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Effect of Plant-Derived Molecules on Acinetobacter baumannii Biofilm on Abiotic Surfaces

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Honors Thesis

"Effect of Plant-Derived Molecules on *Acinetobacter baumannii* Biofilm on Abiotic Surfaces"

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ABSTRACT

The emergence of multiple drug resistant (MDR) *Acinetobacter baumannii* has lead to an increased interest in finding alternative antimicrobial compounds for controlling the pathogen. The present study investigated the effect of three plant-derived antimicrobials, namely eugenol (EUG), carvacrol (CAR), and thymol (THY), on *A. baumannii* biofilms. Using concentrations equal to or greater than the minimum bactericidal concentration (MBC) of each compound, the ability of EUG, CAR, and THY to inactivate the mature biofilms of three strains of MDR *A. baumannii* on polystyrene microtiter plates and stainless steel coupons was examined. All three plant-derived compounds significantly inactivated the bioflims of all three *A. baumannii* strains tested ($P < 0.05$). After 10 min of incubation on polystyrene microtiter plates at 23°C, all three compounds eliminated all viable biofilm-associated *A. baumannii* cells, reducing the biofilm-associated bacterial population by >7.0 log CFU/ml. Eugenol and CAR exerted a similar effect at 37°C, while THY reduced the viable biofilm-associated bacterial population to 0.45 log CFU/ml. On stainless steel coupons incubated at 23°C, all three compounds reduced the viable biofilm-associated bacterial population by 6.5 log CFU/ml. Results indicate that EUG, CAR, and THY could potentially be utilized to control biofilms of *A. baumannii* on abiotic surfaces, thus preventing its persistence in the hospital environment. However, detailed follow up studies are needed.

INTRODUCTION

Acinetobacter baumannii is a Gram-negative, nosocomial pathogen (58). It has been documented as the causative agent in cases of ventilator-associated pneumonia, wound infection, urinary tract infection, and catheter-associated septicemia (5, 25). *A. baumannii* has also been implicated in cases of endocarditis, osteomyelitis, and corneal perforation (66). In recent years, *A. baumannii* has emerged as a prevalent nosocomial pathogen in military field hospitals in Iraq and Afghanistan (57).

The emergence of multiple drug resistant (MDR) strains of *A. baumannii* constitutes a significant public health threat. *A. baumannii* strains resistant to major classes of traditional antibiotics including β-lactams, aminoglycosides, quinolones, tetracyclines and glycylcyclines, polymyxins, trimethoprim-sulfamethoxazole, and chloramphenicol have been isolated (58). A wide variety of mechanisms of antibiotic resistance have been identified in *A. baumannii*, including drug-inactivating enzymes, efflux pumps, alteration in outer membrane proteins (OMPs) and binding sites, and mechanisms of ribosomal protection (28, 58). The ability of *A. baumannii* to rapidly acquire foreign genetic elements has contributed to the development of multiple drug resistance in the bacterium (17, 31). The acquisition of mobile genetic elements such as plasmids (18), transposons (70), and integrons (2, 18, 69, 71) has been reported.

The ability of *A. baumannii* to form biofilms also contributes to its pathogenesis. Biofilm formation contributes to increased persistence in the hospital environment (58). *A. baumannii* has been shown to survive for up to 26.5 days on desiccated abiotic surfaces (38). It has been isolated from hospital suctioning equipment, washbasins, bed rails, bedside tables, ventilators,

sinks, pillows, mattresses, and resuscitation equipment (65). A comprehensive literature review conducted by Moultrie et al. (51) found that 73% of studies evaluated identified the environment as the source of *A.baumannii* infection. *A. baumannii* strains which form substantial biofilms have also been found to have greater resistance to both antibiotics (43, 64, 78) and disinfectants (70).

Plant-derived essential oils are traditionally used as food additives, both to preserve foods and enhance flavor (39). Components of several such essential oils include eugenol (EUG) and trans-cinnamaldehyde found in clove and cinnamon leaf oils, respectively (23). Similarly, carvacrol (CAR) and thymol (THY) are found in the oil from oregano (13) and thyme (74). Previous studies have demonstrated the antimicrobial properties of several plant-derived compounds. Eugenol, CAR, and THY have been shown to exert antimicrobial effects against the Gram-negative bacteria *Salmanella* Enteritidis and *Campylobacter jejuni* (31). These plant-derived compounds have also been shown to be effective against biofilm-associated bacterial populations in particular. Carvacrol and THY have demonstrated the ability to inactivate the biofilms of *Staphylococcus aureus* and *Staphylococcus epidermidis* (56), while EUG and CAR have been shown to inactivate biofilms of *Listeria monocytogenes* and *Escherichia coli* O157: H7 (59). However, no studies to our knowledge have been conducted to investigate the antibacterial effects of these compounds on MDR *A. baumannii*. Therefore, the current study was conducted to investigate the ability of EUG, CAR, and THY to inactivate mature *A. baumannii* biofilms for potential use as disinfectants to reduce the persistence of *A. baumannii* on abiotic surfaces.

LITERATURE REVIEW

History

The current genus description and name *Acinetobacter* were first used in 1954 to differentiate between motile and non-motile organisms of the genus *Achromobacter* (10, 58). The designation was accepted more widely following the work of Baumann et al. in 1968 (4, 58). *A. baumannii* was first identified through DNA-DNA hybridizations studies conducted by Bouvet and Crimont in 1986 (9).

Biology

Acinetobacter spp. are gram-negative, aerobic, catalase-positive, oxidase-negative, nonmotile, nonfermenting coccobacilli (58) which typically grow at 20-30°C on traditional laboratory media (27). They are short gram-negative rods that are difficult to destain, leading to possible misidentification as gram-negative or gram-positive cocci (58). Attempts to phenotypically differentiate *A. baumannii* from other *Acinetobacter spp.* have proven ineffective (31, 58). *Acinetobacter* genomic species 13TU, *Acinetobacter* genomic species 3, *Acinetobacter calcoaceticus,* and *A. baumannii* are often referred to as either the *A. calcoaceticus-A. baumannii* (ACB) complex or the *A. baumannii-A. calcoaceticus* (ABC) complex (57). Molecular methods of differentiating these species are labor-intensive, expensive, and largely conducted only by a few reference laboratories (58). The most accepted of these methods are DNA-DNA hybridization (9), amplified 16S rRNA gene restriction analysis (ARDRA) (77), and high-resolution fingerprint analysis by amplified fragment length polymorphism (AFLP) (37).

