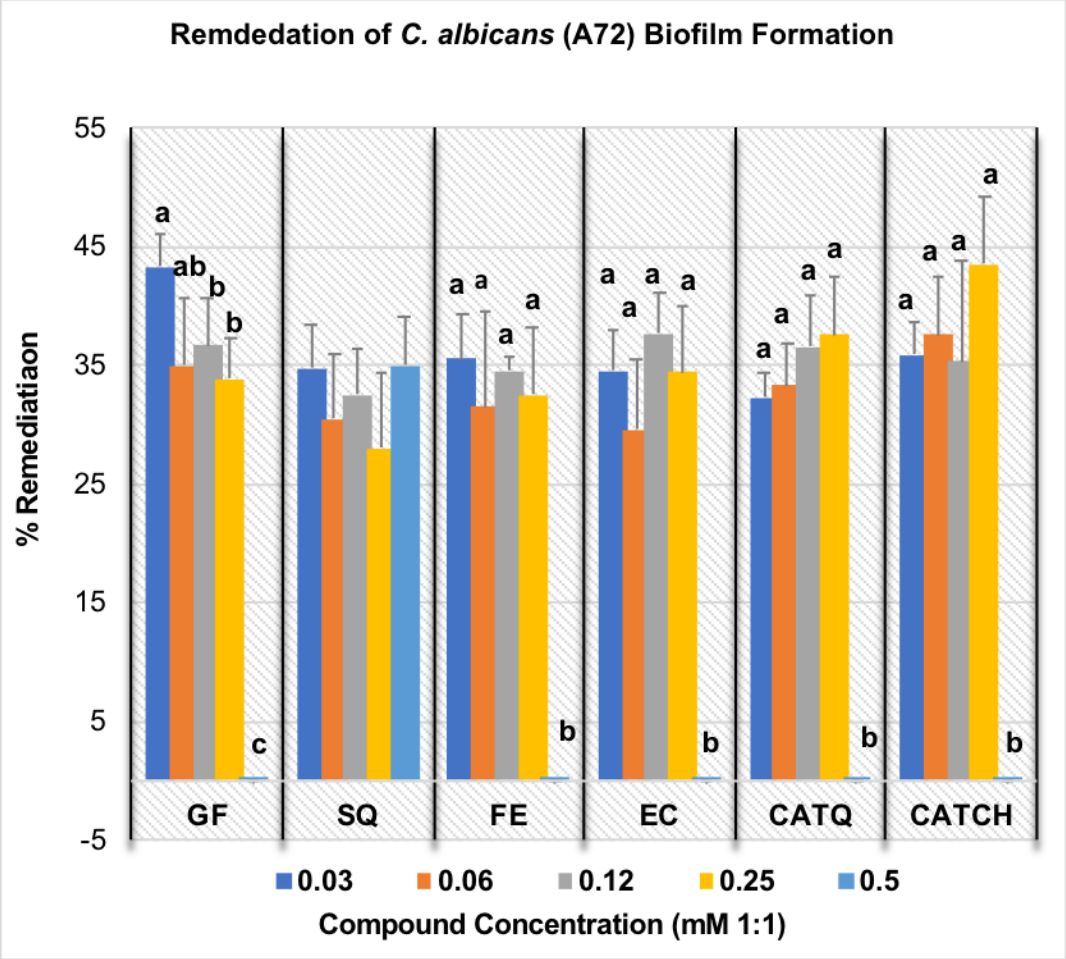

Figure 2.6: Percent remediation of *C. albicans* (A72) treated with different sets of dual compounds. Each set of bars represents remediation results for cited treatment levels ranging specified at for each compound combination. G-F: Gallic Acid-Ferulic Acid, S-Q; Sinapic Acid-Quercetin, F-E; Ferulic Acid-Epicatechin E-C; Epicatechin-Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-CH: Catechin-Chlorophyll. (Different letters shown for a given concentration indicates statistical difference ($p > 0.05$) in cellular adhesion). Bars show the mean % remediation +/- standard deviation (n=4).

Table 2.3: The FIC index shows the results of the two phenolic treatments to remediate *C. albicans* (A2) where FIC < 0.5 (synergistic), 0.5> FIC < 1 (partial synergy), and FIC >1.0 (antagonistic).

	G-F	S-Q	F-E	CAT-Q	CAT-CH
FIC	0.5> FIC <1	0.5>FIC < 1	0.5> FIC < 1	0.5> FIC < 1	0.5> FIC < 1

G-F: Gallic Acid-Ferulic Acid, S-Q; Sinapic Acid-Quercetin, E-C; Epicatechin-Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-CH: Catechin-Chlorophyll

Phenolic compounds as antimicrobial agents have been shown to alter the physiological surfaces, surface charge, and cytoplasmic membrane of cells. For example, the addition of G to F increased the cell hydrophilic properties of *P. aeruginosa* bacteria, while the same combination increased the hydrophobic properties for *L. monocytogenes.* This effect was attributed to the addition of G to the cells; whereas F had a lower impact on modulating the physiological surfaces (Borges et al., 2013). Moreover, 100 µg/ml of G-F was reported to damage 60% of cytoplasmic cell membranes of *P. aeruginosa* bacteria. Due to cell membrane damage, the intracellular K^+ was released (Borges et al.,2013). The antibacterial hydroxybenzoic acids, such as G, are more polar than hydroxycinnamic acids, i.e., F and C, which enable these molecules to be more easily transported through the cell membrane (Campos et al., 2003; Nohynek et al., 2006). This phenomenon may partially explain the high remediation in both the biofilm formation and adhesion (Section 2.3.1) of G-F, and the type of microorganisms and the chemical nature of cell membranes are also factors that might affect their ability to reduce biofilm formation (Borges et al., 2013). Moreover, another study performed with catechin demonstrated that the number of hydroxyl groups on the B-ring was associated with its antimicrobial activity (Kajiya et al., 2004). Therefore, the structural function of these compounds has impacted the antimicrobial activity and thereby their mode of interaction with the *C. albicans* biofilm formation.

2.4.2b *Remediating* C. albicans *(SC5314) biofilm formation when concurrently exposed to 2 natural compounds:* The effect of the treatment of two phenols in a combination on remediating *C. albicans* (SC5314) was also determined to see whether the responses would vary based on strain. The same combinations that were screened previously on remediating the A72 biofilm were applied with the exception of CAT-CH.

Instead, CAT-C was used because it showed more potency than CAT-CH. As shown (Chapter 1), these 7 phenols and 2 non-phenolic compounds have exposed a sort of remediation as isolates. Only G, F, S, and E were capable of treating biofilm formation when tested alone at low concentrations (0.03- 0.05 mM). However, the high variability in percent remediation, ranging from ~ 40 to > 70%, hinders their use as anti-biofilm treatments. Therefore, six treatment combinations were evaluated for their ability to treat biofilm formation of SC5314 at concentration levels ranging 0.03-0.5 mM. Again, 0.5 mM concentration did not result in any reduction for any treatments with the exception of S-Q, which exhibited a remediation of 20.06 +/- 6% (Figure 2.7). At the lowest concentration, 0.03 mM, CAT-C was significantly different from F-E and E-C, while the other combinations were not different from one another. Specifically, CAT-C exposure resulted in high percent of remediation (38.03 +/- 4%) while FE gave 19.54 +/- 2 % and EC gave 15.49 +/- 2 % at 0.03 mM. The phenolic treatment E-C trended differently at 0.25 mM than CAT-C and F-E, whereas the later combinations were statistically similar. Percent remediation was ~ 40 ± 7% CAT-C, while the remediation effect decreased to 34.8 ± 1% for F-E and 12.87 ± 4% for E-C at 0.25 mM. Again, E revealed a positive remediation effect when combined with F and C. However, at the higher concentration of 0.12 mM, E contributed a different impact in combination with either C and F. These results again indicated that the compounds could elicit different responses within a combination rather than its counterpart and it may be the same upon combining with another compound. It should be noted that C had low impact (~ 6% 0.06 mM) on remediating *C. albicans* SC5314 when tested alone (Chapter 1).

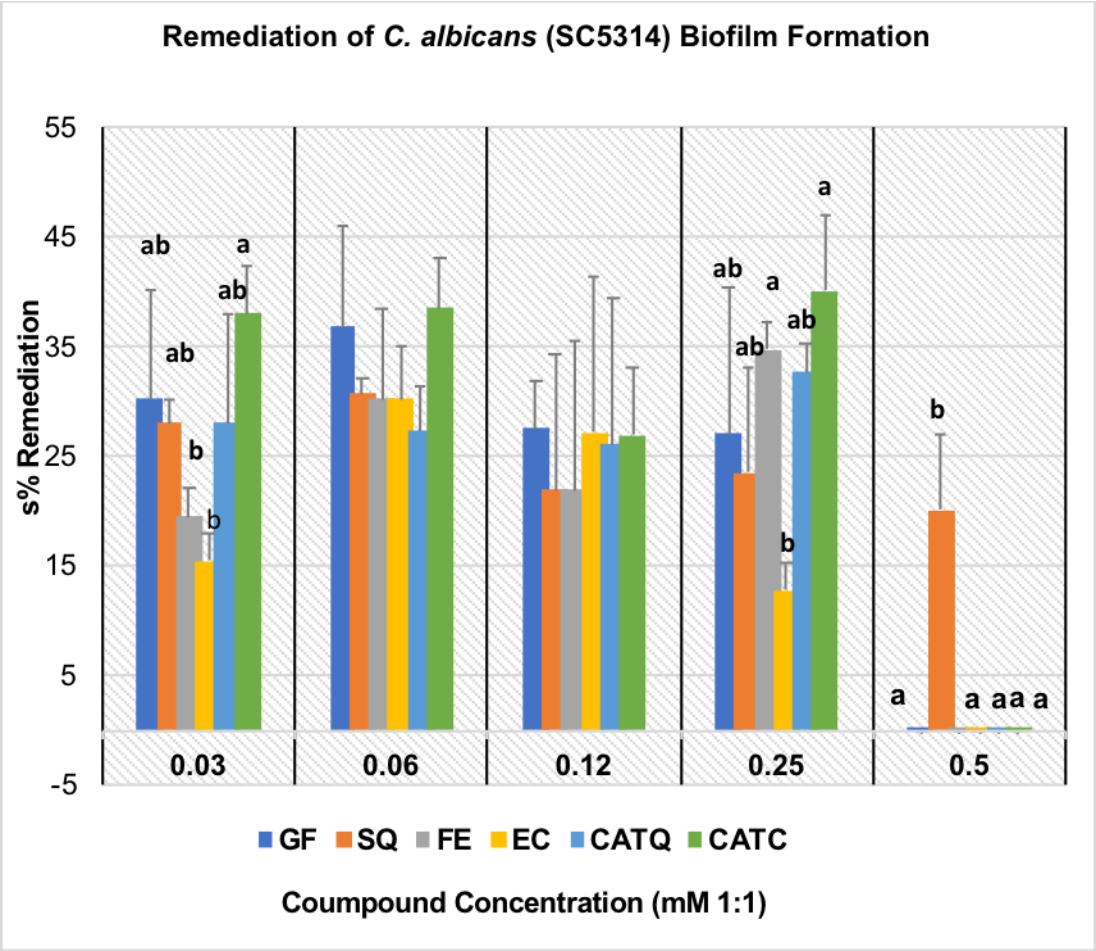

Figure 2.7: Percent remediation of C. albicans (SC5314) treated with different sets of dual compounds. Each set of bars represents remediation results for cited compound ranging at specified concentration provided for each set. G-F: Gallic Acid -Ferulic Acid, S-Q; Sinapic Acid-Quercetin, F-E; Ferulic Acid-Epicatechin, E-C; Epicatechin- Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-C: Catechin-Coumaric Acid. (Different letters shown for a given compound combination for each treatment level indicate statistical difference ($p > 0.05$) in cellular adhesion). Bars represent the mean % remediation +/- standard deviation (n=4).

On the other hand, most of the treatment combinations were not significantly different among concentrations (Figure 2.8), with exception of S-Q was significantly different at 0.5 mM, while the other combinations did not exhibit any remediation effect. Also, S-Q at 0.5 and 0.06 mM was trending differently since it exhibited percent inhibition ranging from a low 20.09 +/- 6% and a high of 30.78 +/- 2%, respectively.

The combination of E-C trended differently among concentrations, and the percent remediation ranged from a low of ~12 +/- 4 % at 0.25 mM to a high of ~ 30 +/- 4% at 0.06 mM. Interestingly, CAT-C was not statistically similar to E-C. it elicited a remediation effect that ranged from a low of 26.96 ± 6 % at 0.12 mM and a high of 38 +/- 4 % at 0.03 and 0.06 mM to ~ 40 +/- 7 % at 0.25 mM. Also, the FIC values were calculated to determine whether the combinations act as synergistic/antagonistic or as additives (Table 2.4). All the FIC values were 0.5> FIC <1 which provides more evidence that these treatment combinations act at least as partial synergists to remediate *C. albicans* SC5314 biofilm formation.

