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Resolving the mechanism of bacterial inhibition by plant secondary metabolites employing a combination of whole-cell biosensors

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ABSTRACT

Tightening regulations regarding the use of biocides have stimulated interest in investigating alternatives to current antimicrobial strategies. Plant essential oils and their constituent compounds are promising candidates as novel antimicrobial agents because of their excellent ability in killing microbes while being non-toxic to humans at antimicrobially-active concentrations. Allyl isothiocyanate (AIT), carvacrol, cinnamaldehyde (CNAD), citral, and thymol were investigated for their antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The five compounds were screened via disc diffusion assay and broth microdilution method, by which inhibition zone diameters, minimum inhibitory concentrations (MICs), and minimum bactericidal concentrations (MBCs) were determined. AIT and CNAD displayed the greatest inhibitory effects against all species tested, with AIT yielding MICs of 156.25 mg/L and MBCs of 156.25 to 312.5 mg/L, and CNAD yielding MICs of 78.125 to 156.25 mg/L and MBCs of 78.125 to 312.5 mg/L. Based on these results, AIT and CNAD were selected for closer examination of their toxic effects. Two complementary bioluminescence-based bacterial biosensors, *E. coli* HB101_pUCD607_*lux* and *Acinetobacter baylyi* ADP1_*recA*_*lux*, were employed to examine the dose-response relationships and mechanism of action of AIT and CNAD. This is the first reported study to employ a *lux*-based biosensor assay coupled with parallel plate count experiments to demonstrate that AIT and CNAD not only damaged cell membranes, but also disrupted cellular metabolism and energy production in bacteria. It is also the first to use genotoxicity-sensing whole-cell bioreporters to demonstrate that neither AIT nor CNAD induced expression of the universal DNA repair gene, *recA*. This suggests that AIT and CNAD were not genotoxic. As an antimicrobial agent, it is advantageous that the compound be genetically non-damaging so that toxicity towards higher multicellular organisms and resistance development can be minimized. Thus, AIT and CNAD may be of high value as novel antimicrobial agents.

1. Introduction

Plant essential oils (EOs) have been used as antiseptic, medicinal, and food preservation agents for thousands of years (Jones, 1996). EOs and their constituent active compounds could potentially serve as effective alternatives or complements to conventional antimicrobial agents. It has been demonstrated that these plant-derived substances and their constituent chemicals display antibacterial (Ouattara et al., 1997; Prabuseenivasan et al., 2006), antimycotic (Kurita et al., 1981; Suhr and Nielsen, 2003), antiviral (Aruoma et al., 1996), anticancer (Sylvestre et al., 2006), and antiparasitic (Monzote et al., 2007) properties without conferring resistance in targeted microbes (Chao et al., 2008). Furthermore, they are generally safe and non-toxic to the environment since they occur abundantly in nature. Interest in employing natural compounds to combat microorganisms is gaining momentum in areas of alternative medicine (Chao et al., 2008; Sylvestre et al., 2006), pharmacology (Sherry et al., 2001), and food preservation (Burt, 2004; Ouattara et al., 1997).

In this study, two aldehyde terpenes, cinnamaldehyde (CNAD) and citral, and two phenolic terpenes, carvacrol and thymol, were selected as test compounds due to their reported effectiveness in inhibiting microbes (Gallucci et al., 2009; Inouye et al., 2001; Pei et al., 2009; Tunc et al., 2007). Terpenes are major constituents of EOs. Several studies have ranked aldehyde and phenolic terpenes highest in antimicrobial activity compared to other terpenoid classes (Dorman and Deans, 2000; Gallucci et al., 2009; Mayaud et al., 2008). Additionally, allyl isothiocyanate (AIT), a potent organosulfur of the essential oil of mustard, was also included in the panel of compounds to be tested. All five test compounds have been shown to inhibit a range of microorganisms in separate studies under varying experimental parameters (Ouattara et al., 1997; Palaniappan and Holley, 2010), but no work has been done in comparing their efficacy in a single study using the four bacterial species presently selected.

Biosensors are sensitive biological materials that interact with analytes to provide a quantifiable signal via a suitable transducer. Of particular interest in this study are whole-cell bacterial biosensors

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that assess acute toxicity of analytes using bioluminescence genes (*luxCDABE*) as the transducer (Close et al., 2012; Simpson et al., 1998). Although certain antimicrobial modes of attack by plant compounds have been proposed (Mayaud et al., 2008), the complex mechanism of action by such compounds has not been fully elucidated. Whole-cell assays reflect the multi-dimensional response to toxic stimuli launched by a host strain that is representative of target bacterial communities. Therefore, such assays can provide a dynamic perspective on the antibacterial mechanism of plant compounds.

In this study, two complementary bioluminescence-based bacterial biosensor systems were employed to measure the cytotoxic and genotoxic effects of AIT and CNAD. In the first system, *E. coli* HB101 transformed with the multi-copy pUCD607 plasmid containing the *luxCDABE* gene cassette from *Vibrio fischeri* (Belas et al., 1982; Tiensing et al., 2001) was used to detect impairment of cellular metabolism in bacteria. The *lux* bioreporter genes are constitutively expressed, resulting in the production of visible light, which is a direct reflection of the level of cellular respiration in the bioreporter cells (Close et al., 2012; Meighen, 1991). The overall reaction is summarized in Fig. 1. Since NADPH and ATP are needed for the regeneration of the aldehyde substrate required in the light-emitting reaction, the bioluminescence of the bioreporter cells is intrinsically tied to their metabolic activity. Thus, any injury to cellular metabolism caused by a test compound is reflected in the decrease of cellular light output, in which the degree of toxicity is directly proportional to the reduction in light intensity (Close et al., 2012; Kurvet et al., 2011; Simpson et al., 1998; Tiensing et al., 2001).

The second bioluminescence-based biosensor used in this study employed the strain *Acinetobacter baylyi* ADP1_*recA_lux*, in which *luxCDABE* genes cloned from *Photorhabdus luminescens* were chromosomally integrated into *recA* of *A. baylyi* ADP1(Warnke et al., 2009). *RecA*, an essential gene for DNA repair and the bacterial SOS response, is induced after DNA damage occurs. With the *lux* operon inserted under the *recA* promoter, *A. baylyi* ADP1_*recA_lux* cells are activated to produce light in the presence of DNA damaging toxicants, and the intensity of light emitted is directly proportional to the degree of *recA* expression.