Role as a Nosocomial Pathogen

A. baumannii poses a significant threat as a nosocomial pathogen. In survey studies from 75 countries, *A. baumannii* caused approximately 9% of ICU infections (81). It has been implicated as the causative agent in cases of ventilator-associated pneumonia, wound infection, urinary tract infection, and catheter-associated septicemia (5, 25). Less frequently, infections have also resulted in meningitis, endocarditis, osteomyelitis, and corneal perforation (66). Current data indicate that critically ill patients with ventilator-associated pneumonia and bloodstream infections caused by *A. baumannii* experience increased mortality and prolonged lengths of stay in the hospital (38).

Once present, *A. baumannii* is capable of persisting in the hospital environment for extended periods. It has been isolated from hospital suctioning equipment, washbasins, bed rails, bedside tables, ventilators, sinks, pillows, mattresses, and resuscitation equipment (65). Persistent environmental contamination in the rooms of patients suffering *Acinetobacter spp.* infections has been documented up to 13 days after discharge from the hospital (5). Jawad et al. (38) showed that 22 strains of *A. baumannii* isolated from 8 hospital outbreaks in the German metropolitan area were capable of surviving an average of 26.5 days on a desiccated abiotic surface, namely glass cover slips. The hospital environment has been demonstrated as a mode of transmission. A comprehensive literature review conducted by Moultrie et al. (51) found that 73% of studies evaluated identified the environment as the source of *A.baumannii* infection. Hospital outbreaks have been traced back to various surfaces (5), including machinery used for respiratory therapy (14), countertops (51), bedding (82), and improperly autoclaved reusable needles (40). Martínez-Pellús et al. (49) identified environmental sources as frequent bacterial

reservoirs of *A. baumannii* in a Spanish ICU. The study also reported frequent patient colonization, as 35% had skin colonization, 43% had tracheal colonization, and 31% had rectal colonization during their stay. Patient colonization can also contribute to further dissemination of the pathogen through contact with healthcare providers. Morgan et al. (50) found that 20.5% of healthcare worker interactions with patients in 6 different ICUs resulted in the contamination of healthcare workers' gowns or gloves. MDR *A. baumannii* accounted for 32.9% of these contaminations, the most abundant pathogen detected during the study. Healthcare workers served as the source of infection in 36% of studies reviewed by Moultrie et al. (51). The presence of *A. baumannii* in air samples collected from hospitals has also been reported. A university hospital in Turkey identified 3 different genotypes of *A. baumannii* in air samples from various locations throughout the facility (1). The fact that 1 of these genotypes was also detected from clinical samples suggests that airborne transmission may contribute to infection.

A. baumannii in Modern Military Conflict

War wound infections by former *Achromobacter spp.* were first described as early as the Korean War (47). In recent years, *A. baumannii* has emerged as a prevalent nosocomial pathogen in military field hospitals in Iraq and Afghanistan (57). Approximately 14,000 American and British soldiers have been evacuated to medical facilities in their own countries following wounds in Afghanistan and Iraq since 2001 (57). Consequently, the prevalence of *A. baumannii* in domestic military medical facilities has increased as well. The United States Centers for Disease Control and Prevention identified 102 cases of *A. baumannii* bloodstream infections in 5 American military field medical facilities in Iraq and Afghanistan during the period from 2002 to 2004, compared to 3 during the period from 2000 to 2002 (15, 57). Peterson

et al. found that *Acinetobacter spp.* accounted for 36% of wound and blood stream infections in trauma victims evacuated from Iraq to the naval hospital ship USNS Comfort during a study period in 2003 (62). In a study of *Acinetobacter spp.* infection at the National Naval Medical Center in Bethesda, MD, *A. baumannii* was isolated in 96% of cases (60).

Acinetobacter spp. are ubiquitous in the environment, commonly isolated from the soil, water, and the skin and gastrointestinal tracts of healthy individuals (3, 28, 57). However, *A. baumannii* has not been isolated from these environmental sources as frequently. Berlau et al. (6) reported that, although 40% of healthy individuals had skin colonized with *Acinetobacter spp.*, only 1 of 192 individuals was found to have skin colonized with *A. baumannii*. Dijkshoorn et al. (20) isolated *A. baumannii* from fecal samples in only 1% of healthy individuals examined in the UK, and only 0.7% of healthy individuals examined in the Netherlands. In the case of military personal in Iraq and Afghanistan, infection directly from these sources is unlikely to be a significant factor (57, 60). Of 49 soil samples collected by Scott et al. (67) around 7 field hospitals in Kuwait and Iraq, only 1 contained ABC complex. Murray et al. (52) cultured samples from 61 open wounds in American military casualties immediately upon arrival at a field hospital in Baghdad, none of which were colonized with *A. baumannii,* and only 3 of which were colonized with any *Acinetobacter spp*. Current evidence suggests that non-U.S. personnel admitted to U.S. military field medical facilities have served as a reservoir for ABC complex (57). From November 2006 to October 2007, 97.5% of patients in an American Army combat hospital in Baghdad from which *A. baumannii* was isolated were Iraqi natives (33). From September 2007 to August 2008, 91% of patients positive for multi-drug resistant (MDR) Gram-negative bacteria at the largest U.S. military field hospital in Afghanistan were Afghan (72).

MDR *Acinetobacter spp.*, including *A. baumannii*, accounted for 20% of these samples, making it the second most common group of MDR Gram-negative bacteria isolated.

Emergence of Antibiotic Resistance

Emergence of MDR *A. baumannii* in recent years constitutes a significant public health threat. Definitions of multiple drug resistance vary across regions of the world and different epidemiological studies (58). Peleg et al. (58) defined multiple drug resistance as resistance to 3 or more of the following five drug classes: antipseudomonal cephalosporins, antipseudomonal carbapenems, ampicillin-sulbactam, fluoroquinolones, and aminoglycosides. Strains of *A. baumannii* have shown resistance to β-lactams, aminoglycosides, quinolones, tetracyclines and glycylcyclines, polymyxins, trimethoprim-sulfamethoxazole, and chloramphenicol (58). In a study of 102 *A. baumannii* strains isolated from infections in service members injured in Iraq, Kuwait, and Afghanistan, a significant amount of antimicrobial resistance was found, including resistance to all drugs tested found in 4% of isolates (15, 28). Jawad et al. (38) investigated the susceptibility of *A. baumannii* strains isolated from 8 hospital outbreaks to several antimicrobials. These susceptibilities were also compared to sporadic strains. They found that outbreak strains were significantly more resistant to aminoglycosides, amoxicillin-clavulanate, mezlocdillin, cefepime, cetriaxone, cefotaxime, and ciproflaxacin than sporadic strains. Currently utilized antimicrobial treatments for MDR *A. baumannii* include the aminoglycosides amikacin and tobramycin, polymixin E (colistin) and polymixin B, tigecycline, the tetracyclines minocycline and doxycycline, and a combination of the β-lactamase inhibitor sulbactam and the β-lactam ampicillin (26). A combination therapy including multiple antimicrobials is usually required to successfully treat MDR *A. baumannii* infections (5).