The data indicated that these combinations were more highly effective in treating cellular adhesion of *C. albicans* SC5314 and A72 in comparison to biofilm formation. Several factors might explain the high antifungal resistant of *C. albicans* biofilms because biofilm is a 3D structure that consists of yeast, hyphae, and pseudohyphae bounded by exopolymer matrix (mainly carbohydrates and proteins) (Kumamoto et al., 2005). These exopolymers provide a protection of the structure of biofilm by hindering antifungal invasion to immune system components (LaFleur et al., 2006). A subpopulation of highly tolerant cells (persister) produced by *C. albicans* biofilm is proposed to be responsible for the high resistance because these cells are able to grow in the presence of antifungal drugs and under different concentrations higher than MIC (LaFleur et al., 2006). However, more studies are needed to confirm this hypothesis

relative to the strains which were used. Moreover, it was noted that membrane sterol alteration could account for biofilm antifungal resistance and altering the membrane characteristics thereby reducing the drug permeability (Kumamoto et al., 2005). For example, amphotericin B targets ergosterol to alleviate mature biofilms, azoles hinder the biosynthesis of ergosterol, (Mukherjee et al., 2003; Garcia et al., 2004; Kumamoto et al., 2005) and echinocandins prevent cell wall β-glucan biosynthesis (Datry et al., 2006). In studies performed with þ-coumaric, caffeic, p-hydroxybenzoic, protocatechuic, vanillic, and syringic acids; and thymol, eugenol, and carvacrol, these compounds increased the permeability of the cell cytoplasmic membrane causing cell constituent (proteins, nucleic acids, and inorganic ions such as potassium or phosphate) leakage (Ultee et al., 2002; Walsh et al., 2003; Puupponen-Pimia et al., 2005; Campos et al., 2009).

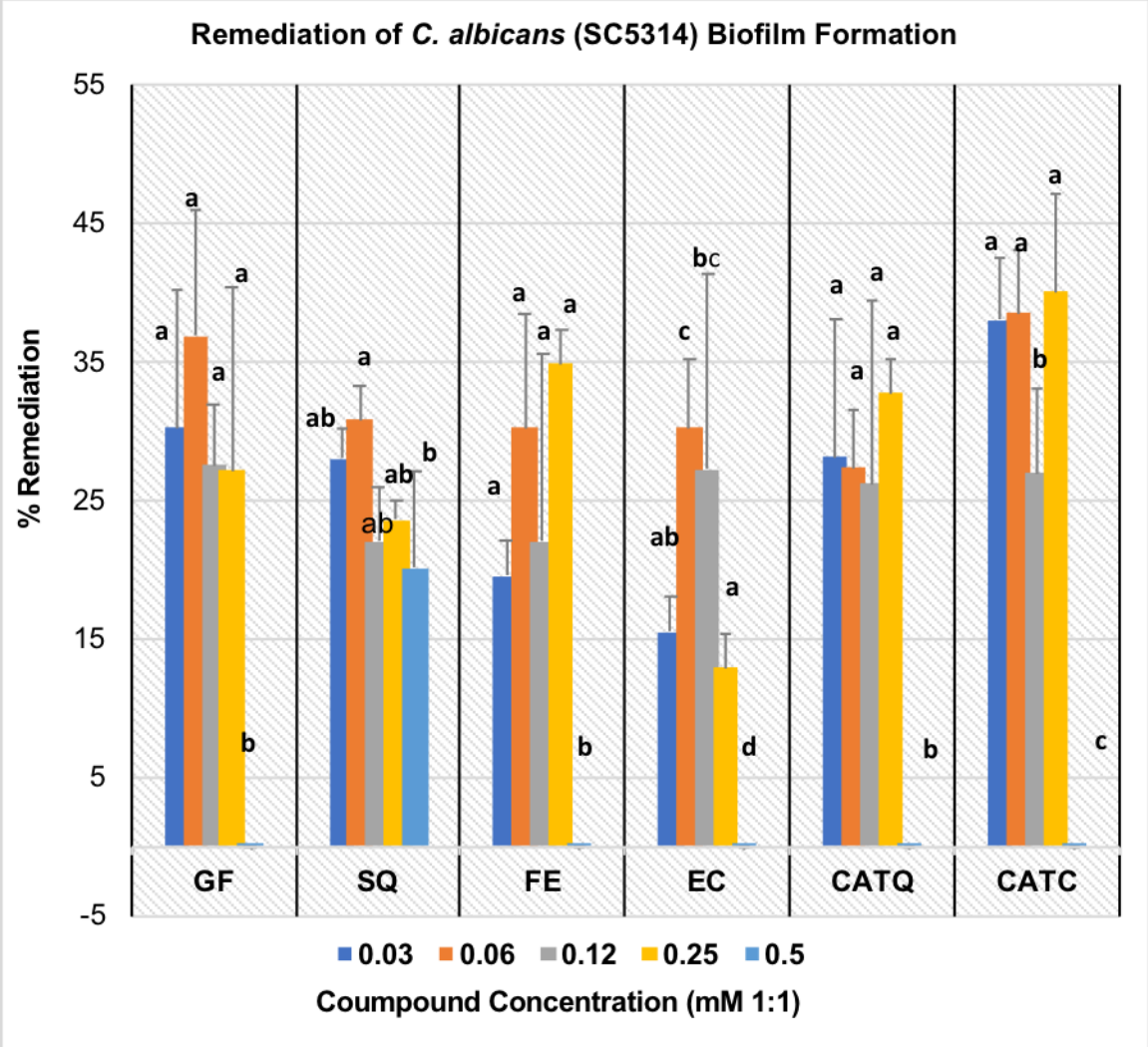

Figure 2.8: Percent remediation of C. albicans (SC5314) treated with different sets of dual compounds. Each set of bars represents remediation results for cited treatment levels ranging specified at for each compound dual. G-F: Gallic Acid -Ferulic Acid, S-Q; Sinapic Acid-Quercetin, F-E; Ferulic Acid-Epicatechin E-C; Epicatechin- Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-C: Catechin-Coumaric Acid. (Different letters shown for \ a given concentration indicates statistical difference ($p > 0.05$) in cellular adhesion). Bars show the mean % remediation +/- standard deviation (n=4).

Table 2.4: Results of FIC index show the results of the two phenolic treatments to remediate *C. albicans* (A2) where FIC < 0.5 (synergistic), 0.5> FIC < 1 (partial synergy), and FIC >1.0 (antagonistic).

	G-F	S-Q	F-E	CAT-Q	CAT-C
FIC	0.5> FIC < 1	0.5> FIC < 1	0.5> FIC < 1	0.5> FIC < 1	0.5> FIC < 1

G-F: Gallic Acid -Ferulic Acid, S-Q; Sinapic Acid-Quercetin, E-C; Epicatechin-Coumaric Acid, CAT-Q; Catechin-Quercetin, CAT-C: Catechin-Coumaric Acid

Another study demonstrated that the action of p-coumaric and ferulic acid as antibacterial agents was due to dynamic alteration of phospholipid chains (Ota et al., 2011). Also, (-)-epigallocatechin gallate caused fluorescent probe calcein leakage due to formation of large pores on the lipid membranes (Tamba et al.,2007). The phenolic compounds can be acting on multiple targets explained herein thereby remediating biofilm formation. Another study demonstrated that the synergistic effect of epigallocatechin-gallate combined with the antifungal agents (itraconazole or ketoconazole) was caused by blocking the ergosterol biosynthesis pathway (Navarro-Martinez, et al., 2006). Also, several studies reported that the synergistic effect of phenolic compounds (i.e. curcumin) with fluconazole against *C. albicans* occurred because of high production of ROS, which induced apoptosis (Sharma et al., 2010; Fu et al., 2011). Therefore, this study focused on studying the synergistic potential of phytochemical compounds on remediating *C. albicans* biofilm formation. This information is significant for developing natural interventions alternative to current antifungal approaches. However, more studies are needed to explain the mechanism of action and how these compounds interact with *C. albicans* to elicit such an effect.

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Chapter 3

Coumaric Acid and Ferulic Acid Present in Supina Grass Interact Synergistically to Remediate Adhesion and Biofilm Formation of *Candida albicans* (A72 and SC5314)

3.1 Abstract

Candida albicans cells' adhesion to a surface, whether mammalian or synthetic, is the first step in its pathogenic phase followed by a morphological change from the yeast to hyphae phenotype (the virulent state). Moreover, *C. albicans* biofilm formation is becoming a common occurrence on catheters and other types of intravenous devices, which if not surgically replaced, can lead to life threatening systemic infections. Yet, resistance is increasing to limited anti-fungal agents currently used to combat these *Candida* virulence factors. Therefore, the objective of this study was to determine the potential synergistic interplay of the phenols present in supina grass to remediate the adhesion and biofilm formation of *C. albicans* (A72 and SC5314). An extract of supina was prepared and characterized for phenolic content, which confirmed the presence of primarily ferulic, coumaric, and lower levels of caffeic acids. The extract was then used to treat cell adhesion and biofilm formation established by *C. albicans* (A72 and SC5314) at incubation times of 1, 3, 5 and 24 h and 4 concentrations ranging from 0.72-7200 ng/g. The extracts were able to remediate cellular adhesion and biofilm formation for both *C. albicans* strains at time points 3 and 6 h by 50-70% at primarily the lower dosages and were mostly effective at all the doses, but not in a dose dependent manner. Upon combining the extract with different phenol levels of ferulic and coumaric acids, *C. albicans* adhesion and biofilms were induced even further, i.e., 50-70%, (6 h incubation. Moreover, these compounds acted synergistically with the grass matrix based on calculation of the fractional inhibitory values, which were below 0.5.

Keywords: Candida, phenols, synergism, biofilm formation, cellular adhesion

3.2 Introduction

Candida albicans inhabits the gastrointestinal tract as a typical benign commensal member but can become an opportunistic pathogen when the host microflora is compromised (Teodoro et al.,2015). *Candida* infections (candidiasis) can be life threatening, particularly in individuals who are critically ill, (immunodeficiency syndrome, hematological malignancy) causing mortality rates of over 30-40% (Morgan et al., 2005). Adhesion to a surface, whether biological or synthetic, is the first step in its pathogenic phase followed by a morphological change from the yeast to hyphae phenotype (the virulent state) (Han et al., 2011).

Moreover, *C. albicans* biofilm formation is becoming a common occurrence on catheters and other types of intravenous devices, which if not surgically replaced, can lead to life threatening systemic infections (Nobile et al.,2006; Bauter et al., 2002). The estimated costs of treating such infections exceed a billion dollars a year in the United States alone (Miller et al., 2001). The increasing *C. albicans* infection rate has been attributed to the emergence of strains resistant to commonly used antifungal agents. Moreover, considering that infectious diseases are causing ever increasing mortality rates among the human population, pathogens appear to have a greater ability to transform and attain resistance to antimicrobial drugs (Sakagami et al., 2002; Nascimento et al.,2000) thereby necessitating the development of innovative and multiple targeted anti-fungal agents.

Phenolic compounds present in grasses (such as supina) may exert such anti-fungal properties due to their ability to complex proteins, disrupt microbial membranes or act as cell signaling agents (Papadopoulou et al., 2005; Hirasawa et al., 2004). In fact, as secondary components in plants, these compounds function as antifungal and

antibacterial agents to provide natural protection. As such, plants have been used to treat and prevent diseases over thousands of years. Reports have shown the health-promoting benefits of plant phenolics even though their mechanisms and mode of actions are not fully understood (Maciel, 2006). However, such research will require an interdisciplinary approach due to the rich levels of phenols present in our environment coupled with their chemical diversity, which in turn affects their solubility, stability, desolation and absorption, which all influence their release that potentially affects antimicrobial potency (Negri et al., 2014). Additionally, as phenolic compounds are largely available in all of plants, and thus typically consumed as a part of large matrix in our diets, it is a challenge to identify their specific health-promoting benefits, but such compounds have properties that protect against cellular oxidation, cellular inflammation, energy dysfunctions, cancer, heart disease, diabetes, to name a few identified in the studies conducted thus far. In particular, phenols have been effectively used as antimicrobials in multiple studies (Alzoreky et al., 2003; More et al., 2008; Shinobu et al*.,* 2011; Pessini et al., 2003; Tempone et al., 2008; Rajeh et al.,2010; Kumar et al, 2005). Although most of these studies have been conducted in with isolated phenols, humans consume a richly diverse composition of phenols on a daily basis via plant-based food intake. As such, it is only reasonable to hypothesize that phenols from plants act together to provide their health benefits, and thus the development of novel drugs from these components should take this approach (Wagner and Ulrich 2009).

The *Poaceae* or grass family is among the most abundant and renewable plant families on the planet that may offer a novel source of phenols (Margorie et al., 1999; Thompson & Thompson, 2010; Odey et al., 2012). The cereal species in particular (corn, rice, and wheat) are staple foods that are widely consumed on a global basis, and these species have been recognized as the primary nutraceutical sources within the

Poaceae family (Thompson and Thompson, 2010). However, *Poa supina* is also an interesting species among grass family due to its turf characteristics and it is a native species to the European Alps (Leinauer, et al., 1997). Despite its potential to contain chemically diverse phenols, the supina grass is typically disposed of in the landfills as grass clippings. Therefore, the objective of this project is to determine the ability of a supina extract to remediate *C. albicans* (A2 and SC5314) colonization and possible synergistic interactions of the phenols present in this plant. Thus, an extract of supina was characterized for the presence of multiple components, including the phenols, to provide a point of reference of its composition pending a positive impact. Moreover, this grass was selected as it is easy to cultivate in short time span thereby providing sustainability as a source of extractable phenols from a drug-related perspective. Also, natural plant extracts have demonstrated a superior metabolic power, which might be attributed to the balance of phenolics present in plant extracts (Ray et al., 2004). As a result, it is expected that this project will provide information on whether this sustainable co-product stream could be a source of anti-fungal phenol synergists and if a more complex matrix of enriched phenols is more effective in targeting *C. albicans* virulence phenotypes.

3.3 Materials and Methods

3.3.1 *Supina grass extraction*

Supina grass was provided by Dr. Roch Gaussoin from the Agronomy and Horticulture Department at the University of Nebraska-Lincoln. Clippings of the grass were sequentially extracted with 25:75 water:methanol followed up by 75:25 water: methanol, 25:75 water:ethanol followed up by 75:25 water: ethanol and ethanol using the same pellet after each extraction in order to recover the chemically diverse polyphenols. Approximately 0.5 g of finely ground supina grass was extracted with 10

ml of a solvent for ~ 1 h and centrifuged for 15 min. The supernatant was collected, and the pelleted residue was extracted with the next solvent. Each supernatant was analyzed for total phenols, flavonoids, anthocyanins, chlorophyll, and then the values were added to obtain the final concentrations.