To confirm the correlation between *recA* induction and the degree of bioluminescence of this biosensor, *recA* expression levels were monitored by quantifying the abundance of *recA* mRNA transcript in cells after exposure to test compounds. This was achieved by targeting and amplifying *recA* mRNA via reverse transcription polymerase chain reaction (RT-PCR), using RNA isolated from *A. baylyi* ADP1_*recA_lux* cells after various treatments as template. RT-PCR products can subsequently be quantified spectrophotometrically. This molecular-based validation study provided an added dimension in determining the degree of genotoxicity of CNAD and AIT.

The aims of this study were threefold. The first objective was to evaluate the antimicrobial activity of five different natural compounds against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*. The second aim was to employ two complementary *lux*-based whole-cell biosensors to elucidate the mechanism of the two most potent antibacterial compounds studied (AIT and CNAD). To our knowledge, this is the first study to combine two complementary bioluminescence-based bacterial biosensors to assess the cytotoxic and genotoxic effects of potential biocides and certainly plant secondary metabolites. Thirdly, the aim was to corroborate findings from the *A. baylyi* ADP1_*recA_lux* biosensor through transcriptional analysis of *recA* using RT-PCR.

2. Materials and methods

2.1. Chemicals and antimicrobial agents

Analytical grade allyl isothiocyanate (AIT) (95% purity), carvacrol (99%), cinnamaldehyde (CNAD) (>93%), citral (>95%) and thymol (99.5%) were selected as test antimicrobial agents. The solvent dimethyl sulfoxide (DMSO) (≥99.9%) was used to dissolve the natural compounds in aqueous solution when appropriate. Streptomycin, sodium hypochlorite (NaOCl), and ethidium bromide (EtBr) were used as positive reference standards when appropriate. All chemicals were supplied by Sigma-Aldrich Company Ltd. (Dorset, UK).

2.2. Bacterial cultures

Two Gram-negative bacteria, *E. coli* (National Collection of Industrial, Food and Marine Bacteria (NCIMB) 8879) and *P. aeurginosa* (NCIMB 950), and two Gram-positive strains, *S. aureus* (NCIMB 6571) and *B. subtilis* (NCIMB 3610), were tested for their ability to grow and survive in the presence of the five selected natural compounds. Cultures of all four strains were prepared by streaking frozen glycerol stocks onto Mueller-Hinton agar (MHA) (Sigma-Aldrich Company Ltd., Dorset, UK) and were left to grow for 16 h at 37 °C until visible colonies appeared. One colony of each strain was then picked to start primary seed cultures in 5 ml sterile Mueller-Hinton broth (MHB) (Sigma-Aldrich Company Ltd., Dorset, UK). Seed cultures were added to 100 ml sterile MHB, incubated at 37 °C with 150 rpm agitation until cultures reached mid-exponential growth phase. Resulting cultures were harvested, washed twice with phosphate buffered saline (PBS) (Sigma-Aldrich Company Ltd., Dorset, UK), and then resuspended in PBS. The turbidity of each bacterial suspension was adjusted to match 0.5 McFarland standard (OD_{600} = 0.132), which corresponds to bacterial density of 10Close et al., 2012 CFU/ml. The resulting bacterial suspensions were used in both the disc diffusion assay and broth microdilution test.

Stock suspensions of the *E. coli* HB101_pUCD607_ *lux* (Belas et al., 1982; Simpson et al., 1998) biosensor were prepared as follows. Five ml of an overnight culture was added to 100 ml of sterile lysogeny broth-Miller with 1 g/L glucose (LBG) with 50 μg/ml ampicillin. The culture was incubated at 37 °C at 150 rpm agitation for 8 h when the cells reached mid-exponential growth phase. The culture was immediately placed on ice to prevent further growth, and then centrifuged at 3000 rpm at 4 °C for 20 min. The resulting cell pellet was resuspended in sterile *Mist dessicans*, which is composed of 1:3 (vol:vol) solution of LBG with additional 10 g/L glucose: horse serum. One ml of this suspension was dispensed into each sterile freeze-drying vial, partially sealed with rubber stoppers, frozen at −80 °C overnight, then freeze-dried for 24 h at −55 °C.

Stock suspensions of the *A. baylyi* ADP1_*recA_lux* biosensor (Song et al., 2009) were prepared as follows. One colony was inoculated into 100 ml of lysogeny broth-Miller (LB) and incubated at 37 °C at 150 rpm agitation for 14 h until the cells reached mid-exponential

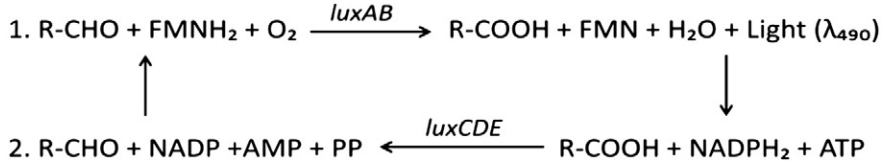

Fig. 1. Bioluminescence reactions catalyzed by enzymes encoded by the *luxCDABE* operon. The *luxAB* genes convert an aldehyde substrate to a carboxyl group, generating visible light (Equation 1). The *luxCDE* genes use NADPH2 and ATP to generate the aldehyde (Equation 2).

growth phase. Cells were then harvested by centrifugation at 3000 rpm for 20 min. The resulting cell pellet was freeze-dried in the same procedure as described above. The resulting freeze-dried products of both *E. coli* HB101_pUCD607_ *lux* and *A. baylyi* ADP1_*recA_lux* were sealed and stored at −20 °C until use in the biosensor assays.

2.3. Susceptibility tests

Both the disc diffusion assay and broth microdilution test were performed in accordance with the procedures outlined by the Clinical and Laboratory Standards Institute. All experiments were performed using MHA and MHB as growth media.