Mechanisms of Antibiotic Resistance

A. baumannii has acquired a wide variety of antibiotic resistance mechanisms, including drug-inactivating enzymes, efflux pumps, alteration in outer membrane proteins (OMPs) and binding sites, and mechanisms of ribosomal protection (28, 58). It produces a wide variety of β-lactamases, both chromosomally and plasmid mediated (7), including chromosomally encoded AmpC cephalosporinases, referred to as *Acinetobacter*-derived cephalosporinases (ADCs) (8, 58) and both narrow-spectrum β-lactamases (NSBLs) (79) and extended-spectrum β-lactamases (ESBLs) (54, 62). Carbapenemases, including serine oxacillinases (OXAs) and metallo-β-lactamases (MBLs) (63) pose particular difficulty to treatment (59). Multidrug efflux pumps, such as AdeABC and AbeM (48, 58, 70), function against β-lactams, aminoglycosides, quinolones, and tetracyclines and glycyclines (79). Tetracycline-specific efflux pumps have also been identified (33). Alterations in many OMPs have been associated with increased resistance to β-lactams (58). The decreased expression or loss of such OMPs as at 22kDa (7), 29 kDa (CarO) (46, 54), and 33-36 kDA (17) have been implicated in carbapenem reistance. Resistance to quinolones has been attributed to mutations in *gyrA* and *parC* leading to altered binding sites on topoisomerase IV and DNA gyrase (35, 80). Decreased affinity or expression of penecillin-binding proteins (PBPs) has further contributed to β-lactam resistance (31, 58). Ribosomal protection mechanisms mediated by *tetM* and *tetO* have been identified in tetracycline-reistant strains (18), as well as in strains with 16S rRNA methylation leading to aminoglycoside resistance (3).

The acquisition of foreign genetics by *A. baumannii* has contributed to the development of multi-drug resistance (18). *A. baumannii* is extremely capable of rapidly acquiring

antimicrobial-resistance genes (31) via mobile genetic elements including plasmids (18),

transposons (70), and integrons (2, 18, 69, 71). While examining the genetics of the 86-kb

resistance island AbaR1 found in an epidemic strain of *A. baumannii* isolated in France, Fournier

et al. (28) predicted that 82 of 88 open reading frames originated from other Gram-negative

bacteria, including *Pseudomonas spp., Salmonella spp., and E. coli*. (27, 58). Class I and class II

integrons have been associated with multiple-antibiotic resistance among nosocomial isolates (2,

18, 28, 70). Poirel et al. (63) found that 7 clonally related isolates of *A. baumannii* recovered

from Valenciennes Hospital in France contained a gene encoding the Ambler class A ESBL

VEB-1 on a class I integron previously identified only in *Pseudomonas aeruginosa* isolates

recovered in Southeast Asia. Although data regarding the natural competency of *A.baumannii* is

lacking (58), other *Acinetobacter spp.* have shown a high frequency of natural transformation,

including *Acinetobacter spp.* BD413 (58).

Biofilm Formation

One of the characteristics of *A. baumanii* which contributes to its extended survival in the

environment is its ability to form bioflims, or communities of intercommunicating bacterial cells

associated with a surface and encased in an extracellular matrix of carbohydrates, nucleic acids,

proteins, and other macromolecules (29, 72). Tomaras et al. (75) described the processes

involved in the surface attachment and biofilm formation on polystyrene and polypropylene

utilizing the strain 19606. After inoculating petri dishes and Teflon strips and incubating them at

room temperature for 10-15 minutes, they found a high level of adherence to these surfaces after

washing with tap water. Overnight incubation in Luria-Bertani (LB) broth at 37°C in both

polystyrene tubes and square petri dishes also resulted in adherence. They noted that the densest

cell aggregates formed at the liquid-air interface of the culture, and grew upward onto the walls of the containers. At this interface, channels in between stacks of cells were observed, which would allow for the acquisition of nutrients and removal of waste. Light microscopy of cells attached to the bottom surface of petri dishes revealed discrete cell clumps rather than a monolayer, even after several days of incubation. Areas without cells were also found within cellular aggregates, as has also been described in *P. aeruginosa* (43). Fluorescence microscopy also identified the presence of exopolysaccharides, present between cell stacks as well as covering the top later of cells above the broth.

A variety of factors contributing to biofilm formation in *A. baumannii* have been identified. Tomaras et al. (75) found that cells were linked through pili-like extracellular appendages. Genetic analysis of a mutant strain in the same study revealed that the absence of a protein very similar to that encoded by the *Vibrio parahemolyticus csuE* gene resulted in an inability to form surface pilli and a subsequent inability to form substantial cell clusters, characteristics which were restored upon reintroduction of the *csuE*-like gene. This pilli formation likely contributes to the initial steps of biofilm formation by allowing bacterial cells to adhere to abiotic surfaces and initiate the formation of microcolonies that continue to fully develop into bioflims. The *abal* autoinducer synthase gene *A. baumannii* has been shown to produce the signaling molecule acyl-homoserine lactone (AHL), a molecule which regulates quorum sensing (29). Niu et al. (55) reported that mutants of the M2 strain which lacked *abal* failed to produce AHL and had subsequent decreases in biofilm formation of 30-40%. Biofilm-forming capabilities were restored upon the addition of purified M2 AHL, indicating the importance of cellular communication to biofilm maturation in *A. baumannii*, as with other

prominent bacterial pathogens (29).   Loehfelm et al. (47) found that the absence of a homolog to

the 854 kDa staphylococcal biofilm-associated protein (Bap), identified in *A. baumannii* strain

307-029 and conserved across 98 strains, resulted in an inability to maintain volume and

thickness of mature biofilms, suggesting that Bap supports the structure of mature biofilms. Choi

et al. (16) determined that the cell-associated polysaccharide poly-beta-(1,6)-N-

acetylglucosamine (PNAG), regulated by the *pgaABCD* locus, contributed to the biofilm

phenotype of 30 clinical isolates of *A. baumannii*. Mutant strains with a *pga* locus deletion lost

PNAG production and experienced subsequent loss of the strong biofilm phenotype, which was

restored through complementation.