3.3.2 *Total phenols*

Total phenolic content of the extract was determined by the Folin-Ciocalteu method (Singleton and Rossi 1965). Extract aliquots (100 µl) were treated with 100 µl Folin-Ciocalteu reagent and 4.5 ml of nanopore water. After 3 min of mixing, 0.3 ml of 2% (w/v) sodium carbonate was added and the samples were incubated at room temperature for 2 h with intermittent shaking. The absorption at wavelength 760 nm was measured with a Beckmen Coulter DU 800 Spectrophotometer (Fullerton, CA). The sample data was expressed as the means +/- standard deviation calculated to mg gallic acid equivalent g^{-1} GT (dry weight).

3.3.3 *Total flavonoids*

Total flavonoids were determined by the method according to Adom and Liu (2002). Extracts (125 µl) with proper dilution was added to 37.5 µl of 5 % (w/v) sodium nitrite and 0.625 ml of nanopore water. After 4-6 min of incubation at room temperature, 75 µL of 10 % (w/v) aluminum chloride was added to the sample. Following an additional 5-7 min of incubation, 0.25 ml of 1.0 M sodium hydroxide and 0.4 ml nanopore water were added to the mixture. The samples then were vortexed and monitored at 510 nm. Total flavonoids were expressed as mg catechin equivalents g^{-1} grass clipping (dry weight) of triplicate analyses.

3.3.4 *HPLC profile*

The extracts were hydrolyzed following Devananad et al., (2006) by weighing 200 mg of sample into 50 ml conical plastic tubes. 2 N Sodium hydroxide was added (5

mL) in water containing 10 mM EDTA and 1% ascorbic acid followed by thorough mixing. After the mixture was stirred for 30 min at 40 to 45 ^{o}C, 1.4 ml of 7.2 N hydrochloric acid in water were added and vortexed for 10 s. The free phenolics were extracted from the samples by adding 6.4 mL of ethyl acetate and centrifuged at 3000 rpm until a clear supernatant was obtained. The organic layers were transferred to another 50 ml tube, and the extraction was repeated again, and the extracts combined. The extracts were dried under a steady stream of liquid nitrogen until the residue was completely dried, which was then diluted in 1 ml of methanol: water (80:20) and vortexed 3 times at 30 s per vortex to dissolve the residue. The samples were filtered through PVDF syringe filter (0.45 µm) and analyzed by HPLC.

Phenolic profiling was completed on extract that showed high potency by using a reverse-phase HPLC system coupled with a C18 column (5 µm, 250 x 4.6 mm) and a photo-diode array detector. The method reported by Lin et al. (2008) was adopted for resolving the phenolic acids. In brief, the mobile phase consisted of a combination of A (0.1% formic acid in water) and B (acetonitrile) with a flow rate of 1 ml/min. The gradient was varied linearly from 10–26% B (v/v) in 40 min, to 65% B at 70 min, and finally to 100% B at 71 min and held at 100% B to 75 min maintaining a flow rate of 1 ml/min. The UV-vis spectra from 190 to 650 nm was collected using a photodiode array detector. The resolved peaks were identified and quantified with external standards and expressed as the mean +/- standard deviation of µg per g of clipped grass for triplicate analyses.

3.3.5 *Preparation of C. albicans yeast stock culture*

C. albicans strains (SC5314 and A72) were obtained from Kenneth Nickerson, University of Nebraska-Lincoln. A stock culture was grown to the stationary phase. i.e., no visible budding was observed which was typically 24 -30 h post inoculation, in 500 ml

of yeast extract (5 g), peptone (2.5 g), dextrose (10 g) medium (YPD). Aliquots of the media (25 ml) were then added to 125 ml Erlenmeyer flasks, along with a ½ loopful of *C. albicans* (A72 and SC5314) which had been maintained on YPD agar. The inoculated flasks were incubated in a shaking water bath at 30°C for 22 h (or until cells achieved stationary phase, i.e., no visible signs of budding). The cells were then washed three times with potassium phosphate buffer (pH 6.5) followed each time with centrifugation until a clear supernatant was obtained. The ensuing pellet was then re-suspended in 7.5 ml of PBS and maintained at 8-10 ^{o}C until use.

3.3.6 *Virulent cell induction and treatment*

Serum media from Atlanta Biological was thawed at room temperature for 5 min, and then 5 ml of the serum was dissolved in 45 ml of potassium phosphate buffer (pH 6.5) prior to use. The 25:75 and 75:25 methanol and ethanol extracts were combined, and 1.5 ml was concentrated under a steady stream of liquid nitrogen. After the sample was completely dried, the residue was dissolved in 1 ml of 100% ethanol as a stock solution. Different concentrations (0.72, 7.2, 72, 720, and 7200 ng/g) of the extracts were prepared by re-diluting in 2% ethanol. The stock solutions of þ-coumaric and ferulic acids were prepared by preparing 300 mM of each phenol in 100% of ethanol until the solids had completely dissolved. Then, the concentrations of 0.03, 0.06, 0.125, 0.25, 0.5, 1, and 3 mM were prepared by diluting the stock solution into 2% ethanol. Preliminary experiments were completed that showed an organic solvent was needed to ensure complete solvation of the phenols. Methanol at any concentration affected the adhesion and biofilm formation assays, while ethanol at concentrations below 2% did not hinder induction of the two virulence factors (data not shown).

3.3.7 *Remediation experiments*

C. albicans strains (A72 and SC5314) were added at 5 x 10^6 cell per ml in each well of Immunol 2HB 96 well plates containing 140 μl serum. The plates were covered with aluminum and incubated at 37 °C. After the 24 h incubation, 60 μl of the extracts at a given concentration that ranged from 0.72-7200 ng/g were added to each well. The, the plates were covered again with aluminum and incubated at 37 °C for 6 h. Adhesion and biofilm remediation were analyzed at 1, 3, 6 and 24 h. The 6 h point was selected for further analysis as preliminary studies showed cellular adhesions and optimal biofilm formation occurred at this time point.

3.3.8 *Adhesion assay*

The adhesion assay was performed according to Pierce *et al.* (2008). Briefly, the media from each well was carefully removed and 50 μl of crystal violet was added to the wells. The plates were covered again and incubated at room temperature for 45 min. After incubation, each plate was rinsed gently with 400 μl of ice cold water 5-10 times. The plates were inverted onto a paper towel to remove any non-adherent cells and water. The plates were then incubated for another 30 min at room temperature after adding 200 μl of 75% methanol to each well. The absorbance was determined at 590 nm using a microtiter plate reader (Fazly et al., 2013).

3.3.9 *Biofilm assay*

Biofilm formation was determined by using the (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) XTT kit according to the manufacturer's direction (Sigma-Alorich). (The XTT kit consists of XTT labeling reagent and electron coupling reagent. This assay relies on yellow tetrazoluim XTT salt cleavage to yield an orange formazan through the active metabolic cells, which indicates the viable cells; the method is based on procedures cited by Pierce *et al.,* 2008 & Sudjana,

et al., 2012). The XTT labeling reagent and electron coupling reagent were thawed in a water bath set at 37 ^{o}C, and then 0.1 ml of electron coupling reagent was added to 5 ml of XTT labeling reagent to be activated prior to use. The XTT mixture (100 μl) was added to each well and incubated for 2 h. The absorbance was determined at 450 nm using a microtiter plate reader.

3.3.10 *Percent Remediation calculations*

Remediation of biofilm formation or *C. albicans* adhesion in already established films were defined as % remediation for both cases, which was determined by the following equation (Romano *et al.,* 2009):

$$\% \text{ Remediation} = ((A_{control} - A_{sample})/(A_{control}))*100$$

Where: $A_{control}$ is the absorbance of cells without a treatment

A_{sample} is the cells with the treatment.

3.3.11 *Synergistic Interaction*

Synergism was determined by calculating the fractional inhibitory concentration FIC_A, which is the activity of phenolic compound A in the presence of B/activity of phenolic compound B alone. FIC_B is the activity of phenolic compound B in the present of A/activity of phenolic compound A alone. The equation $FIC_{index} = FIC_A + FIC_B$ was used to determine if the compounds acted as synergists, additives or antagonists. The synergist values were considered in the range of FIC_{index}, whereas values <1, 0, or > 1 indicated synergists, additives or antagonists, respectively (Romano et al., 2009).

3.1.12 *Statistical analysis*

The biofilm/adhesion experiments were completed on 3-9 replicates for each treatment/concentrate used and the time point monitored. After data outliers were removed by the Grubs test at a 5% confidence interval, the final results were reported as the mean +/- standard deviation of the one-way ANOVA, which was used to determine

whether that various treatments differed in terms of % remediation at 95% confidence interval ($p < 0.05$) using Tukey's honest significant difference. Grass clipping characterization analyses were completed in triplicate and the results expressed as the mean +/- standard deviation. The statistical analyses were obtained with Minitab 17.

3.4 Results and discussion

Several studies have been conducted on the antimicrobial activity of plant extracts. For example, extracts from *Ruta graveolens* and *Zingiber officinale*, used in Asia, exhibited an inhibitory effect against *Bacillus cereus* strains (Alzoreky et al., 2003). In another study, extracts of six plants from South Africa (*Annona senegalensis, Englerophytum magalismontanum, Dicerocarym senecioides, Euclea divinorum, Euclea natalensis*, and *Parinari curatellifolia* were tested against human oral cavity pathogens, such as *Actinobacillus actinomycetemcomitans, Actinomyces naeslundii, Actinomyces israelii, Candida albicans, Porphyromonus gingivalis, Prevotella intermedia and Streptococcus mutans; E. natalensis* showed some inhibitory effect against *C. albicans* in a disc diffusion assay (More et al., 2008).

Moreover, several types of plant extracts demonstrated antimicrobial activity against *Candida*, including those from *Curcuma zedoaria, Psidium guajava, Plectranthus amboinicus, Aristolochia cymbifera, Plectranthus barbatus, Lippia alba, Hydrocotyle bonariensis, Hydrocotyle bonariensis, Justicia pectoralis var. stenophylla, Herreria salsaparilha, Mentha X piperita, Eleutherine bulbosa, Baccharis trimera, Calamintha adscendens, Albizia inundata, Bauhinia forficata, Cymbopogon citratus, Plectranthus grandis,* and *Euphorbia hirta (*Shinobu et al., 2011*;* Pessini et al., 2003; Tempone et al., 2008; Rajeh et al.,2010). A study by Polaquini et al. (2006) showed the effect of a crude extract of Neem (*Azadirachta indica*) on inhibiting *Candida* adhesion; however, it did discuss *Candida* biofilm formation inhibition. *Crossandra infundibuliformis* and *Labisia*

pumila extracts also demonstrated potential in inhibiting *Candida* spp growth and filamentous antifungal activity (Madhumitha et al., 2011; Karimi et al., 2013). The extracts also showed from *Bauhinia racemosa* showed antimicrobial activity against *Candida albicans* (Kumar et al, 2005).

A study demonstrated a high anti-adherent potential of *Schinus terebinthifolius* and Croton *urucurana* extracts on in vitro *C. albicans* biofilm formation (Barbieri et al., 2014). The dried bark of *Acacia catechu* was also able to suppress microbial growth and enhance the immune system to face the invading antigens of organisms. *Acacia catechu* has shown potency as antimicrobial agent due to its taxifolin and active chemical ingredients including catechin, epicatechin, epigallocatechin, epicatechin gallate, and quercetin (Lakshmi et al., 2006).

Strawberry, raspberry, and cloudberry extracts demonstrated potential effect on suppressing *C. albicans* growth (Liisa et al., 2006). Another study investigated the effect of propolis on *C. albicans* virulence factors. Propolis is a substance from plant sources collected by honeybees that yielded dramatic reduction of *C. albicans* adhesion, yeast-mycelial conversion, and hyphae length at 0.22 mg/ml. (D'auria et al., 2003). Common to these studies is the use of plant extracts, which all contain phenolic compounds and chlorophyll. Phenolic and flavonoid compounds have been associated with the potential of plant extracts to act as antimicrobial agents. A study by Rauha et al., (2000) reported that purple loosestrife (*Lythrum salicaria*) extract was very active against *Candida albicans*, while white birch (*Betula pubescens*), pine (*Pinus sylvestris*) and potato (*Solanum tuberosum*) extracts significantly inhibited the gram-positive bacterium *Staphylococcus aureus*. However, although grasses are the most abundant plant in the world, studies related to their ability to protect against *C. albicans* virulence factors, among other health promoting benefits, are non-existent to our knowledge, which has

made supina grass one of the most underutilized agriproducts in modern medicine (Wenger, 2011).

3.4.1 *Characterization of Supina Grass*

3.4.1a: Total Phenols: Phenolic compounds such as ferulic, caffeic, p-hydroxybenzoic, protocatechuic, p-coumaric, vanillic, and synergic acids are typically present in cereal grains (Cowan, 1999). These phenolics are present as conjugates, bound with sugars, fatty acids, or proteins (White and Xing, 1997). In this work, total phenols of supina grass extracts were quantified by Folin-Ciocalteu method. The result indicated that total phenolic content of supina grass extracts was 0.96 ± 0.05 mg/g, shown in Table 1. In a study by Wenger (2011), the total phenolic content of supina grass was 0.89 ± 0.13 mg/g. These differences may be attributed to the extraction methods used as preliminary data shown in our lab have shown that even the solid to water ratio substantially affects the phenolic level.