2.3.1. Disc diffusion method

The disc diffusion assay is conventionally used to determine relative antibacterial efficiency between test compounds (Burt, 2004). In this study, the assay was performed by streaking each bacterial suspension (turbidity adjusted to 0.5 McFarland units as described earlier) onto MHA with a sterile cotton swab to obtain uniform lawn growth. Series of two-fold dilutions of each natural compound, ranging from 100 to 6.25 mg/ml, were prepared with DMSO sterilized by filtration through a 0.22 μm membrane filter. Under aseptic conditions, sterile filter discs (Whatman no. 5, 6 mm diam; Fisher Scientific Ltd., Loughborough, UK) were saturated with 20 μl of each respective test compound at five serially diluted concentrations (100, 50, 25, 12.5, and 6.25 mg/ml) and placed onto inoculated agar with sterilized tweezers. For positive reference standard, the same was done using streptomycin at the same concentrations. A second reference standard, 15% NaOCl solution, was also included. DMSO served as vehicle control since it was used to dissolve the natural compounds. Concentrations of each reference standard were chosen to be relevant to levels used commercially and industrially today.

Once the moistened filter discs were placed onto the seeded agar, they were sealed with parafilm and left at room temperature for 30 min to allow for the diffusion of the natural compounds, and then incubated at 37 °C for 16 h. After incubation, the zones of inhibition were measured and rounded to the nearest 0.5 mm. Studies were performed in biological and technical triplicates at the least, and the diameters of inhibition zones were averaged. The test compound that yielded the largest zone of inhibition was taken to be the most effective antibacterial agent, and others were ranked accordingly.

2.3.2. MIC and MBC determination

Based on the previous screening, citral demonstrated the lowest overall antimicrobial activity and therefore was not included in further experiments. The minimum inhibitory concentrations (MICs) and maximum bactericidal concentrations (MBCs) of AIT, CNAD, carvacrol, and thymol were determined using the broth microdilution method. Stock solutions of 5000 mg/L of each natural compound were prepared with DMSO (20%)-MHB solution as solvent when testing against *E. coli*, *B. subtilis*, and *S. aureus*. Each well was then inoculated with 100 μl of the bacterial suspensions prepared as described earlier (turbidity adjusted to 0.5 McFarland units). Series of two-fold dilutions of each compound ranging from 2500 to 4.88 mg/L final concentration were prepared across sterile 96-well microtiter plates. MHB with no inoculum served as negative control. DMSO(20%)-MHB with no test compound served as vehicle control, MHB with inoculum served as positive control. Plates were sealed with microporous tape sheets (AirPore® Tape Sheets, Qiagen, UK) and incubated at 37 °C for 16 h at 150 rpm agitation. Experiments were performed in biological and technical triplicates at the least. Using a Synergy HT Multi-detection Microplate Reader (Bio-Tek, UK), inhibition of growth was assessed by comparing absorbance values before and after incubation. The MIC is defined as the lowest concentration inhibiting any visible growth (Andrews, 2001).

After incubation, the contents in the clear wells of the microplate were diluted 20-fold. Three μl of such diluted samples were spread onto fresh MHA plates using sterile glass beads (3 mm diam; VWR, Leicestershire, UK) and then incubated at 37 °C overnight to allow for colonies to form. The MBC is defined as the lowest concentration at which 99.9% of bacteria are killed (Andrews, 2001). The concentration at which the number of colonies corresponded to a 1000-fold reduction as compared to the colony count of the start inoculum was determined to be the MBC.

2.4. Biosensor assays

2.4.1. E. coli HB101_pUCD607_lux

The *E. coli* HB101_pUCD607_*lux* biosensor assay was performed at respective MICs of the two compounds for *E. coli* (156.25 mg/L AIT and 78.125 mg/L CNAD; determined previously via broth microdilution method). In addition, treatment with 156.25 mg/L CNAD (2MIC) was also included in order to directly compare the cytotoxic effects of AIT and CNAD when used at the same concentration. Freeze-dried stock suspensions of *E. coli* HB101_pUCD607_*lux* cells (prepared as described earlier) were resuscitated by dispensing 2 ml of sterile deionized water (DW) into each vial and incubating at 25 °C for 2 h. The cell suspensions were then centrifuged at 3000 rpm for 5 min, followed by removal of supernatant and resuspension in 2 ml of sterile DW. The resuscitated cells were diluted 10-fold in solutions of 156.25 mg/L AIT (corresponding to 1MIC for *E. coli*), 78.125 mg/L CNAD (1MIC), 156.25 mg/L CNAD (2MIC), and sterile DW which served as the control. The bioreporter cells were exposed to the designated concentrations of AIT and CNAD for 30 min and then transferred to a 96-well black microplate (Nunc), with 200 μl of sample dispensed in each well. Light output was measured using a Synergy HT Multi-detection Microplate Reader (Bio-Tek, UK). Luminescence values were expressed as relative light units (RLU), and the intensity of light emitted by the treated bioreporter cells was expressed as percentages of the untreated control. Sixteen readings (two rows of a 96-well microplate) were taken for each sample during each run, and the experiment as a whole was repeated at least three times.

After exposure to the natural compounds, serial 10-fold dilutions of the *E. coli* HB101_pUCD607_*lux* samples from the bioluminescence study were immediately prepared and subcultured onto LBG agar with 50 μg/ml ampicillin. Inoculated agar plates were incubated at 37 °C for 16 h and visible colonies were subsequently counted to determine the CFU/ml resulting from exposure to specified concentrations of AIT and CNAD. In order to directly compare RLU values from the bioluminescence study with colony forming unit (CFU)/ml values from plate count experiments, colony counts were normalized to percentages of the control wells. Plate counts for each sample were performed in biological and technical triplicates at the least.