Biofilm formation can vary noticeably across different strains of *A. baumannii* (70).

When examining 2 different strains isolated from the same patient, one mucoid and highly

virulent in a mouse pneumonia model (AB-M) and the other non-mucoid and moderately

virulent in the same model (AB-NM), Kempf et al. (41) found that the AB-NM strain had cells

with rougher surfaces formed in larger clusters, while the AB-M strain had smooth cell surfaces

and formed smaller clusters. Calcofluor white staining showed that the AB-NM strain formed

more biofilm than the AB-M strain, resulting in 40 times more adherence to silicone catheter

material. Strong biofilm formation has been identified in clinical isolates associated with cather-

related urinary tract infections, wound infections, septicemia, and shunt-related meningitis (28,

70).

Biofilm Formation and Antimicrobial Resistance

Biofilm formation in *A. baumannii* has been correlated its to increased antimicrobial resistance (78). Vidal et al. (78) examined the effects of biofilm formation on the efficacy of sulbactam and imipenem. While both antibiotics were strongly bactericidal against young biofilms, the efficacy of sulbactam was significantly reduced in aged biofilms and the efficacy of imipenem was reduced to some extent. Rao et al. (64) found that biofilm-producing *A. baumanii* strains had comparatively higher resistance to amikacin, cephotaxime, ciproflaxacin, and aztreonam than non-biofilm-forming strains. Lee et al. (43) found that isolates which had the *blaPER-1* gene coding for the blaPER-1 ESBL had greater capacity for biofilm formation than strains which lacked the gene. Studying clinical strains of *A. baumannii* isolated from 2 Ohio hospitals, Srinivasan et al. (70) found that surface disinfectants, such as benzalkonium chloride, chlorhexidine gluconate, and Virkon-S were significantly less effective against strains which produced strong biofilms compared to those which produced weak biofilms.

Plant-Derived Antimicrobial Compounds

Plant-derived essential oils are a group of natural antimicrobials that are traditionally used as food additives, both to preserve foods and enhance flavor (39). This study utilized three such compounds with previously demonstrated antimicrobial properties, including eugenol (EUG), carvacrol (CAR), and thymol (THY). All these plant-derived molecules are classified as generally recognized as safe (GRAS) by the United States Food and Drug Administration (EUG: 21CFR582.60; CAR and THY: 21CFR172.515) (39).

Eugenol (4-Allyl-2-methoxyphenol) is a colorless liquid phenol with the chemical formula $C_{10}H_{12}O_2$ (22, 23). It is a component of clove oil, isolated via extraction in hydroxide solutions and subsequent distillation (23). Eugenol is currently used as a food flavoring, analgesic in dental medicine, and production of synthetic vanillin (23).

Carvacrol (5-Isopropyl-2-methylphenol) is a colorless to yellowish liquid phenol with the chemical formula $C_{10}H_{14}O$ (12, 13). It is a major component of the essential oils of various plants, chiefly oregano (13). It is also produced synthetically from p-cymene (13).

Thymol (2-Isopropyl-5-methylphenol) is a crystalline phenolic compound also of the chemical formula $C_{10}H_{14}O$ (73, 74). It is a major component of the essential oil of *Origanum*, like CAR, as well as thyme (74). It is also produced synthetically from p-cymene, piperitone, or m-cresol (74).

The antimicrobial effects of these three plant-derived molecules have been reported. Our laboratory previously investigated the antibacterial effects of EUG, CAR, and THY on 2 Gram-negative bacteria, namely *S.* Enteritidis and *C. jejuni*, in chicken cecal contents *in vitro* (31). Eugenol and CAR reduced *S.* Enteritidis by >5.0 $\log_{10}$ cfu/mL while THY, although the least effective of the 3 molecules, also showed a concentration-dependent reduction. All concentrations of CAR (10, 20, and 30 mM), the 2 higher concentrations of EUG (20 and 30 mM), and the highest concentration of THY (30 mM) reduced *C. jejuni* to undetectable levels 15 seconds after addition of the molecules. The antimicrobial effects of these compounds with particular focus on the inactivation of biofilms have also been reported. Nostro et al. (56) examined the effects of CAR and THY on biofilms produced by 6 strains of *S. aureus* and *S.*

epidermidis. At high concentrations, ranging from 0.125% to 0.5% depending on the strain, both molecules were capable of completely inactivating the biofilms of both bacteria. Pérez-Conesa et al. (59) investigated the effects of EUG and CAR (encapsulated in non-ionic surfactant solution) on biofilms formed by *L. monocytogenes* and *E. coli* O157: H7 on polycarbonate membranes. Although *E. coli* O157:H7 strains were more susceptible to both compounds than *L. monocytogenes* strains, inactivation of colony biofilm was observed in each. Three of the 4 *E. coli* O157:H7 strains had viable cell numbers decreased below detectable levels after 3 hours of treatment with the molecules. The effect on *L. monocytogenes* varied more across strains, and 2 of the 4 strains were more susceptible to CAR than EUG. However, both showed excellent potential for inactivation of biofilm produced by these two bacteria.

The availability of new, more effective methods to inactivate *A. baumanii* biofilms could significantly contribute to the control of this increasingly threatening nosocomial pathogen, reducing its persistence in hospital environments. The objective of this study was to investigate the efficacy of EUG, CAR, and THY in inactivating mature biofilms of *A. baumannii* at 23 and 37°C on polystyrene microtiter plates, as well as at 23°C on stainless steel coupons.