3.4.1b: Total Flavonoids: Flavonoids are polyphenolic compounds that are also abundant in the plant kingdom, and mainly include flavones, flavonols, and anthocyanins (Papadopoulou et al.,2005). They are known for several biological activities with an emphasis on their antioxidant properties as they can scavenge free radicals, chelate reactive metals, activate antioxidative enzymes such as super oxidase dehydrogenase, or inhibit pro-oxidant enzymes, such as xanthine oxidase (Wegner, 2011; Havsteen, 1983; Bylka et al., 2003). Flavonoids possess health promoting properties and may be considered a health promoter, which has the potential to prevent or treat human chronic diseases (Bylka et al., 2003). There is a growing interest of flavonoids' antipathogenic properties due to the increasing resistance of pathogens to available drugs (Wegner, 2011). Therefore, in this study total flavonoids present in supina grass extracts were determined and the results showed that extracts contained 0.49 ± 0.01 mg/g (Table 3.1).

3.4.1c: HPLC Profile: A reverse-phase HPLC system was used for identification and quantification of phenolic compounds present in supina grass extracts (Table 3.1 and Figure 3.1). The most abundant phenols were caffeic acid (2.3 ± 0.03 mg/ g), coumaric acid (1.592 ± 0.09 mg/ g) and ferulic acid (2.8699 ± 0.09 mg/ g). In another study, ferulic acid was the most predominant phenolic in supina grass extracts, which supports our results (Wegner, 2011). However, ferulic acid quantities were lower in the latter study (30.9 ± 1.99 mg/100 g).

Table 3.1: Total Phenolics content (TPC), Total Flavonoids (TFC), Ferulic acid, Coumaric acid, and Caffeic acid content.

TPC	0.96±0.05 mg/g
TFC	0.49±0.01 mg/g
Ferulic acid	2.8699 ± 0.09 mg/g
Coumaric acid	1.592 ± 0.09 mg/g
Caffeic acid	2.3 ± 0.03 mg/g

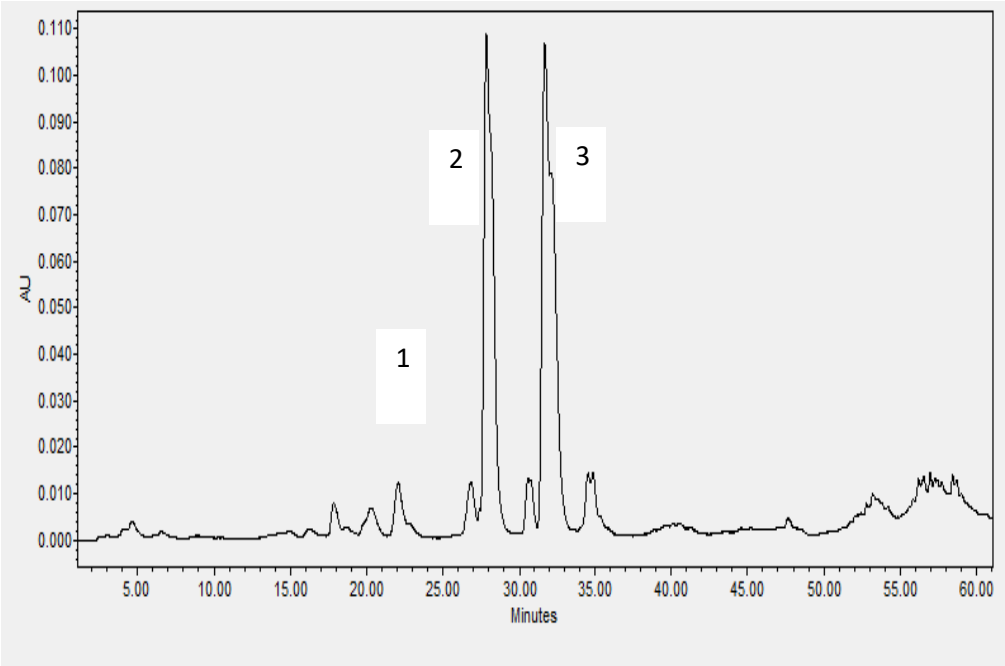

Figure 3.1: HPLC Chromatogram showing peaks of (1) Caffeic acid, (2) P-coumaric acid, and (3) Ferulic acid at 23 mg/100 ml, 159.19 mg/100 ml, and 286 mg/100 ml, respectively.

3.4.2 *Effect of Supina grass extracts to remediate* C. albicans *(SC5314 and A72) cellular adhesion:*

Use of plants as traditional medicines that can potentially prevent and treat diseases has been a common practice in antiquity but has increased in intensity during the modern age (Chaudhari et al., 2016; Silva et al., 2010; Cowan, 1999). In terms of *C. albicans* infections, herbal approaches are an ideal strategy to prevent cell pathogenicity and thus control biofilm formation due to the biodiversity of plants, cost-efficiency and sustainability. Indeed, studies completed in our laboratory showed that phenols are more potent in combination (Chapter 2) than the sum of the individual components (Chapter 1). Therefore, supina grass may be a promising, underutilized agro-product for reducing adherence of already established *C. albicans* cells to several surfaces (Barbieri et al, 2014) and thus offer a potentially novel alternative to currently used anti-adhesion approaches while averting resistance to such treatments (Martins et al.,2015).

Therefore, in the present work, the remediation effect of a supina grass extract was determined at four time points (1, 3, 6 and 24 h) using five different concentrations (0.72, 7.2, 72,720, and 7200 ng/g). The effectiveness of the extracts was analyzed by statistically comparing the four time points that showed remediation at a given concentration (Figures 3.2 and 3.4) and a given time point across concentrations (Figures 3.3 and 3.5) for two strains of *C. albicans*, A72 and SC5314.

3.4.2a Remediating C. albicans *(A72) cellular adhesion:* In the case of *C. albicans* (A72), % remediation of cellular adhesion was significantly different among time points at the given concentrations ($P>0.05$) (Figure 3.2). At the low concentration of 0.72 ng/g, the % remediation was significantly high (> 50% at 6 h) and decreased

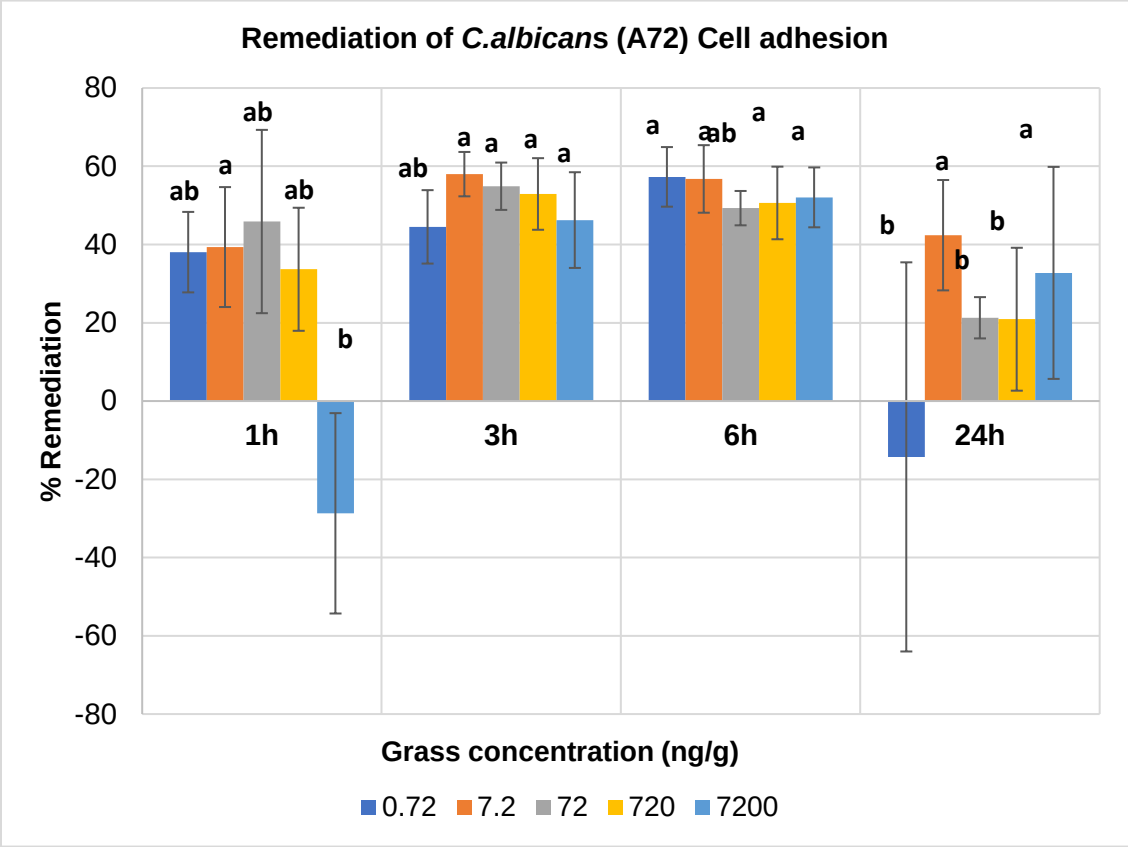

Figure 3.2: Effect of supina grass extracts on remediating *C. albicans* (A72) cellular adhesion 1, 3, 6, and 24 h post exposure of a given treatment concentration. Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within the same time having different letters are significantly different, $p < 0.05$.

significantly to -14.27% by 24 h. Additionally, reduction of cellular adhesion at 3 and 24 h were significantly different when the cells were exposed to 0.72 and 72 ng/g. The 3 h incubation with the 72 ng/g treatment exerted a high of 54.89 %, while the remediation decreased significantly to 21.28% by 24 h. When the concentration increased to 72 ng/g, the cellular adhesion was significantly different at 24 h relative to 3 and 6 h but were the latter they were not statistically different from one another. Percent remediation at 24 h was the lowest at 20.90%, while the effect at 3 and 6 h was much higher: 52.92 and 50.62%, respectively. The % remediation at 1 h was significantly different from that at the other time points at 7200 ng/g, which resulted in a remediation effect of -28.69%. The results indicated that the 1 h exposure to 7200 ng/g and 24 h to 0.72 ng/g of the extract has the lowest effect on remediating cellular adhesion to *C. albicans* A72 cells. The values obtained from these time points most likely were due, in part, to the high variability as evidenced by the large error bars (Figure 3.2), as the results were significantly similar at least for the 0.72 ng/g extract for time points 1, 3 and 24 hr. Cellular adhesion can be difficult to interpret as it is not uncommon to have imprecise results when monitoring this effect. This could be due to the different targets that may be affected by the compounds or the assay itself.

Figure 3.3 shows the differences between each time point across different concentrations. Remediation at the highest concentration, 7200 ng/g, and 1 h (-28.69%) was significantly different from that at any other concentration or time point. However, the reactions produced in response to different concentrations of extracts were not significantly different among time points, which ranged from low of ~ - 28 % to a high of > 50%. Again 1 h (7200 ng/g) and 24 h (0.72 ng/g) had high variability among concentrations (Figure 3.3).

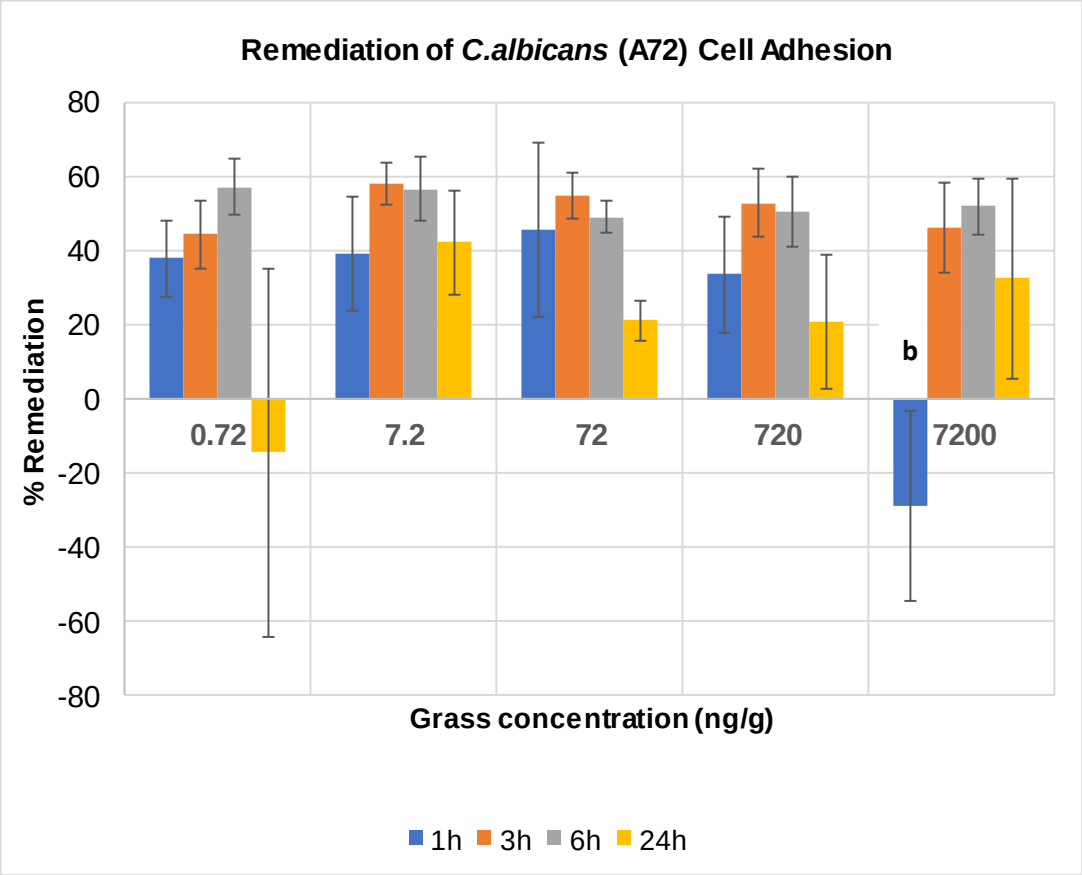

Figure 3.3: Effect of supina grass extracts on remediating *C. albicans* (A72) cellular adhesion at diffrent concentration (0.72,7.2, 72,720, 7200 ng/g). Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within each response concentration having different letters are significantly different, P< 0.05.