2.4.2. A. baylyi ADP1_recA_lux

Resuscitated *A. baylyi* ADP1_*recA_lux* cells were diluted 10-fold in solutions of AIT (at final concentrations of 2 mg/L, 0.2 mg/L, and 0.1 mg/L) and CNAD (at final concentrations of 4 mg/L, 2 mg/L and 0.2 mg/L) in a 96-well black microplate (Nunc, UK), with 20 μl of cell suspension and 180 μl of treatment in each well. Cells were also similarly diluted in sterile DW, which served as negative control, and in 2 μM EtBr and 0.001% NaOCl, which served as positive reference controls. Light output was measured immediately after sample preparation using a Synergy HT Multi-detection Microplate Reader (Bio-Tek, UK) at 10-min intervals for 4.25 h and at 15-min intervals for 3 h thereafter. The intensity of light emitted by the treated bioreporter cells was expressed as percentages of the untreated negative control. Sixteen readings (two rows of a 96-well microplate) were taken for each sample during each run, and the experiment as a whole was repeated at least three times.

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2.5. Transcriptional analysis of recA

A. baylyi ADP1_*recA_lux* bioreporter cells were grown to mid-exponential growth phase in LB, then washed and resuspended in PBS so that final OD of the suspension was 0.3. One ml aliquots of the bacterial suspension were diluted 10-fold in solutions of 0.2 mg/L AIT, 2 mg/L CNAD, 2 μM EtBr, 0.001% NaOCl, and sterile DW. Cells treated with EtBr and NaOCl served as positive reference controls; cells in DW served as negative control. Samples were incubated at 20 rpm on an orbital rotator at room temperature and total RNA was extracted after 0, 5, and 7 h exposure using RNeasy® Mini Kit (Qiagen Ltd., West Sussex, UK). To remove any contaminating genomic DNA from the RNA samples, on-column DNase digestion was performed using DNase I (RNase-Free DNase Set, Qiagen Ltd., UK). Experiment was repeated three times. Extracted RNA samples were stored at −20 °C.

Based on the coding DNA sequence of *recA* for *A. baylyi* ADP1 (GenBank accession number L26100.1, nucleotide positions 403 to 1452), forward primer (5′-TTG GTG GCT TAC CTA AAG GGC GTA-3′) and reverse primer (5′-ACA GGT ACC ACC TGC TTT CTG ACA-3′) were designed and synthesized (Integrated DNA Technologies, London, UK) to target *recA* mRNA and to amplify its cDNA.

RT-PCR was performed using OneStep RT-PCR Kit (Qiagen Ltd., West Sussex, UK). For all samples, 1 μl of extracted total RNA was used as template. RT-PCR reactions were carried out as described below:

1. Reverse transcription	50 °C for 30 min
2. Initial PCR activation	95 °C for 15 min
3. Denaturation	94 °C for 30 s
4. Annealing	55 °C for 30 s
5. Extension	72 °C for 1 min
Repeat steps 3–5 ten times	
6. Final extension	72 °C for 10 min

Using a 50 bp DNA ladder (New England BioLabs Inc., Hitchin, UK) as a reference, RT-PCR products were visualized via 1.8% agarose (Invitrogen, Paisley, UK) gel electrophoresis for amplicon size verification. The gel was stained after electrophoresis with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen, Paisley, UK) diluted 10,000-fold in 1X Tris/Borate/EDTA (TBE) for 15 min at 12 rpm on an orbital rotator, then visualized using a Visi-Blue™ Transilluminator (UVP Ltd., Cambridge, UK). Once amplicons were verified to contain the expected number of base pairs, RT-PCR products were quantified using NanoDrop® 2000 Micro-Volume UV–Vis Spectrophotometer (Thermo Scientific, Nortumberland, UK).

2.5.1. Statistical analysis

F-test, *t*-test and ANOVA followed by Tukey's test were applied when appropriate. *P* values of 0.05 and 0.01 were used to determine statistical significance.

3. Results

3.1. Susceptibility tests

The antibacterial activity of five selected natural compounds against four bacterial species is summarized in Table 1. All five compounds exhibited inhibitory effects of varying magnitudes against *E. coli*, *B. subtilis*, and *S. aureus*. *P. aeruginosa* was susceptible to only AIT and CNAD. There was no inhibition observed with the vehicle controls (DMSO) in either assays.

Overall, AIT performed much better than the other selected compounds in inhibiting all four bacterial strains. It produced the largest zones of inhibition in all cases except at concentrations ≤25 mg/ml against *B. subtilis* and *P. aeruginosa*. AIT was the only compound to completely inhibit growth of all four species on the entire agar plate (86 mm diam) at one or more concentrations used in this study. However, the activity of AIT abruptly dropped to zero as its concentration decreased by one order of magnitude, demonstrating maximum inhibitory capacity at 12.5 mg/ml and then suddenly no inhibition at all at 6.25 mg/ml against *E. coli* and *S. aureus*.

CNAD also displayed excellent antibacterial activity, generating the largest inhibition zones against *P. aeruginosa* at concentrations ≤25 mg/ml. Otherwise, in most cases CNAD produced the second-largest zones of inhibition following AIT (except in the case against *B. subtilis*, in which citral performed second-best).

Carvacrol and thymol displayed moderate and similar levels of inhibition against three bacterial strains, but had no detectable effect on *P. aeruginosa*. Although citral showed good inhibition against *B. subtilis*, it displayed the poorest antibacterial activity against all other strains. Because of its overall low efficacy, citral was not included in the broth microdilution test.

Results of the broth microdilution method for AIT, CNAD, carvacrol, and thymol against four bacterial species are presented in Table 2. CNAD exhibited the greatest inhibitory effect against all bacterial strains, yielding the lowest MICs ranging from 78.125 to 156.25 mg/L and the lowest MBCs ranging from 78.125 to 312.5 mg/L. AIT displayed the second-highest activity, demonstrating MICs of 156.25 mg/L and MBCs of 156.25 to 312.5 mg/L. Carvacrol and thymol generated very similar MIC and MBC values except with *P. aeruginosa*, in which case carvacrol resulted in MIC of 1250 mg/L and MBC of 2500 mg/L, while thymol's MIC and MBC were beyond the highest concentration used in this study (>2500 mg/L).

The results from the disc diffusion assay and the broth microdilution indicated that of the five compounds tested, AIT and CNAD displayed the greatest antimicrobial activity against the broadest range of bacteria. Therefore, AIT and CNAD were selected as test compounds in the two subsequent biosensor assays.