MATERIALS AND METHODS

Bacterial strains, media, and plant-derived molecules. Three strains of *A. baumannii* (173795B, 474030, and 260177B) were used for in this study. All strains of *A. baumannii* were kindly provided by Daryl Hoban, International Health Management Associates (IHMA), Schaumburg, IL, USA. All bacteriological media used were obtained from Difco (Difco, BD,

Sparks, MD). Each strain was cultured separately in 10 ml of sterile tryptic soy broth (TSB) in 30 ml screw-cap tubes at 37°C for 24 h with agitation (150 rpm). Following incubation, the cultures were sedimented by centrifugation (8,000 x g for 10 min), washed twice, and resuspended in 10 ml of sterile phosphate-buffered saline (PBS), pH 7.2. The bacterial population in each culture was determined by plating 0.1 ml portions of diluted culture on duplicate tryptic soy agar (TSA) plates and incubated at 37°C for 24 h. The efficacy of EUG, CAR, and THY (Sigma Chemical Co., St. Louis, MO) for inactivating *A. baumannii* biofilm was investigated on polystyrene microtiter plates and stainless steel coupons..

Inactivation of *A. baumannii* biofilm by EUG, CAR, and THY. EUG, CAR, and THY levels used in this experiment were equal to or greater than minimum bactericidal concentrations (MBCs) of the molecules, the minimum concentration at which *A. baumannii* is killed. The MBCs (EUG: 0.2%; CAR and THY: 0.075%) were selected based on preliminary experiments conducted in our laboratory.

Preparation of Media. Tryptic soy broth (pH 7.0) and TSA were prepared according to the manufacturer's directions. Glass beads (435 to 600 μm in diameter; Sigma-Aldrich) were added to PBS to facilitate removal of adherent *A. baumannii* cells from the surfaces of stainless steel coupons.

Inactivation of *A. baumannii* biofilm on microtiter plates. *A. baumannii* strains were grown to stationary phase in TSB at 37°C for 24 h. The cultures were separately centrifuged (8,000 x g for 10 min), resuspended in TSB. 200 μl of washed culture was used as inoculum (~6.0 log CFU). Sterile 96-well polystyrene tissue culture plates (Falcon, Franklin Lakes, NJ)

were inoculated with 200 µl of the cell suspension and allowed to incubate at 23 or 37°C for 24 h without agitation to facilitate biofilm formation. The antibiofilm effect of 0.2%, 0.5%, and 0.75% EUG, 0.075% and 0.15%, and 0.3% CAR or THY was tested with an exposure time of 0, 2, 5, and 10 min. The surviving bacterial population in the biofilm was enumerated by scraping and plating the biofilm on duplicate TSA plates. Triplicate samples were included for each treatment, and the experiment was replicated two times.

Inactivation of *A. baumannii* biofilm on stainless steel coupons. Stainless steel coupons (type 304; 5 by 2 cm; surface area, 20 cm^2) with no. 4 finish were used. *A. baumannii* strains separately grown to stationary phase in TSB at 37°C for 24 h were centrifuged (8,000 x g for 10 min), and cells were resuspended in 40 ml TSB (~6.0 log CFU). A volume of one ml of the resuspended culture (~6.0 log CFU) was used as the inoculum in 12-well cell culture plates (Falcon, Franklin Lakes, NJ). The coupons described before were deposited separately into wells containing inoculum and incubated at 23°C for 24 h without agitation for biofilm formation. The coupons were gently washed in 1 ml sterile PBS three times and resuspended in 1 ml of TSB. The antibiofilm effect of 0.2% and 0.75% EUG, and 0.075% and 0.3% CAR or THY was tested with an exposure time of 0, 2, 5, and 10 min. The coupons were then transferred to 50 ml centrifuge tubes containing 2 ml PBS and 1g glass beads. The tubes were vortexed for 5s. The PBS suspension was serially diluted in PBS, surface plated (0.1 ml) on TSA, and incubated at 37°C for 24 h. *A. baumannii* colonies formed on the plates were then enumerated. Triplicate samples were included for each treatment, and the experiment was replicated two times.

Statistical Analysis. Data from the four independent replicate experiments were pooled. The effect of different concentrations of EUG, CAR and THY, sampling time, temperature and their interaction on bacterial counts was analyzed using PROC MIXED of SAS (SAS Institute, Cary, NC, U.S.A.). Variation among replicates was used as the error term. Data were expressed as least squares means; differences were considered significant at $P < 0.05$.

RESULTS

Since EUG, CAR, and THY were found to be equally effective in inactivating all the three strains of *A. baumannii*, only the results obtained from 173795B strain are presented here. The efficacy of EUG, CAR and THY for inactivating fully formed *A. baumannii* biofilms on polystyrene microtiter plates at 23 and 37°C is depicted in Figures 1 to 6. The mean biofilm-associated population of control samples without any antimicrobial at 23 and 37°C were ~7.4 log CFU/ml and 7.7 log CFU/ml, respectively. At both 23 and 37°C, 0.75% EUG eliminated all viable cells in the biofilm after 10 min of incubation, as did 0.3% CAR and 0.3% THY after 10 min of incubation at 23°C. Since there was no difference in the antibiofilm effect of the molecules between the two temperatures at which *A. baumannii* biofilms were grown ($P < 0.05$), only the results obtained from *A. baumannii* biofilms grown at 23°C are described here. The antibiofilm effect of the molecules was both time-dependent and concentration-dependent ($P < 0.05$). The bacterial population in control samples did not change during the 10 min interval. Eugenol at 0.2%, 0.5%, and 0.75% reduced the biofilm-associated *A. baumannii* population to ~1.8 log CFU/ml, 0.3 log CFU/ml, and undetectable levels immediately after addition of the

molecule, respectively. After 2 min of incubation with the molecule, 0.2% EUG reduced the biofilm-associated *A. baumannii* population to ~0.9 log CFU/ml, while 0.5% and 0.75% EUG eliminated all viable *A. baumannii* cells. After 10 min of incubation with the molecule, 0.2% EUG had also eliminated all viable *A. baumannii* cells from the biofilm-associated population. Antibiofilm effects of CAR and THY were also both time and concentration-dependent ($P < 0.05$). However, only 0.3% CAR or THY eliminated all viable cells of *A. baumannii* biofilm. Carvacrol at 0.075% and 0.15% decreased the biofilm-associated *A. baumannii* population to ~1.8 and ~ 1.2 log CFU/ml after 10 min of incubation, respectively. Similarly, thymol at 0.075% and 0.15% reduced the biofilm-associated *A. baumannii* population to ~0.6 and 0.3 log CFU/ml after 10 min of incubation, respectively.