As was confirmed by HPLC analysis shown in Figure 3.2, supina extracts contained high quantities of phenolic compounds including coumaric (C), ferulic (F), and caffeic acid, which are hydroxylated derivatives of cinnamic acid, but the hydroxyl groups' numbers and position on the aromatic ring and substitution type results in potential differences on phenolic characteristics (Borges et al.,2013).

Phenolic antifungal extracts target the fungal membrane and its components as a common mechanism of action (Martins et al., 2015). Jothy et al. (2012) investigated the anticandidal mechanism of action of methanol extract of *Cassia fistula*. The extracts entered and disrupted the plasma membrane. The extracts accumulated in the plasma membrane, which caused mitigation of cell growth. Several studies support that F, CA, and C effectively mitigated fungal infections by disrupting the cell cytoplasmic membrane. (Campos et al,2009; Walsh et al., 2003; and Ultee et al., 2002).

3.4.2b *Remediating* C. albicans *(SC5314) cellular adhesion:* In contrast to *C. albicans* strain A72, the % remediation of *C. albicans* SC5314 cellular adhesion was significantly similar among time points at concentrations 0.72, 720 and 7200 ng/g. For example, these concentrations detached established adhered cells by percentages of 30.24, 54.54, and 32.11 at 24 h; 47.12, 49.99, and 47.88 at 1 h; 55.86, 52.59 43.80 at 3 h; and 47.88, 46.33, and 47.29 at 6 h respectively (Figure 3.4). The responses produced when incubated for 24 h were significantly different from 1 and 3 h, while the latter time points were similar at 7.2 ng/g (24 h). When the concentration increased to 72 ng/g, incubation at 24 h was significantly different from 3 and 6 h, causing a reduction of bound cells by 42.65 % (Figure 3.4). Clearly, the time points affected the two strains differently (Figures 3.2 and 3.4). While the 1 h and 24 h were not as effective on remediating *C. albicans* A72 cellular adhesion, these time points were effective on alleviating cellular adhesion of SC5314 with higher % remediation and less variability.

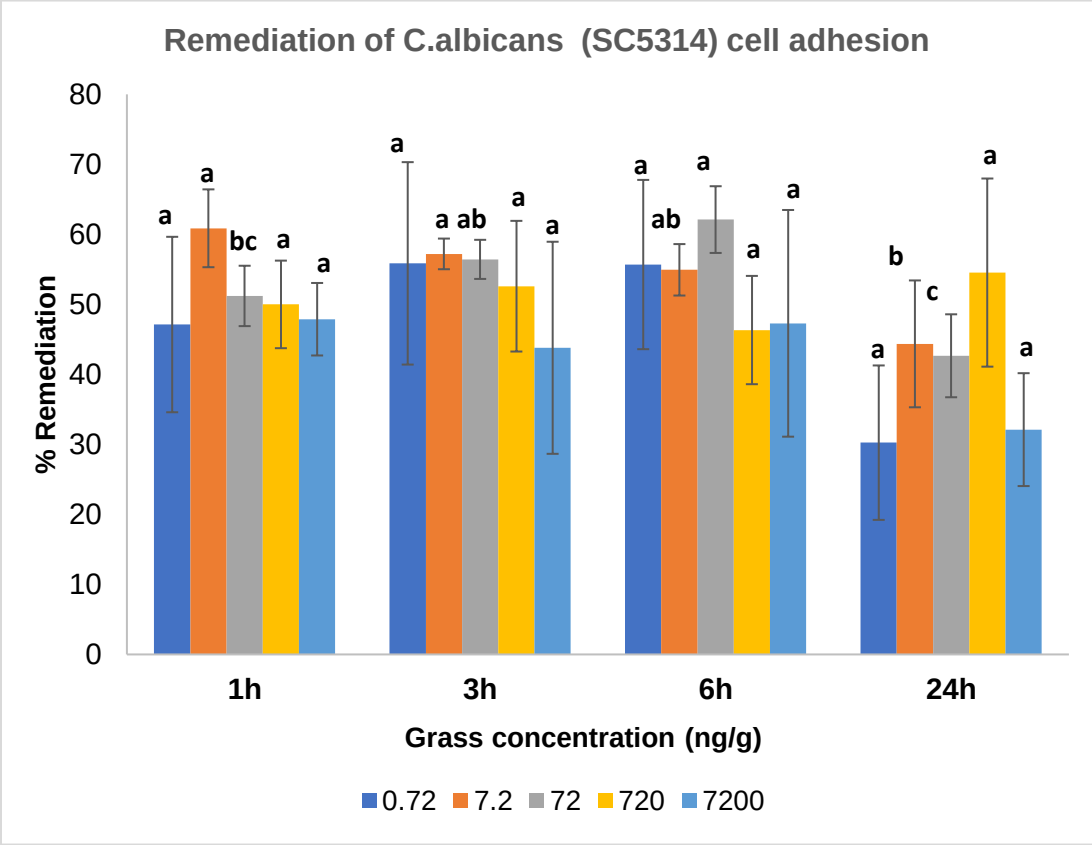

Figure 3.4: Effect supina grass extracts on remediating *C. albicans* (SC5314) cellular 1,3,6,24 h post exposure of a given treatment concentration. Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within the same treatment time having different letter are significantly different, $p < 0.05$.

These results indicate that extracts may be acting on different targets. The adhesion responses were statistically similar for each time point across concentrations (Figure 3.5), except the efficacy at 0.72 and 7200 ng/g were different at 24 h, i.e., 30.24 and 54.54 % at 0.72 and 7200 ng/g, respectively. Different concentrations acted similarly for the two strains at each time points and efficient. Only the 0.72 and 7200 ng/g were not effective at alleviating cellular adhesion of A72 at 24 and 1 h of exposure, respectively.

As plant extracts are the focus of many clinical applications due to their high numbers of bioactive molecules, which might show synergic or antagonistic, and diverse effects and in turn neutralize or reduce side effects and toxicity, supina grass shows efficacy at detaching bound *C. albicans* cells from synthetic surfaces at ng/g levels. In some cases, incubation time periods do contribute to differences in the degree of potency, but for the most part % remediation is consistent at approximately 30-50%. Using supina grass may also prove to useful for human health purposes, as plant extracts tend to target symptoms directly, which lessens side effects and toxicity and thus promote nutritional balance (Martins et al., 2015). Moreover, supina grass may be effective for treating infection by other species/strains of Candida, as the study by D'auria et al., (2003) demonstrated the efficacy of the plant source (propolis) at 0.22 mg/ml to reduce hyphal transition of the following *Candida* strains: *C. albicans, C. glabrata, C. tropicalis, C. guilliermondii, C. parapsilosis, C. krusei, C. humicola,* and *C. intermedia*. Such studies could have important ramifications for the effects of supina grass in mitigating *Candida* cellular adhesion. Of course, more studies are needed to test this this hypothesis.

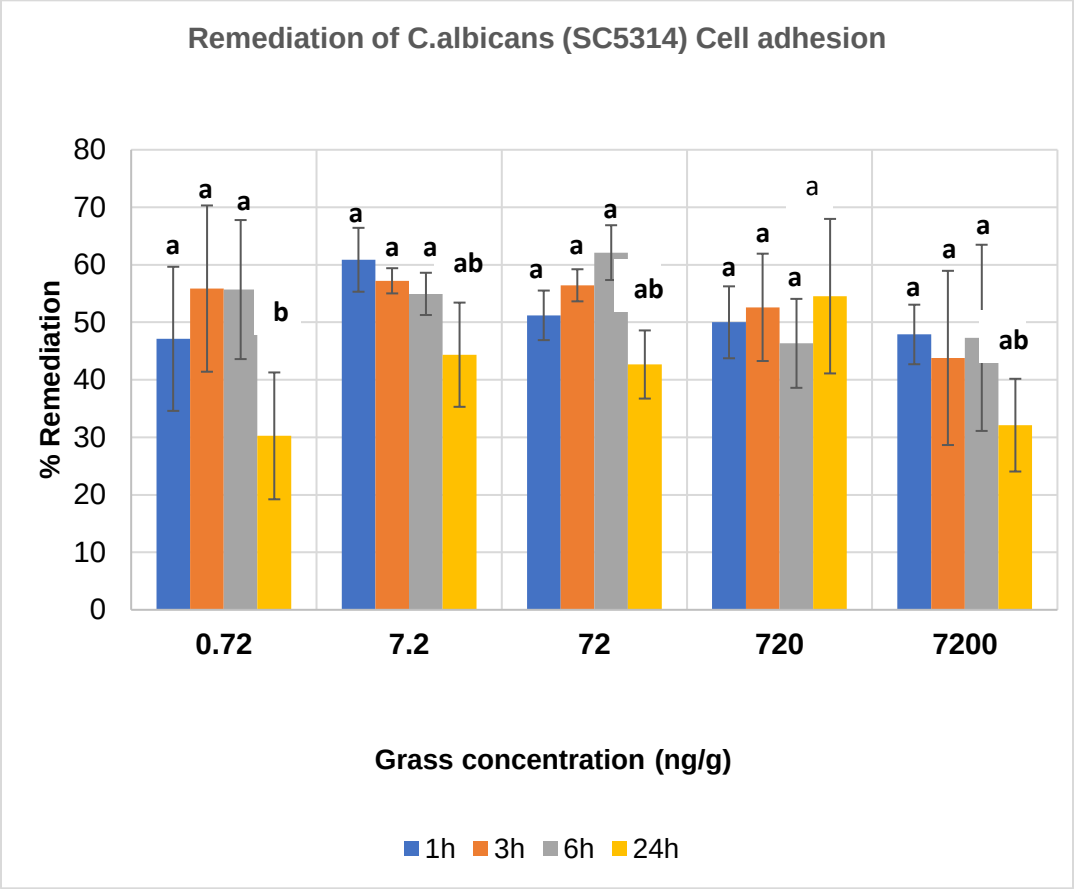

Figure 3.5: Effect of supina grass extracts on remediating *C. albicans* (SC5314) cellular adhesion at different concentration (0.72,7.2, 72,720, 7200 ng/g). Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within each response concentration having different letter are significantly different, ($p < 0.05$).

3.4.2a *Remediating* C. albicans *(A72) biofilm formation:* The extracts were analyzed further by comparing the four time points that showed remediation at a given concentration (Figures 3.6 and 3.7) and a given time point across concentrations (Figures 3.8 and 3.9) for *C. albicans* (A72 and SC5314). For *C. albicans* A72, after incubation with the supina grass, only the 24 h time point resulted in different responses among concentrations as % biofilm remediation ranged from a low -0.29 % (7.2 ng/g) to high of 11.93 % (0.72 ng/g). As the concentration increased to 7.2 ng/g, 24 h had a low of ~ -0 % (24 h) (Figure 3.6).

Moreover, at a 72 ng/g dosage, the responses ranged from a low of 14.85% (24 h) to a high of 44.46% (3 h), and the 1 and 3 h trend were different at the same concentration, i.e., with a % remediation of >40 % (3 h) and ~ 17 % (1 h). Again, it is clear that incubation for 1 and 24 h was not as efficient as incubation for 3 and 6 h at remediating biofilm formation by *C. albicans* A72, similar to the case reported for cellular adhesion remediation (Figure 3.2 and 3.4).

Figure 3.7 demonstrates the effect of different concentrations on remediating biofilm formation at the various treatment incubation times for *C. albicans* (A72). The concentrations performed similarly at mitigating biofilm formation at the 6 h incubation period, which ranged from a low 21.65 % (0.72 ng/g) to a high of 33.06 % (7200 ng/g). The % remediation was significantly different for biofilms exposed to the supina grass extracts after 3 h, with the 720 ng/g dosage exhibiting a biofilm reduction of < 50 %, while the 7200 ng/g resulted in a > 50 % remediation effect. Also, the low concentration of 0.72 ng/g elicits < 30 % remediation.