No obvious differences in susceptibility were detected between Gram-negative and Gram-positive bacteria, although it has been reported that Gram-negative bacteria are generally more resistant to conventional biocides and antibiotics (Denyer and Maillard, 2002). *P. aeruginosa* was the most resistant to antibacterial treatments employed in these two assays, followed by *B. subtilis*. This is in accordance to previous studies suggesting *P. aeruginosa* to be highly tolerant to antimicrobial agents (Burt, 2004; Mayaud et al., 2008). No inhibition of growth was observed with DMSO control samples.

3.2. Biosensors

Results from the *E. coli* HB101_pUCD607_*lux* biosensor assay are shown in Fig. 2. Exposure of luminescent *E. coli* cells to 156.25 mg/L AIT (1MIC), 156.25 mg/L CNAD (2MIC), and 78.125 mg/L CNAD (1MIC) for 30 min resulted in light output that were 2.1%, 8.3%, and 9.4% of control cells, respectively. Parallel plate count experiments demonstrated that when the samples from the bioluminescence study (1MIC AIT-treated, 2MIC CNAD-treated, and 1MIC CNAD-treated cells) were subcultured onto fresh agar media, the resulting colony counts were 14.0%, 23.9%, and 80.0% of the control, respectively. Both the bioluminescence study and the plate count method reflected that AIT is more toxic than CNAD when used at the same concentration (156.25 mg/L) (Fig. 2). Also, these two methods revealed the order of relative toxicity to be 1MIC AIT > 2MIC CNAD > 1MIC CNAD. However, in all three treatments, the results from the bioluminescence study were significantly lower ($P \leq 0.05$) than those from the plate count method performed in parallel. Furthermore, the difference in toxicity observed between 2MIC CNAD and 1MIC CNAD in the bioluminescence study was not significant, whereas in the plate count method, CNAD at 2MIC inhibited approximately four times more cells than did CNAD at 1MIC ($P \leq 0.01$).

The results from the *A. baylyi* ADP1_*recA_lux* biosensor assay are shown in Fig. 3a and b. The intensity of light emitted by the treated

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Table 1
Mean diameters of growth inhibition zones obtained from disc diffusion assay. Values presented in millimeters ± SD of at least three biological and technical replicates. 0 = no observed inhibition. * = positive reference standards.

	E. coli					*P. aeruginosa*				
Concentration (mg/ml)	100	50	25	12.5	6.25	100	50	25	12.5	6.25
Compound										
AIT	≥86	≥86	≥86	≥86	0	≥86	≥86	0	0	0
CNAD	42 ± 0	36 ± 1.7	28.7 ± 0.6	16.7 ± 0.6	8 ± 0	19 ± 0	13.7 ± 0.3	11.2 ± 0.3	8.2 ± 0.7	0
Carvacrol	30 ± 0	21.75 ± 0.4	14.7 ± 0.5	11.3 ± 0.3	0	0	0	0	0	0
Citral	10.5 ± 0.5	11.18 ± 0.6	8 ± 0	8 ± 0	0	0	0	0	0	0
Thymol	33.8 ± 0.6	20.78 ± 0.8	13.8 ± 0.2	12.7 ± 0.6	0	0	0	0	0	0
Streptomycin*	9.3 ± 0.3	1 ± 0	0	0	0	0	0	0	0	0
1% Triclosan*					5.72 ± 0.2					0
15% NaOCl*					10.5 ± 1.3					19 ± 0.3

	S. aureus					*B. subtilis*				
Concentration (mg/ml)	100	50	25	12.5	6.25	100	50	25	12.5	6.25
Compound										
AIT	≥86	≥86	≥86	≥86	0	≥86	≥86	15 ± 0.2	0	0
CNAD	39.8 ± 0.4	32.4 ± 1.3	20.5 ± 0.5	15.7 ± 0.4	0	37.4 ± 4.2	29.3 ± 2.9	14 ± 0	8.3 ± 0.3	0
Carvacrol	34.6 ± 0.8	28.2 ± 0.9	25.5 ± 0.8	9.2 ± 0.4	0	30.8 ± 0.7	22.8 ± 0.3	14.2 ± 0.1	8.5 ± 0	0
Citral	22.2 ± 0.8	15.3 ± 0.5	9.3 ± 0.5	0	0	53.5 ± 3.0	36.9 ± 1.4	24 ± 0	20 ± 1.0	11.3 ± 0.3
Thymol	33.2 ± 0.4	23 ± 0.3	12.8 ± 0.7	8.5 ± 0.3	0	34 ± 0.5	23 ± 0.8	15.4 ± 0.3	9.8 ± 0.1	6.5 ± 0
Streptomycin*	28 ± 0	26 ± 0	14 ± 0	6.8 ± 0.2	0	7 ± 0	0	0	0	0
1% Triclosan*					6.5 ± 0.3					0
15% NaOCl*					21 ± 0.3					21.3 ± 0.3

bioreporter cells was standardized as percentages of negative control. Since *A. baylyi* ADP1_*recA_lux* cells express a constant level of bioluminescence, a decrease in light output below the baseline expression indicated that the bioreporter cells were inhibited or killed, while an induction of bioluminescence above the baseline indicated that the cells had been exposed to DNA damaging toxicants. The baseline expression of bioluminescence was represented as the amount of light emitted by the negative control samples, and thus expressed as 100% in Fig. 3a and b. AIT at 0.1 mg/L and 0.2 mg/L did not stimulate a genotoxic response as the treated cells emitted light at levels consistent with the baseline at all time points, never reaching levels significantly greater than 100% of the control ($P \leq 0.01$). Similarly, 0.2 mg/L and 2 mg/L CNAD did not damage DNA since treatments at both concentrations did not result in bioluminescence levels that significantly exceeding 100% of the control ($P \leq 0.01$) at any time point. At 2 mg/L, AIT significantly reduced light output below 100% of the control ($P \leq 0.01$) and failed to induce expression of bioluminescence above the baseline. Likewise, CNAD at 4 mg/L also caused expression of bioluminescence to drop significantly below 100% of the control ($P \leq 0.01$) and did not induce light output beyond the baseline at any time. This suggests that 2 mg/L AIT and 4 mg/L CNAD successfully inhibited or killed the bioreporter cells, but not by damaging genetic material, since the essential DNA repair gene *recA* was not activated even at sub-inhibitory concentrations of both compounds as discussed earlier. Bioreporter cells treated with CNAD at all three concentrations experienced a steady decline in bioluminescence expression starting at 225 min (Fig. 3b), which implies that the cells proceeded to die gradually after exposure to CNAD sustained for at least 225 min. NaOCl at 0.001% caused the bioreporter cells to emit light at levels that significantly exceeded 100% of the negative control starting at 65 min ($P \leq 0.01$) (Fig. 3a and b). EtBr at 2 μM, serving as positive control, also caused a steady increase in bioluminescence expression that increased beyond the baseline starting at 125 min. This indicates that 0.001% NaOCl and 2 μM EtBr were both damaging to DNA, since *recA* was activated upon both treatments.