The effect of EUG, CAR, and THY for inactivating *A. baumannii* biofilms on stainless steel coupons incubated at 23°C is depicted in Figures 7 to 9. The antibiofilm effect of the compounds was not dependent on the time in which the sample was incubated with the molecule or their concentration ($P > 0.05$). The average initial bacterial population in the control samples was ~7.0 log CFU/ml. The treatment with all concentrations of EUG, CAR, and THY reduced the bacterial population to ~0.9 log CFU/ml immediately after the addition of the molecule.

DISCUSSION

The emergence of MDR *A. baumannii* constitutes a significant public health threat. The ability of the pathogen to resist dessication and form biofilms allows it to survive for long periods of time on abiotic surfaces (58). Subsequently, nosocomial infections resulting in

ventilator-associated pneumonia, wound infection, urinary tract infection, and catheter-associated septicemia have been reported (5, 25). Difficulty controlling MDR *A. baumannii* with traditional classes of antibiotics and disinfectants has increased interest in investigating novel antimicrobial compounds to which the pathogen is susceptible. The current study investigated the efficacy of plant-derived compounds in inactivating the mature biofilms of MDR *A. baumannii.*

Bacterial inactivation by EUG, CAR, and THY has been attributed to their hydrophobicity, which allows them to increase permeability through disrputing the structure of lipids in the bacterial cell and mitochondrial membranes (11). In addition, the hydroxyl group on EUG is thought to bind to proteins, preventing enzymatic action (81). CAR has also been shown to weaken the proton motive force, leading to a subsequent decrease in intracellular ATP (75).

In conclusion, the plant-derived molecules EUG, CAR, and THY were effective in inactivating mature *A. baumannii* biofilms on polystyrene microtiter plates and stainless steel coupons. These results suggest that EUG, CAR, and THY could be potentially used as antimicrobials to inactivate *A. baumannii* biofilms in hospital settings, preventing persistence, transmission, and subsequent infection of the pathogen. However, detailed follow up studies are needed.

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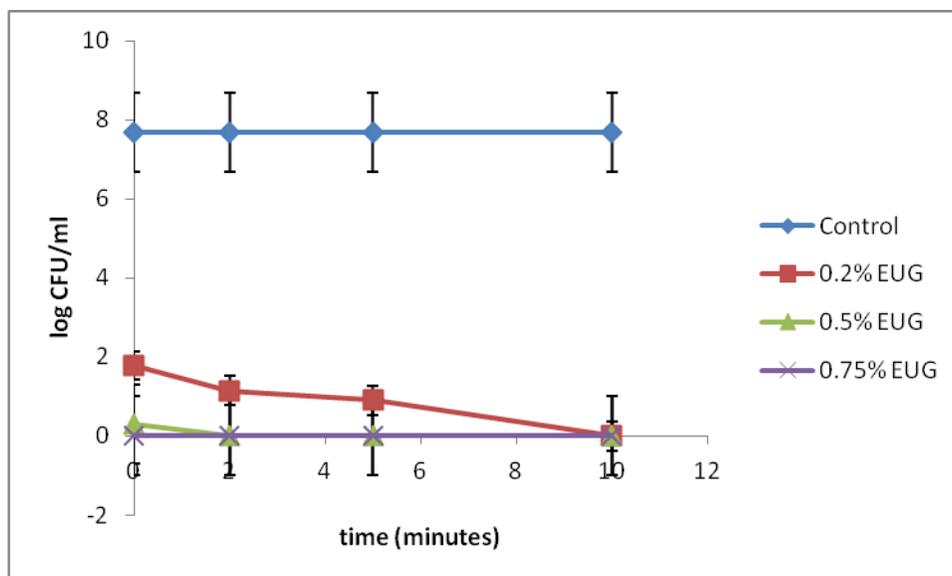
Baghdad, Iraq. Mil. Med. 171:821–825.

FIGURES

FIGURE 1. *Inacvitation of* A. baumannii *173795B biofilm by EUG on polystyrene microtiter plates at 23°C.*
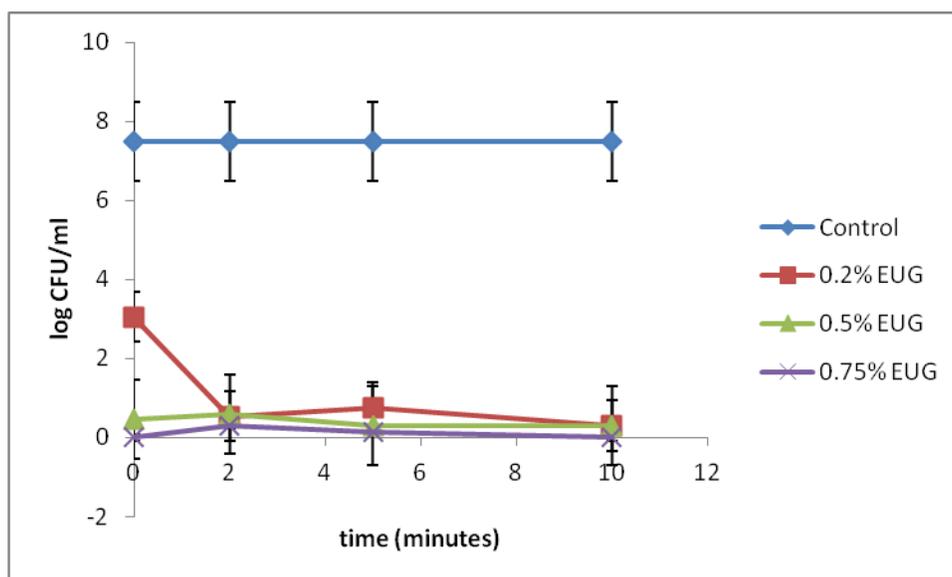