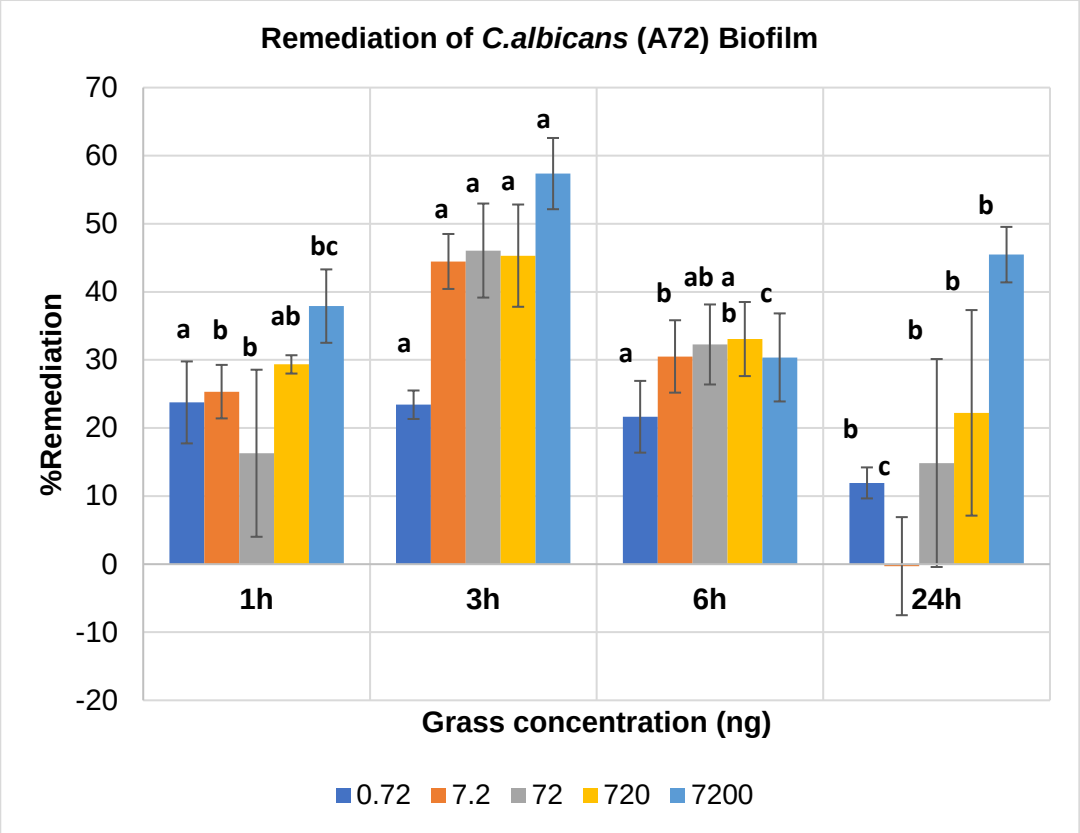

Figure 3.6: Effect supina grass extracts on remediating *C. albicans* (A72) biofilm formation 1, 3, 6, and 24 h post exposure of a given treatment concentration. Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within the same time having different letter are significantly different $p < 0.05$.

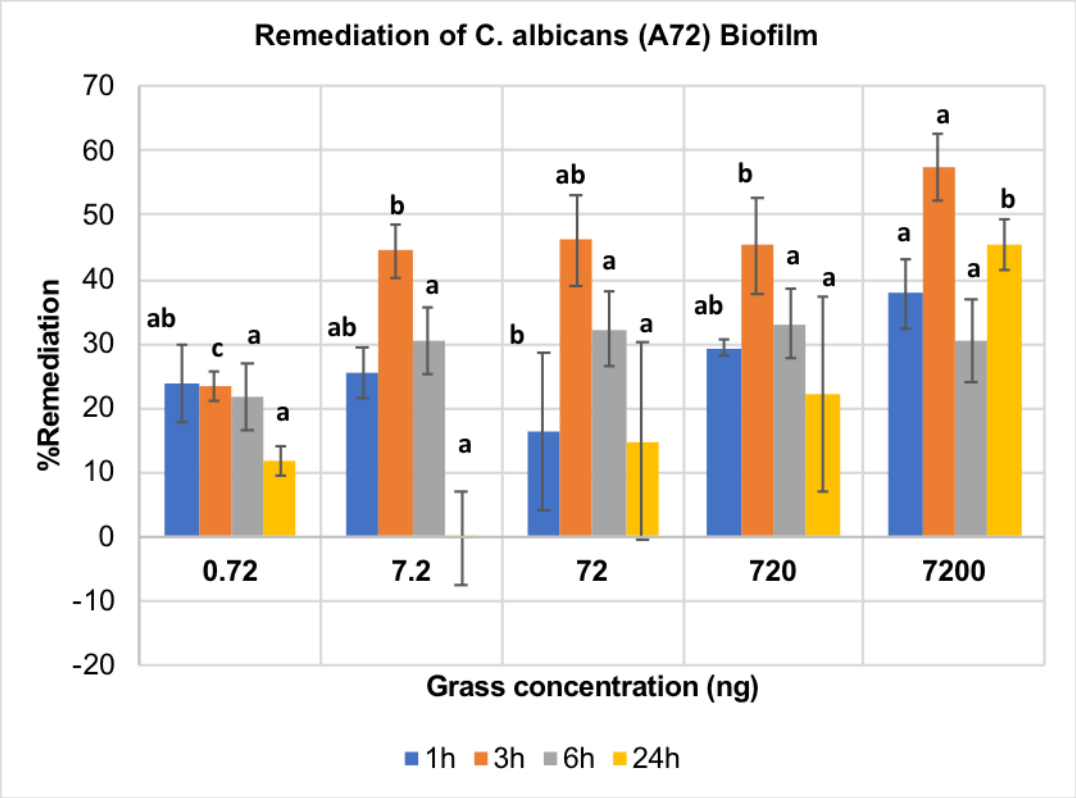

Figure 3.7: Effect of supina grass extracts on remediating *C. albicans* (A72) biofilm formation at different concentration (0.72,7.2, 72,720, 7200 ng/g). Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within each response concentration having different letter are significantly different, $p < 0.05$.

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After 24 h of exposure to the 7200 ng/g treatment, the biofilm decreased by > 40 %, which was different than the lowest concentrations that were able to impact the biofilm reduction. These results indicated that supina grass extracts can potentially act as efficient antifungal agents. The extracts at the different concentrations were able to remediate biofilm remediation more effectively than cellular adhesion at 1 and 24 h in *C. albicans* A72. This remediation action is strongly attributed to the cinnamic derivatives (CA, F, and C), which are commonly present with other polyphenolic compounds with higher overall antioxidant capacity (Cowan, 1999). As proposed by Borges et al (2013) the compounds that exert high antioxidant capacity to scavenge free radicals also represent high antimicrobial activity (Borges et al., 2013), as the harm caused by microbes is due in part by their ability to release abundant reactive oxidative species.

3.3.3b *Remediating* C. albicans *(SC5314) biofilm:* In the case of strain SC5314, the % remediation was significantly different among time points. Case in point, the 24 h exposure treatments were not similar to 1 and 3 h, which in turn were statistically different from one another (Figure 3.8). Also, the 1 h incubation time was

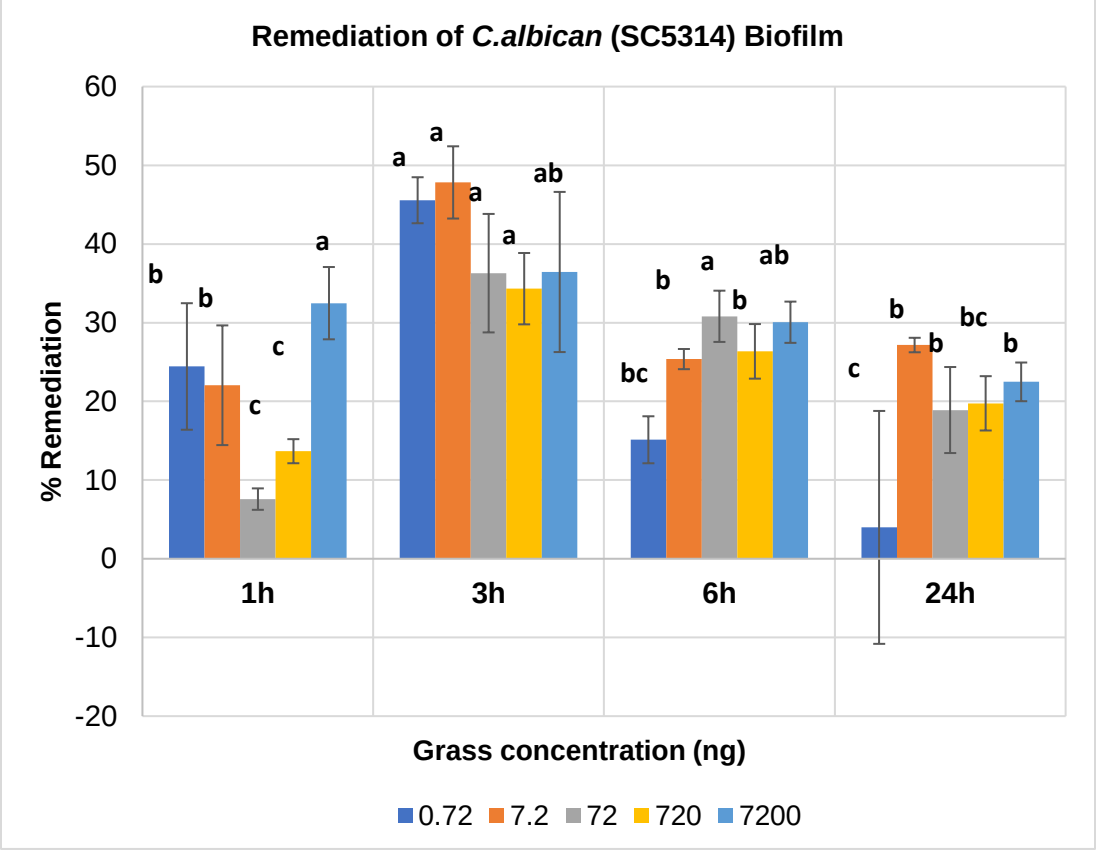

Figure 3.8: Effect supina grass extracts on remediating *C. albicans* (SC5314) biofilm 1,3,6,24 h post exposure of a given treatment concentration. Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within the same time having different letter are significantly different, $p<0.05$.

statistically differently from 3 and 6 h (720 ng/g), and from 24 h (7200 ng/g). It should be noted that 3 and 6 h were the most effective times to reduce established biofilms produced by the SC5314 *C. albicans* strain among concentrations. Accordingly, 1 h is not enough time to allow penetration into cells to alter biofilm formation or to act directly upon it structure, while after 24 h extracts also lose their activity to influence the biofilm formation as % remediation declined relative to the 3 and 6 h time points.

As depicted in Figure 3.9, the differences between each time point among the different concentrations showed that 72, 720, and 7200 ng/g exhibit different responses at 1h: 7.58. 13.66, and 32.47%, respectively. When the time responses increased to 3 and 6 h, the concentrations elicited similar responses, which ranged from 30% to > 47% (3 h) from ~25 to~ 31% (6 h). However, 0.72 ng/g exhibited a different response among other concentrations that ranged from a high of 45% (3h) to a low of ~ 4 % (24 h). The extracts were more efficient at remediating *C. albicans* SC5314 cellular adhesion (> 50%) than biofilm formation, which was < 50 %. The inability to remediate *C. albicans* SC5314 biofilm formation that might be due to other substances present in the extracts. However, this hypothesis needs further investigation.

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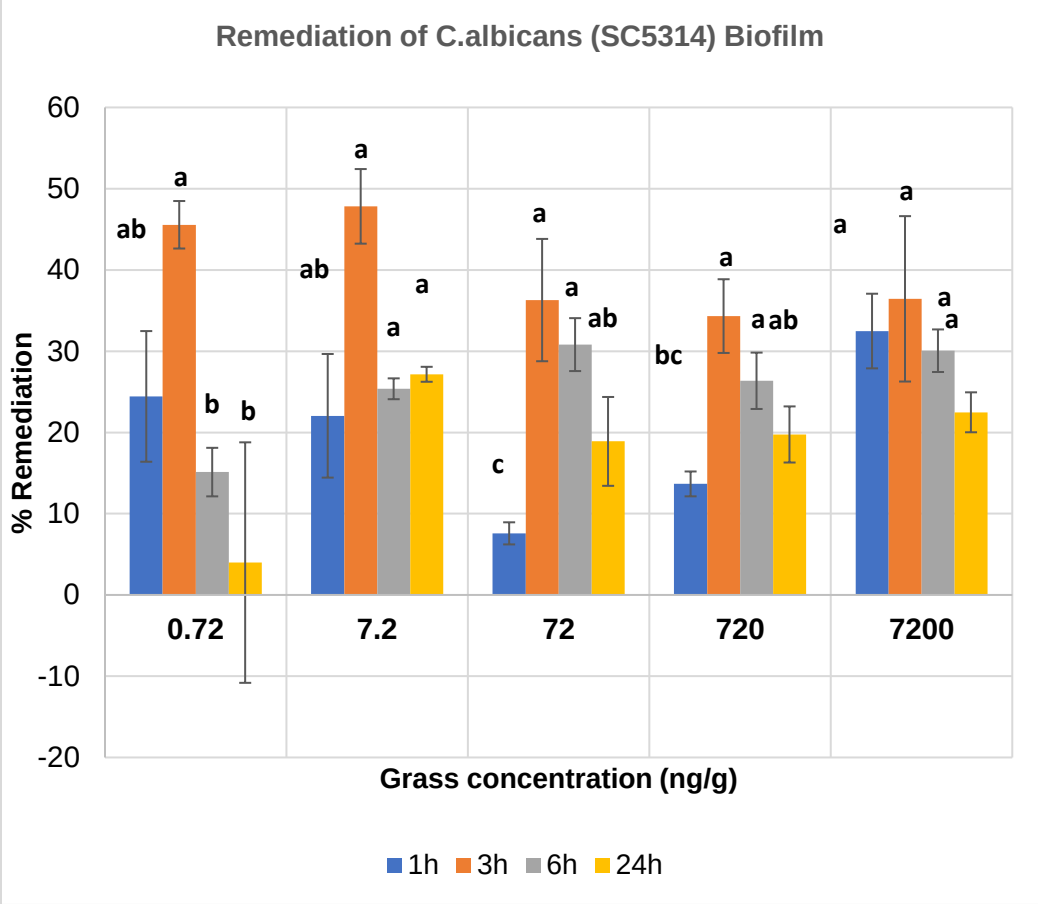

Figure 3.9: Effect of supina grass extracts on remediating *C. albicans* (A72) biofilm formation at different concentration (0.72,7.2, 72,720, 7200 ng/g). . Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within each response concentration having different letter are significantly different, $p < 0.05$.

Supina grass extracts have shown a potential to remediate *C. albicans* (SC5314 and A72) cellular adhesion and biofilm formation via different means. Nonetheless, the extracts significantly remediated cellular adhesion for both strains more effectively than biofilm formation. Indeed, the extracts were effective on remediating cellular adhesion for most time points and concentrations. For biofilm formation, the 24 and 1 h incubations were the least effective, particularly at low extract concentrations for both strains.