3.3. Transcriptional analysis of recA

The present approach employed two techniques (DNA gel electrophoresis and spectrophotmetric quantification) as a cross check to confirm the expression profile of *recA* after various treatments. DNA gel electrophoresis of RT-PCR products resulting from amplification of *recA* mRNA isolated from *A. baylyi* ADP1_*recA_lux* after designated treatment for 7 h is presented in Fig. 4. This visualization via 470 nm blue light transillumination achieved three objectives: (i) confirmed that the amplicons were of the expected size (113 bp) and therefore products were indeed amplified from *recA* mRNA; (ii) demonstrated that no induction of *recA* occurred upon treatment with 0.2 mg/L AIT and 2 mg/L CNAD since there was no visually significant difference among band intensities of the control, AIT, and CNAD samples (Fig. 4); (iii) demonstrated that treatment with 2 μM EtBr and 0.001% NaOCl triggered upregulation of *recA* since band intensities of EtBr and NaOCl samples were relatively darker than those of the control (Fig. 4). The intensity of band fluorescence upon staining with a DNA-intercalating

Table 2
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of four natural compounds, obtained from broth microdilution method and plate count experiments, respectively, for four bacterial species. Units of concentration are expressed in mg/L. Experiment was repeated three times, with eight technical replicates per experiment.

Compound	Bacterial species							
	E. coli		*P. aeruginosa*		*S. aureus*		*B. subtilis*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
AIT	156.25	156.25	156.25	312.5	156.25	156.25	156.25	312.5
CNAD	78.125	78.125	156.25	312.5	78.125	78.125	156.25	312.5
Carvacrol	312.5	312.5	1250	2500	625	1250	625	625
Thymol	625	625	>2500	>2500	625	1250	625	625

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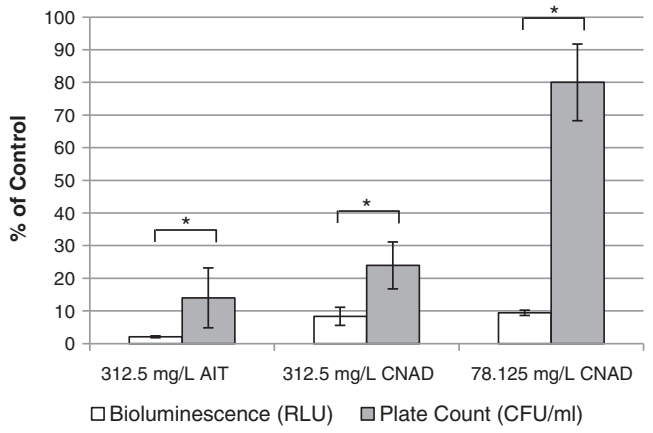

Fig. 2. Bioluminescence output and plate count results for *E. coli* HB101_pUCD607_lux. Values presented are all normalized as percentages of the negative control. Error bars represent ± SD. Asterisks (*) denote significant difference ($P \leq 0.05$).

agent is directly proportional to the quantity of nucleic acid present; therefore, the darker bands of the EtBr and NaOCl samples indicated that higher concentrations of RT-PCR products were present in those lanes than in the lanes of the other samples and the control.

Spectrophotometric (NanoDrop®) quantification of the RT-PCR products is shown in Fig. 5. The results were consistent with the semi-quantitative findings of the DNA gel electrophoresis run: (i) treatment with 0.2 mg/L AIT and 2 mg/L CNAD did not induce *recA* expression above basal level, since the concentrations of RT-PCR products for both samples were not significantly different than those of the control; (ii) exposure to 2 μM EtBr and 0.001% NaOCl caused an increase in *recA* transcription, specifically at 5 h and even more so at 7 h, as shown in the significantly higher amplicon concentrations ($P \leq 0.05$) for both EtBr and NaOCl reference controls as compared to those of the negative control (Fig. 5). Present findings from transcriptional studies of *recA* validate the results from the *A. baylyi* ADP1_*recA_lux* luminescence biosensor study (Fig. 3a and b).

4. Discussion

Natural plant materials are a rich source of valuable compounds for key sectors such as pharmaceuticals and therapeutics, but they are rarely seriously considered as sources of biocides. With the increasing realization that conventional biocides have their limitations, not least indiscriminate impact on human health, there is an increasing drive to find alternative biocides. The European Commission's Biocidal Products Directive (98/8/EC) (Directive 98/8/EC, 2012) seeks to Q3