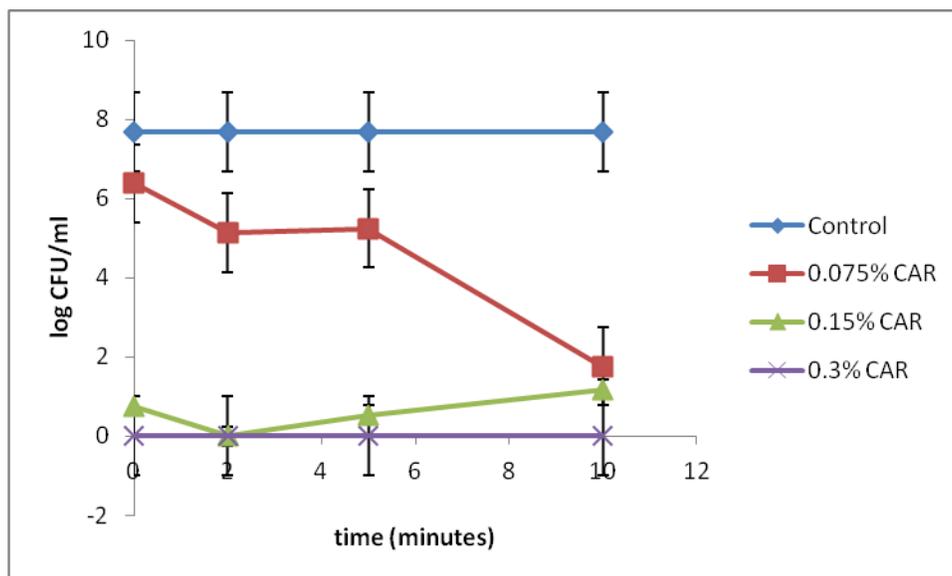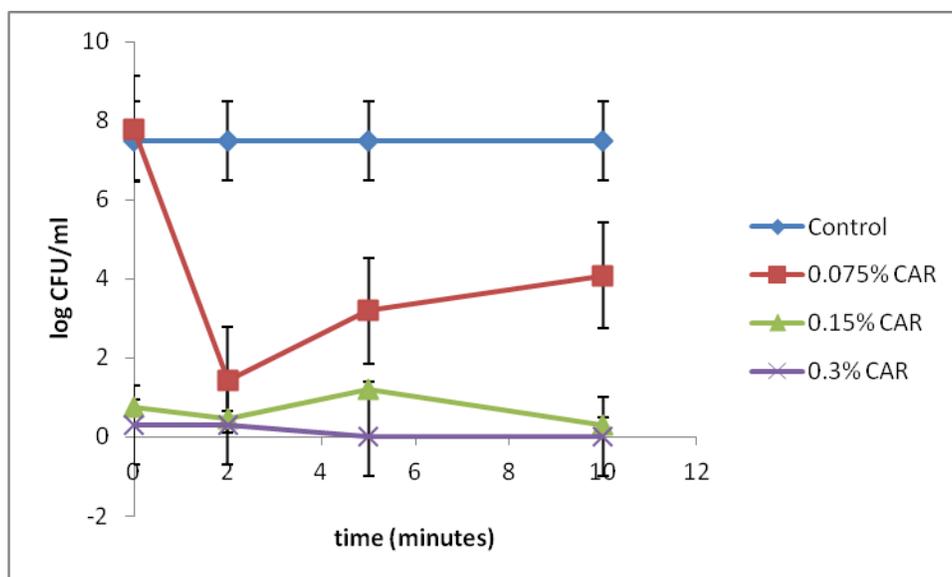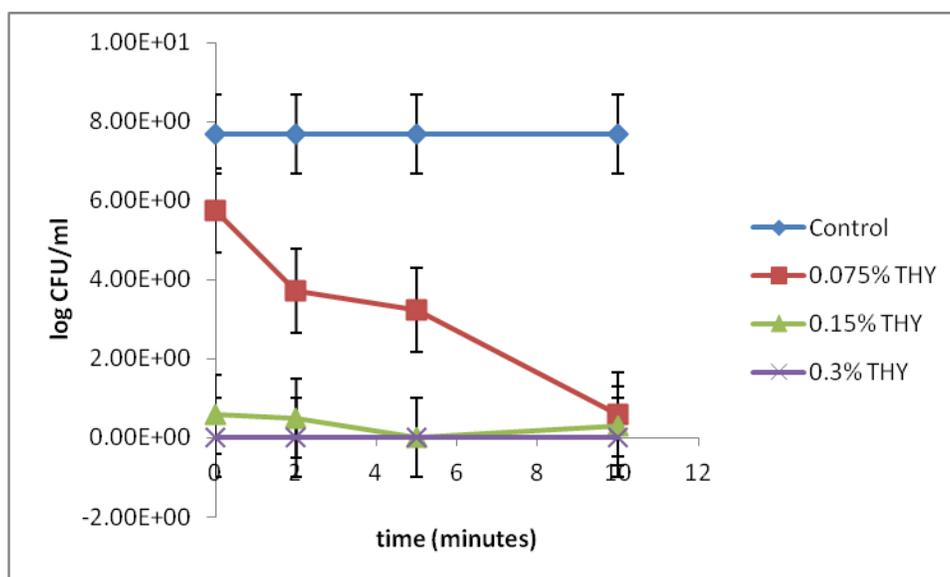FIGURE 2. *Inacvitation of* A. baumannii *173795B biofilm by EUG on polystyrene microtiter plates at 37°C.*