3.2.5 *Synergistic effects of phenolic compounds with supina extracts combined with phenols on remediating* C. albicans *(A72 and SC5314)*

Herbal efficacy is often described as being due to the chemically diverse components present in the natural system, which are distinct in concentrations and constituents. Thus, these components may act synergistically or additively to contribute to such a positive effect (Junio et al., 2011). The literature has demonstrated that mixtures of phytochemicals have a high capacity of acting as synergists rather than in isolation (Spelman et al., 2006; Wagner, 2009). Therefore, in this study, supina grass extracts were combined with ferulic (F) and coumaric (C) acids because they are the main phenolics identified in this system. Five concentrations of supina extract (0.72, 7.2, 72, 720, and 7200 ng/g) were combined with four concentrations of F and C (0.03, 0.06, 0.13, and 0.25 mM) that showed effectiveness in reducing adhesion and biofilm formation either as isolated components (Chapter 1) or when combined with another phenol (Chapter 2). The 6 h response time was studied because it shows the maximum remediation effect in most of the cases.

3.2.4a *Synergistic remediation of* C. albicans *(A72) celluar adhesion:* When supina grass extracts were prepared at the different concentrations used previously were combined with the most predominant phenols present in the extraction (F and C),

significant differences on the remediation effect occurred (P>0.05) (Figure 3.10). The percent remediation ranged from 66.69 % (7200 ng/g /0.03 mM) to 72.47 % (7.2 ng/g/0.03 mM) for supina grass - C and from 46.76 % (7200 ng/g /0.03 mM) to 56.23 % (7.2 ng/g/0.03 mM) supina grass – F. Upon increasing the concentration of phenols C and F to 0.06, the two compounds started to trend the same at the low concentrations of the supina grass (0.72 and 7.2 ng/g).

Increasing the concentration of phenols to 0.13 mM in the mixture of supina grass (7.2, 720, and 7200 ng/g) resulted in percent remediations of 52.77, 48.49, 50.92 supina grass-F to 71.66, 65.55, 68.15% supina grass-C respectively. However, the addition of supina grass extract at small concentrations exerted high % remediation > 70 % in comparison to high concentrations of extracts (720, 7200 ng/g), which exhibited < 70 % remediation for supina grass-C, and > 50% for supina grass-F. These results demonstrated the strongest potential of C on disrupting the cytoplasmic membrane of cells causing ion leakage and proton influx, and potentially reducing cell viability (Campose et al., 2009).

Most importantly, however, these results showed the synergistic interaction with supina grass and the phenols as most of combinations were able to statistically able to inhibit the cellular adhesion at greater levels than when the phenols are not present at the quantities cited. The FIC values also show that a synergistic effect is occurring between the extract and the phenol of interest, as all were below 0.5. Similarly, punicalagin is a small component identified from *Punica granatum* fruit peel extracts, which showed a synergic effect as antifungal agent against *C. albicans* (ATCC 10231) upon combination with fluconazole for 24 h with 0.25 FIC (Endo et al., 2011).

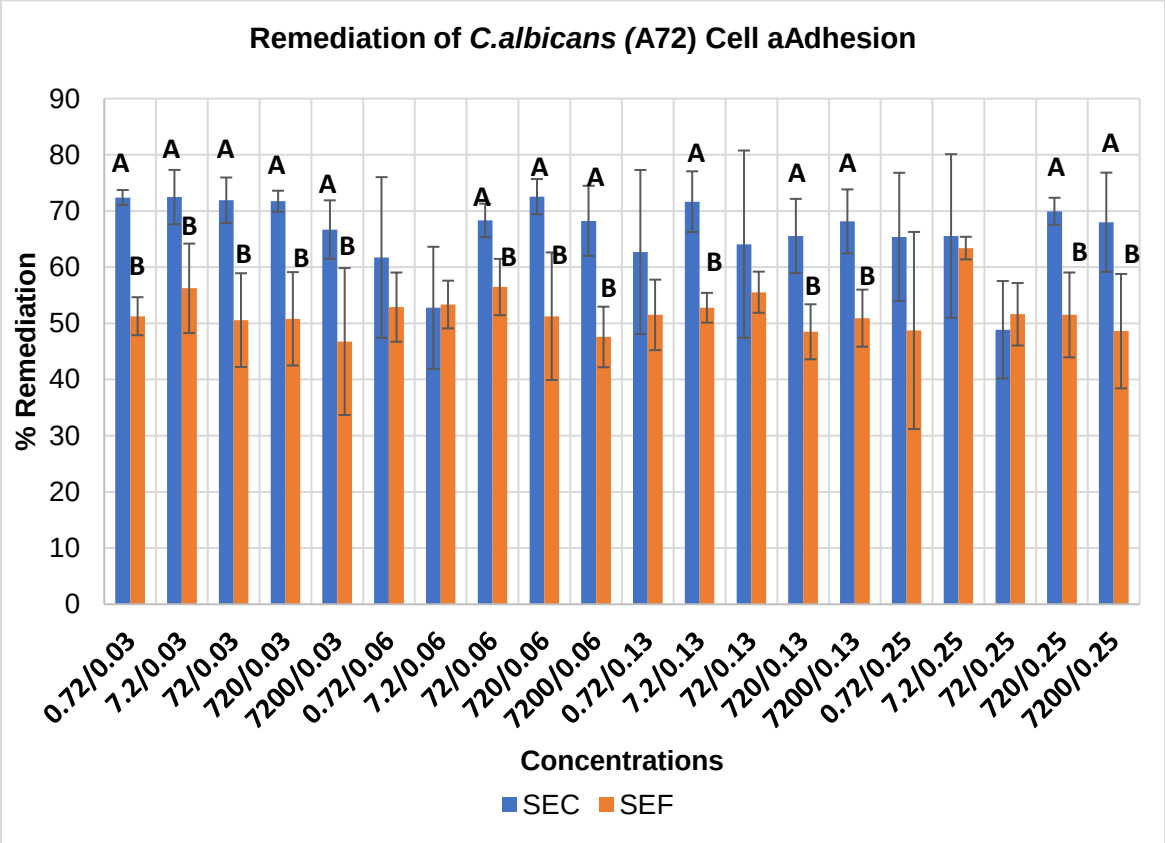

Figure 3.10: Effect of supina grass extracts combined with isolated ferulic and coumaric acid (SEC and SEF) on remediating *C. albicans* (A72) cellular adhesion 6 h post exposure at a given treatment concentration. Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within each compound having different letter are significantly different P<0.5.

3.2.4b *Remediating cellular adhesion of* C. albicans *(SC5314):* For *C. albicans* SC5314, the two compounds supina extract - C and supina extract-F were not statistically different among the concentrations screened (P>0.05). Supina extract - C and supina extract-F exhibit percent remediation > 70 %. However, the two compounds trended differently (P< 0.05) (Figure 3.11) upon combing 7.2 and 7200 ng/g of supina extract with 0.06 mM of C and F. The remediation effect ranged from 54.60 to 64.80 % (SEC) and from 66.69 % to 73.97 % (SEF) respectively. The bioactive compounds might be responsible for the high remediation effect against *C. albicans* SC5314, and again the synergistic effect exerted by the phenols, as evidenced by the FIC values, but F or C did not contribute more than the other in remediating cellular adhesion in *C. albicans* (SC5314) indicating again different mechanisms of action between the two strains, which thus have to be taken into consideration when developing a suitable treatment for these virulent effects.

3.2.4c. *Remediating biofilm formation of* C. albicans *(A72): C. albicans* A72 demonstrated similar remediation effects among compounds supina extract- C and supina extract-F extract at concentrations tested (Figure 3.12), which ranged from ~ 20 % to < 40% for supina extract- C, while remediation by supina extract-F ranged from 12% to > 50%. However, the biofilm remediation effect trended differently at some of the concentrations as 0.72 and 72 ng/g /0.03 mM exhibited ~ 19% (supina extract- C), > 50% (supina extract – F) >20 (supina extract-C), and ~ 45% (supina extract – F), respectively. The addition of 0.06 mM C and F to 72 and 7200 ng/g SE showed a low of ~ 23 to a high of 37.64 % when treated with supina extract- C and a low of ~ 9% to a high of 21.45% remediation effect when exposed to supina extract-F, respectively. As the concentrations

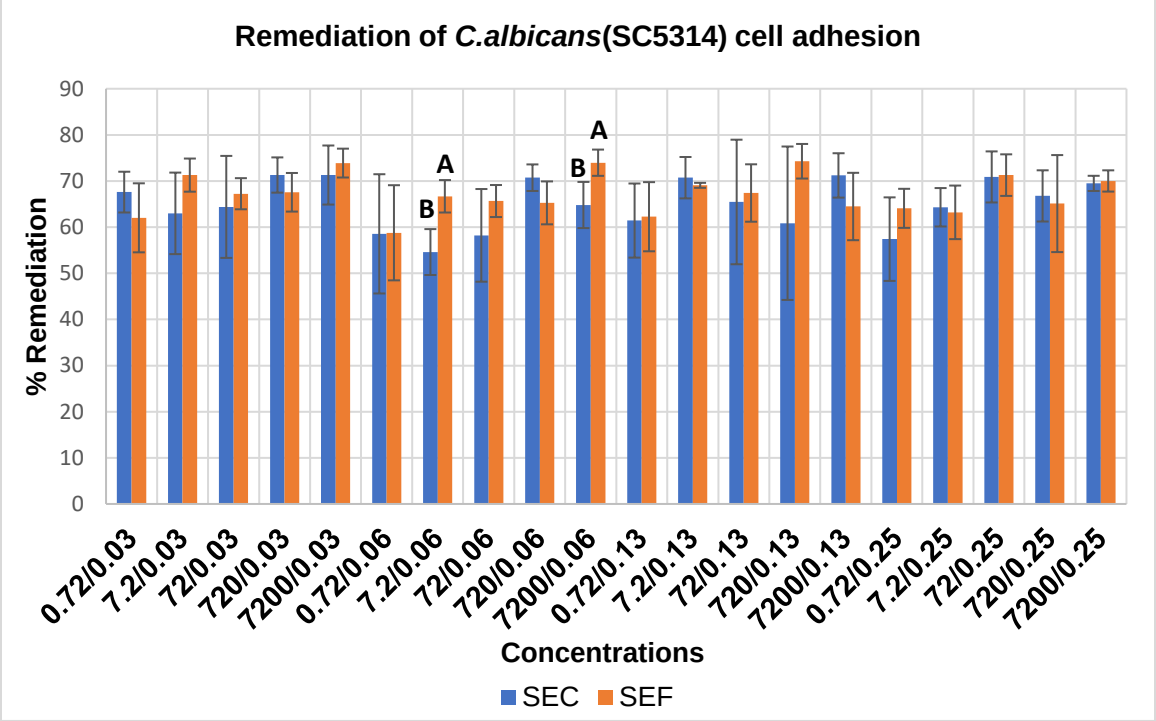

Figure 3.11: Effect of supina grass extracts combined with isolated ferulic and coumaric acids (SEC and SEF) on remediating *C. albicans* (SC5314) cellular adhesion 6 h post exposure at a given treatment concentration. Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within each compound having different letter are significantly different, P<0.05.

of supina extract- C and supina extract- F increased to 7200 ng/g and 0.13 mM, the remediation effect was significantly different as reduction was respectively 37.04% and ~6%.

In a study performed on demonstrating the antimicrobial activity of olive mill wastewater (OMW) in combination with phenolic compounds including ascorbic acid, tyrosol, protocatechuic acid, vanillic acid, caffeic acid, gallic acid, ferulic acid, and p-coumaric acid, results indicated that these combinations demonstrated complete reduction of gram-positive (*Streptococcus pyogenes and Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*); also, the extracts and phenolic compounds had synergic effect (Tafesh et al., 2011). In support of this data, a study completed using *Plantago major* extract and a combination of its two major compounds (aucubin and baicalein) demonstrated strong alleviation of *C. albicans* biofilm formation in dose-dependent manner (Shirley et al., 2015).

3.2.4d *Remediating biofilm formation of* C. albicans *(SC5314):* The remediation potential of supina extract - C and supina extract - F were statistically different in the case of *C. albicans* SC5314 ($P < 0.05$). Supina extract - C showed a high percent of remediation that ranged from ~ 29 % to > 50 % at the concentrations tested (Figure 3.13). On the other hand, supina extract - F exhibited low percent biofilm remediation ranged from ~ 9 % to ~ 26 %. These results again demonstrated the high efficacy induced by the addition of C to supina extract comparison to F.