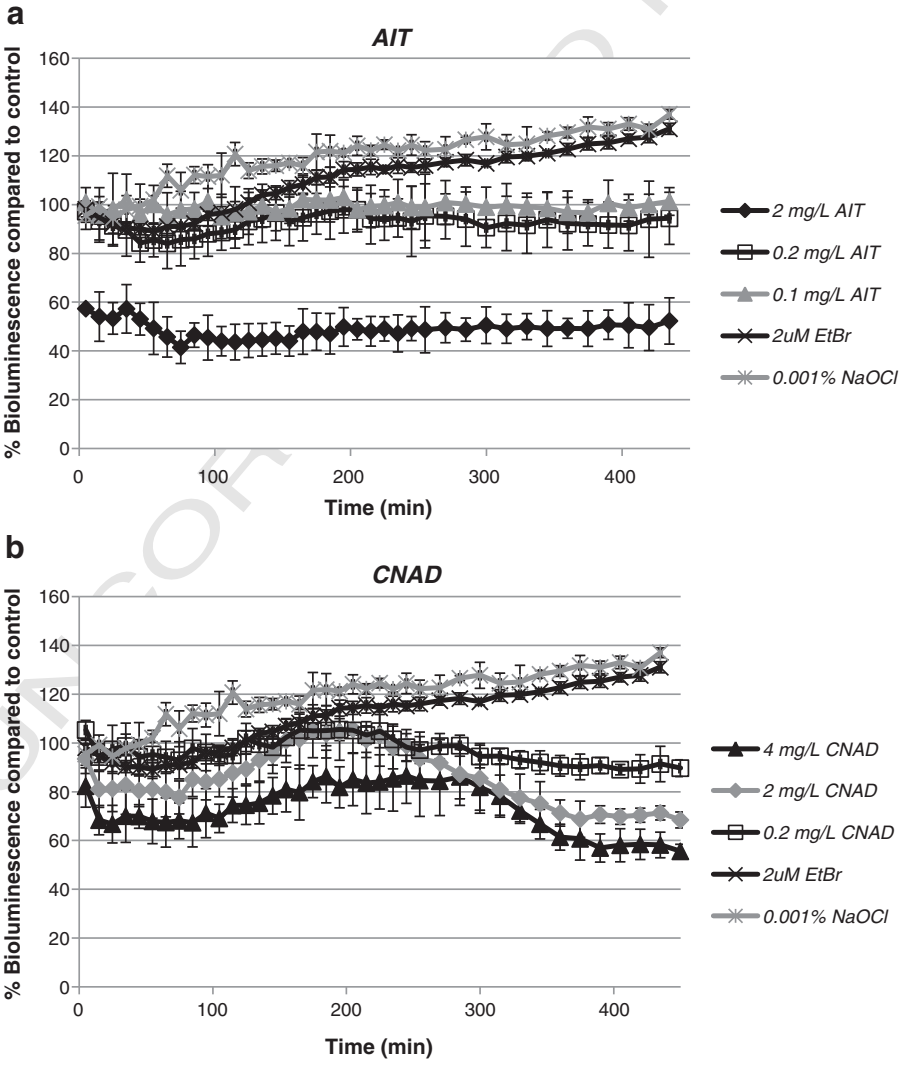

Fig. 3. a & b: Bioluminescence response of *A. Baylyi* ADP1_recA_lux biosensor to different concentrations of AIT (a) and CNAD (b), expressed as percentages of the negative control. 2 μM EtBr and 0.001% NaOCl served as positive reference controls. Error bars represent ± SD. Experiment was repeated three times, with eight technical replicates per experiment.

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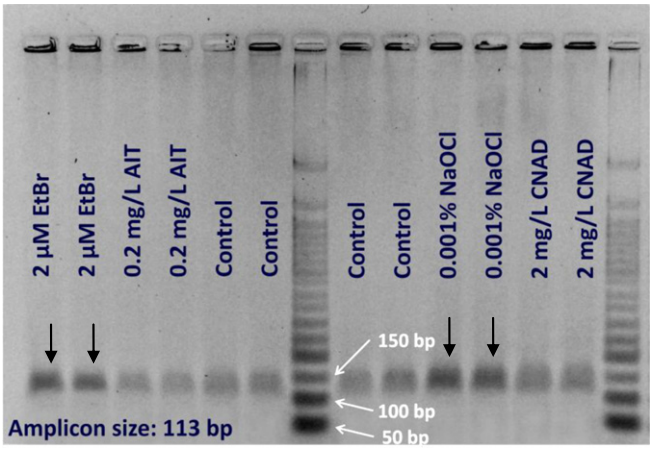

Fig. 4. Gel electrophoresis of RT-PCR products (113 bp) from amplification of *recA* mRNA from *A. baylyi* ADP1_recA_lux after various treatments for 7 h. Black arrows highlight relatively darker bands of EtBr- and NaOCl-treated samples, indicating induction of *recA* above basal level following treatment with 2 µM EtBr and 0.001% NaOCl. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

abolish the most hazardous, non-natural biocides in the short term, and eventually all harmful ones in the long term.

In this study, a combination of complementary techniques was used to assay the toxicity and mode of action of five natural plant-based compounds. Four species of bacteria were challenged with five different plant compounds to determine whether they were effective and more sustainable alternatives to current antimicrobial agents. All five test compounds exhibited inhibitory effects against the selected bacteria at varying degrees, with the antimicrobial order being AIT > CNAD > carvacrol ≥ thymol > citral.

As a general group of chemicals, natural plant-derived compounds were chosen to be investigated in this study for two major reasons. Firstly, these plant secondary metabolites are safer and non-damaging for the natural environment, since they are originally sourced from plant material. Natural compounds may be effective alternatives to current biocides such as chlorine and ozone, which are strong oxidizing agents that indiscriminately react and destroy the surrounding environment (Kim et al., 2009). Secondly, natural compounds are safe for human exposure, unlike current antimicrobial strategies that produce toxic and carcinogenic by-products such as trihalomethanes and halo acetic acids from chlorine treatment and bromate from ozone treatment (Bernhard, 2003; Kim et al., 2009). These natural compounds are registered by the European Commission for use in foodstuffs as they present no risk to consumers (European Commission Decision of 23 January, 2002) and are classified as Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (Center for Food Safety and Applied Nutrition, 2006).

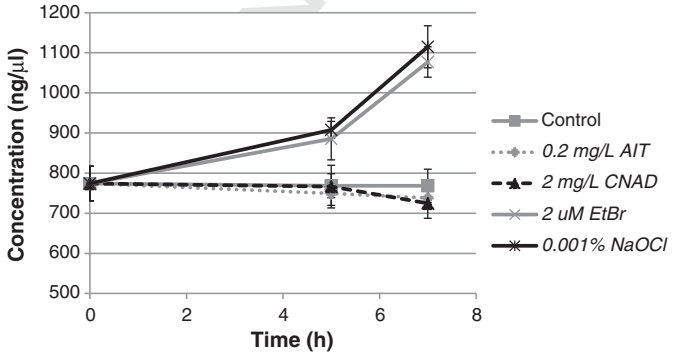

Fig. 5. NanoDrop® quantification of RT-PCR products from amplification of *recA* mRNA from *A. baylyi* ADP1_recA_lux after exposure to various compounds for 0 h, 5 h, and 7 h. Values presented are means ± SD. RT-PCR was performed three times for all samples; four NanoDrop® readings were performed per sample for all RT-PCR runs.