FIGURE 3. *Inacvitation of* A. baumannii *173795B biofilm by CAR on polystyrene microtiter plates at 23°C.*
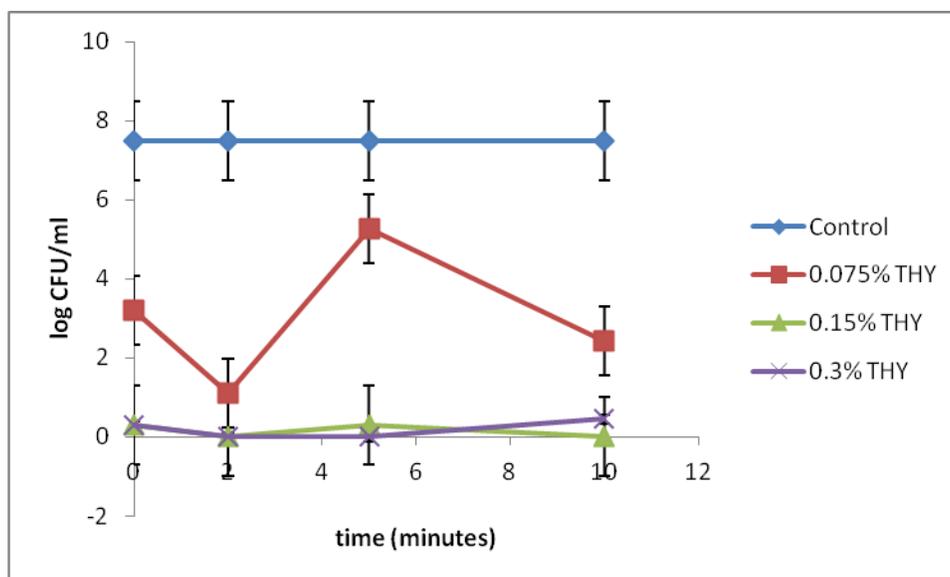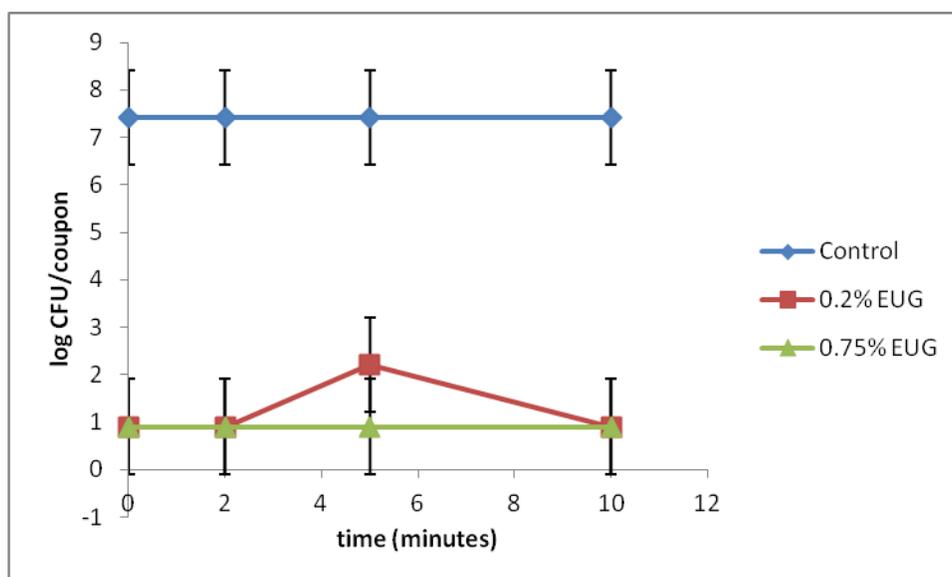

FIGURE 4. *Inacvitation of* A. baumannii *173795B biofilm by CAR on polystyrene microtiter plates at 37°C.*

FIGURE 5. *Inacvitation of* A. baumannii *173795B biofilm by THY on polystyrene microtiter plates at 23°C.*

FIGURE 6. *Inacvitation of* A. baumannii *173795B biofilm by THY on polystyrene microtiter plates at 37°C.*

FIGURE 7. *Inacvitation of* A. baumannii *173795B biofilm by EUG on stainless steel coupons at 23°C.*
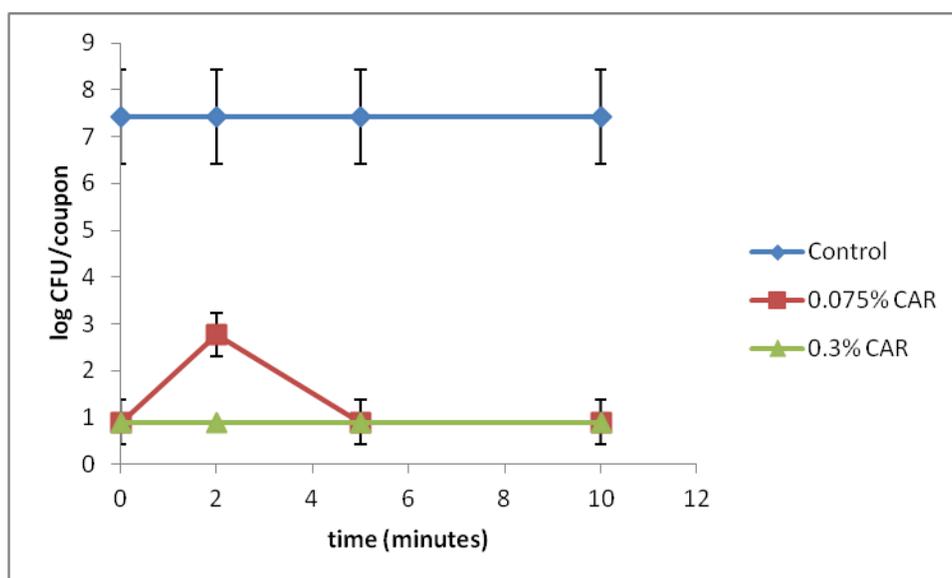

FIGURE 8. *Inactivation of* A. baumannii *173795B biofilm by CAR on stainless steel coupons at 23°C.*
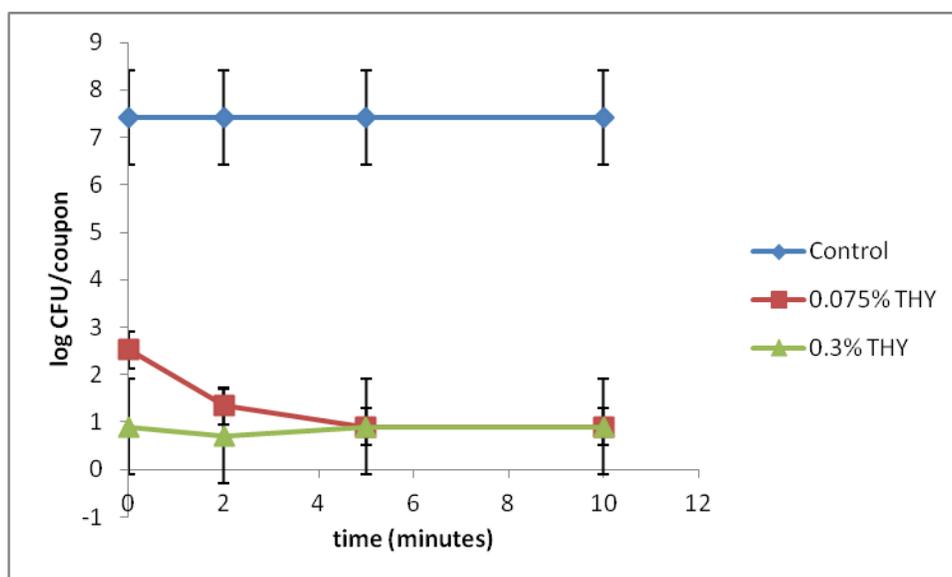
FIGURE 9. *Inactivation of* A. baumanii *173795B biofilm by THY on stainless steel coupons at 23°C.*