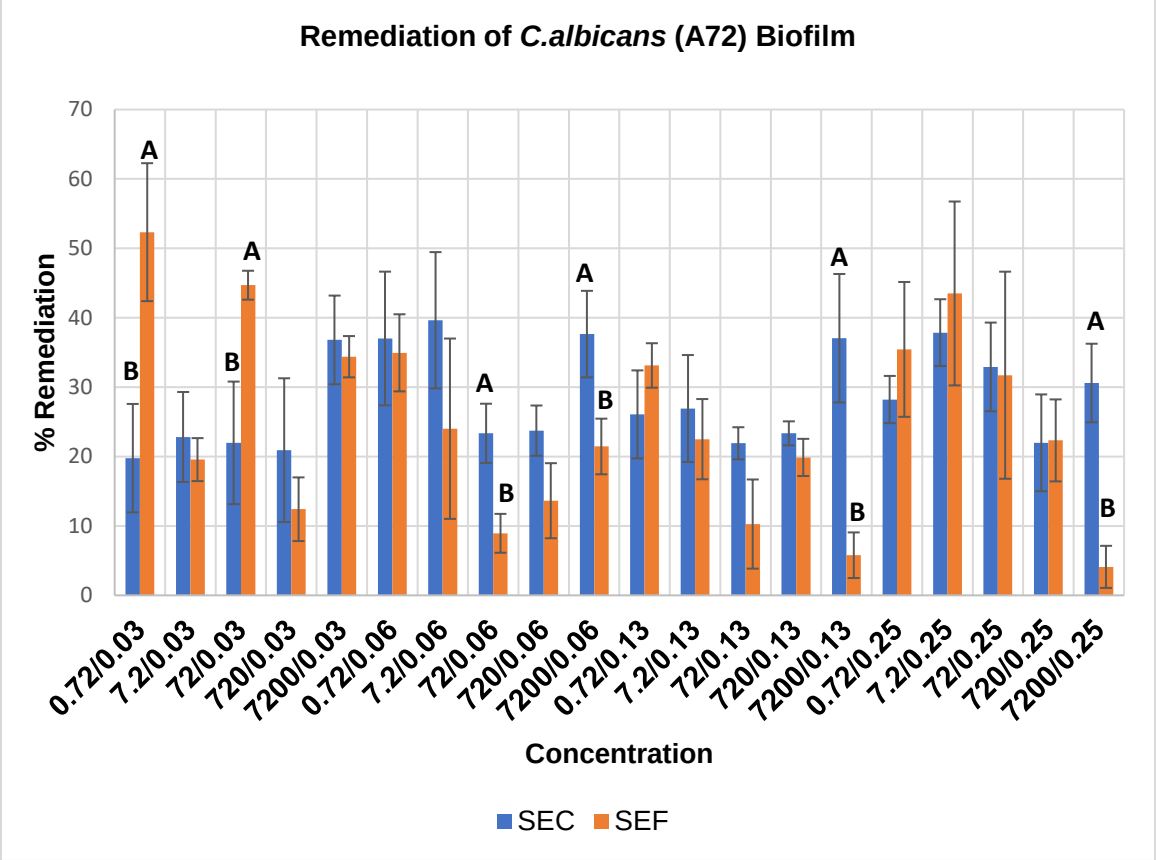

Figure 3.12: Effect of supina grass extracts combined with isolated ferulic and coumaric acids (SEC and SEF) on remediating *C. albicans* (A72) biofilm formation 6 h post exposure at a given treatment concentration. Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within each compound having different letter are significantly different, $P<0.05$.

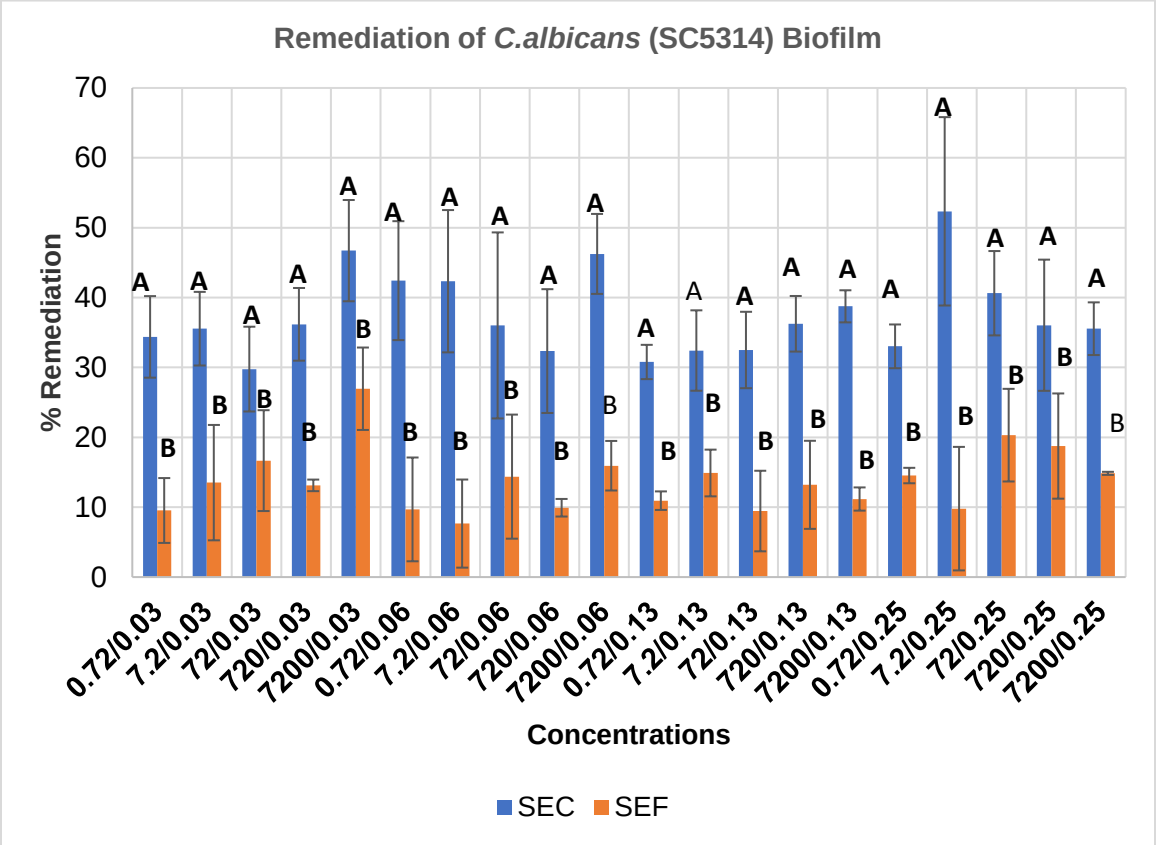

Figure 3.14: Effect of supina grass extracts combined with isolated ferulic and coumaric acids (SEC and SEF) on remediating *C. albicans* (SC5314) biofilm formation 6 h post exposure at a given treatment concentration. Each point and vertical bar represent the mean ± standards deviation of three replicates. The bars within each compound having different letter are significantly different, $P<0.05$.

3.5 Conclusions:

Supina grass extracts were the most effective on remediating the two strains of *C. albicans,* SC5314 and A72, cellular adhesion and biofilm formation at an incubation time of 3 and 6 h. However, upon combining the extracts with C or extracts with F, the remediation effect of cellular adhesion and biofilm formation was higher than 50 % in comparison to biofilm formation remediation (< 50 %), which indicates that the phenols and extracts were acting synergistically, as also supported by the FIC <0.5. Therefore, the significance of this study is that a sustainable co-product stream could be used as a source of anti-fungal agents with phenol synergists most likely playing an important role. Moreover, as complex matrix, supina grass-enriched phenols or phenols extracted from this natural system may be more effective in targeting *C. albicans* virulence phenotypes and also acting on multi-targets thereby aiding in preventing resistance to a potentially novel anti-fungal agent.

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Summary

The overall objective of this study was to screen the ability of several selected isolated phenolic compounds to remediate virulence factors: cellular adhesion and biofilm formation of two strains of *C. albicans* (A72 and SC5314), using synthetic surfaces; to study the synergistic effect of a combination of phenolic compounds targeting *C. albicans* adhesion and biofilm virulence factors; and to determine the potential synergistic interplay of the phenols within a complex matrix, using supina turf grass extracts to remediate *C. albicans* virulence.

Several isolated phenolic compounds were screened for their ability to remediate *C. albicans* (SC5134 and A72) 6 h post cellular adhesion. Among the 7 phenolic compounds and 2 non-phenolic compounds tested only catechin (CAT), chlorophyll (CH) and farnesol (FA), were able to reduce A72 cellular adhesion. At the low concentration range (0.5- 0.06), results were not statistically different, indicating that the three compounds could be used at these low concentrations but the efficacy of each would not be consistent. CAT exhibited a % remediation of > 50% at 2.00 mM. However, these compounds were not statistically different among concentrations.

Unlike A72, the percent remediation of *C. albicans* (SC5314) cellular adhesion was not significantly different among these compounds at the low concentrations 0.06-0.25 mM, but CAT did vary at 0.05 and 1.00 mM compared to CH and FA at these concentrations, but then all three compounds were similar at the high concentrations of 2.00-4.00 mM. Obviously, the compounds act upon the two strains differently. The mid-concentrations (1.00 and 2.00 mM) were more effective in reference to *C. albicans* SC5314, strain while the higher concentrations were the most efficacious on remediating the cellular adhesion of *C. albicans,* A72.

On the other hand, the isolated compounds (G, F, S, E, Q, C, CAT, CH and FA) were able to remediate *C. albicans* A72 biofilm formation. The phenol Q trended differently from the other phenolic compounds at >1.00 mM. At concentrations > 1.00 mM, Q, CAT, CH and FA started to trend differently from the other molecules in most cases with the exception of 4.00 mM. It must be noted that FA, CAT, and CH were the least effective at remediating *C. albicans* A72 biofilm. These results most likely are due to the structure-function effect of the compounds and thereby their mode of interaction with the *C. albicans* biofilm formation.

Again, the compounds G, F, S, E, Q, C, CAT, CH and FA were able to reduce the formation of the corresponding biofilm in the case of *C. albicans,* strain SC5314 at low concentrations (0.5-0.06 mM). Then, the compounds Q, CAT, CH, and FA, started to trend differently at high concentrations > 1.00 mM. The compounds G, F, S, and E were effective on remediating biofilm formation of SC5314 by > 50 %.

The same 7 phenols (G, F, S, E, C, Q, CAT) and 2 non-phenols (CH and FA) used in isolation (Chapter 1) were screened for their potential as synergists to remediate *C. albicans* (SC5314 and A72) 6 h post cellular adhesion and biofilm formation. Each of the combinations (G-F, S-Q, F-E, E-C, CAT-Q, and CAT-C) were equally capable of detaching the bound cells despite compound structural differences and the compound combination in the case of *C. albicans* A72. However, only the CAT-Q treatment resulted in a remediation of cellular adhesion at 0.50 mM. The compound CAT- Q was optimal on remediating cellular adhesion of A72 (30-82 %).

Again, the 6 treatment groups were able remediate cellular adhesion of SC5314 at levels greater than 50% when exposed to the lower concentrations tested (0.03 to 0.25 mM). Only S-Q exerted a % remediation of 52 +/- 18 % AT 0.5 mM. Similar to A72, CAT-Q provided the optimal effect (70 +/- 2 %) 0.03 mM but was also significantly similar

for the 0.12 mM (58 +/- 14%), and 0.25 mM (65 +/- 5%) 0.25 mM treatments. These combinations were acting synergically to remediate cellular adhesion of SC5314 and A72 with FIC < 0.5.

The same combinations used for the adhesion reduced biofilm remediation ~ > 30%. The highest reduction occurred for G-F at 0.03 mM, which was statistically different from the other compounds exhibiting % remediation of 43% +/- 2 % (G-F), while the other combinations S-Q, F-E, E-C, CAT-Q, and CAT-CH ranged from 30-40%. For concentration 0.06, 0.12, and 0.12 mM, biofilm formation was reduced by 25-37%, and 25-50%, respectively. In terms of the 0.25 mM concentration, the highest inhibition occurred among the treatment for CAT-CH, which was 37 +/- 7%. The combination S-Q was again the only combination to reduce biofilm formation at 0.5 mM (30-40%), at which the other treatments showed no inhibition.

In the case of SC5314, CAT-C exposure resulted in high percent of 38.03 +/- 4% remediation among the treatments at 0.03 mM (low remediation was achieved by FE and EC at 0.03 mM, 19.54 +/- 2 % and 15.49 +/- 2 % at 0.03 mM, respectively). The phenolic treatment, E-C trended differently at 0.25 mM than CAT-C and F-E, whereas the later combinations were statistically similar. Percent remediation was ~ 40 ± 7% with CAT-C, while the remediation effect decreased to 34.8 ± 1% for F-E and 12.87 ± 4% for E-C at 0.25 mM. The data indicated that these compounds act at least partially synergistically as the value of FIC was 0.5<FIC >1. These results are significant in the development of potential antifungal drugs to remediate *C. albicans* SC5314 and A72 virulence factors including cellular adhesion and biofilm formation. However, the mechanism of action needs to be further investigated to provide clear understanding.

Supina grass extracts were characterized in term of phenolic and flavonoid content, with total phenolic content 0.96 ± 0.05 mg/g and total flavonoid content 0.49 ±

0.01 mg/g, respectively. HPLC profile showed that supina extracts contain caffeic acid 2.3 ± 3.32 mg /g coumaric acid 1.59.19 ± 9.2 mg / g, and ferulic acid, 2.86 ± 9.43 mg/ g. Supina extracts were effective at remediating cellular adhesion of A72 and SC5314 at 3 and 6 h by > 40% at concentrations tested (0.72- 7200 ng/g). On the other hand, remediation of biofilm formation of the two strains of *C. albicans* was lower (< 40 %) at the same time points. Upon combining the extracts with ferulic or coumaric acids, the remediation effect ranged from 50 to > 70% for remediating cellular adhesion for both strains, and the result indicated that they act synergically (FIC <0.5). however, remediation of biofilm formation for both strains was < 40 % comparison to cellular adhesion for both strains.

Therefore, this project generates a foundation to determine the feasibility of obtaining `synergistic *C. albicans* anti-fungal agents that act upon virulent targets from a readily available agricultural stream (in the short term), which then is expected to facilitate the development of efficacious anti-fungal treatments capable of preventing potentially life-threatening *C. albicans* infections (in the long term). Such knowledge provides alternative marketing opportunities for a sustainable but highly under-utilized co-product stream that is present throughout the world, but typically disposed of.

This project could be extended by studying the synergic effect of phenolic compounds on preventing cellular adhesion and biofilm formation of *C. albicans* using additional strains or different phenolics. Also, different turf grass might be used to the project. The mechanism of action of these phenolic compounds need to be further investigated.