Although several modes of attack employed by plant compounds to kill microbial cells have been proposed, the complex mechanism of action is not well understood. The approach taken in this study was to employ bacterial biosensors to elucidate the method of kill. This novel approach proved to be highly effective in measuring the toxicity of plant compounds and providing a dynamic perspective on their respective antimicrobial action.

4.1. Susceptibility tests

AIT, carvacrol, CNAD, citral, and thymol were selected because they span different classes of plant-derived compounds (aldehyde terpene, phenolic terpene, and organosulfur), each with distinct chemistry. Although the exact antimicrobial mechanism of natural compounds has not been fully determined, it is likely to be attributed to their chemical structure. The reactivity of each compound's functional groups, its aqueous solubility, and its ability to form intermolecular bonds is directly linked to antimicrobial power (Koroch et al., 2007).

Of the five compounds tested, AIT and CNAD displayed the greatest biocidal activity against the broadest range of bacteria used in this study. Carvacrol and thymol exhibited moderate inhibitory activity against three bacterial strains, but were ineffective against *P. aeruginosa*. Lastly, although citral performed well against *B. subtilis*, it was the overall poorest performer against all other strains tested.

AIT exhibited the greatest antimicrobial activity against all four bacterial species by a substantial margin in the disc diffusion assay. AIT has been reported to exhibit higher antibacterial activity than some conventional antibiotics (streptomycin, penicillin G, and polymyxin B) (Lin et al., 2000; Prabuseenivasan et al., 2006) and biocidal agents (sulfur dioxide and ethanol) (Tunc et al., 2007). With a polar isothiocyanate end and a non-polar allyl side chain, AIT is an amphiphilic compound that can pass through the hydrophilic outer cell wall of Gram-negative bacteria and also partition into the hydrophobic phospholipid bilayer of the cell membrane (Lin et al., 2000). AIT possesses a highly electrophilic central carbon atom that can be reactive with amino acids, peptides, and aromatic and aliphatic amines; thus, AIT may be able to alter protein structure and inactivate enzymes via reaction with free amino groups and oxidative cleavage of disulphide bonds (Delaquis and Mazza, 1995; Kawakishi and Kaneko, 1987). In addition, previous studies infer that AIT shunts ATP production and drives ATP hydrolysis inside the cell (Chao et al., 2008; Tiensing et al., 2001).

CNAD produced the second-largest zones of inhibition against three of the four strains in the disc diffusion assay and presented the lowest MIC and MBC values for all four bacterial strains. Several reports have demonstrated that cinnamon oil displayed the greatest efficacy against a range of microbes when compared to a number of other EOs (Chao et al., 2008; Prabuseenivasan et al., 2006; Warnke et al., 2009). CNAD is a phenolic aldehyde terpene that inhibits bacteria largely by disrupting the phospholipid bilayer of the cell membrane. The functional aldehyde group conjugated to a carbon–carbon double bond is highly electronegative, which could explain CNAD's ability to interfere with biological processes involving electron transfer (Knobloch et al., 1986).

AIT did not produce the lowest MIC and MBC values as would have been expected based on the results from the disc diffusion assay. A possible explanation for CNAD performing better than AIT in the broth microdilution test was the difference in volatility between the test compounds. The vapor pressure of AIT (4.6 mm/Hg at 25 °C) is greater than that of CNAD (0.03 mm/Hg at 25 °C), meaning a greater amount of AIT compared to CNAD would have been lost from the media into the atmosphere during the preparation of the microplate or the incubation period, since the microtiter plates were not sealed air-tight. This was not the case for the disc diffusion assay because the petri dishes were sealed with parafilm during incubation. The highly volatile nature of AIT may

also explain the sudden drop in activity within just one dilution step in the disc diffusion assay.

Since AIT and CNAD were the most promising antimicrobial agents, these were selected for more in depth study employing biosensors in order to elucidate their mechanisms of kill.

4.2. E. coli HB101_pUCD607_lux

Results from the *E. coli* HB101_pUCD607_*lux* biosensor assay revealed dose-response relationships of AIT and CNAD against *E. coli*, and also provided some information on the mechanism of kill and cytotoxic effects of the compounds. Findings from both the bioluminescence study and the biosensor plate count method corroborate results from the disc diffusion assay, which indicated that AIT was more toxic to bacteria than CNAD when both were applied at the same concentration. These results from three separate assays increase the confidence level in terms of identifying AIT as the most potent antimicrobial compound in this study.

In this biosensor, light emitted from bacterial cells was directly linked to energy products of cellular respiration, and therefore the significant decreases ($P \leq 0.01$) in bioluminescence of both CNAD-treated and AIT-treated cells compared to the control suggest that the availability of intracellular ATP and/or NADPH in treated samples were <10% that of healthy control samples. ATP and NADPH are coenzymes that transport chemical energy within cells for metabolism and are required in many vital cellular processes (Knowles, 1980). If these two coenzymes were depleted due to the rupturing of the cell membrane alone, then the corresponding number of culturable colonies should have also been <10% of the control since membrane damage to that degree (causing the exit of >90% intracellular ATP and/or NADPH) is likely to have been irreversible. However, in fact, the resulting colony counts from parallel plate count experiments (expressed as percentages of the control) were greater than the light output (also normalized as percentages of the control) for each respective sample. This suggests that the significant decreases in bioluminescence of treated samples were due to significant impairment of metabolic activity reflected in the decline of ATP and NADPH production, and not solely due to the disruption of barrier function of the cell membrane leading to the exit of ATP and NADPH. When the treated samples were subcultured onto fresh growth agar media in which the inhibitory pressures of AIT and CNAD were removed, a fraction of the cells were able to recover from their metabolically stunted state (Fig. 2), eventually producing higher levels of ATP and NADPH to form healthy colonies.

4.3. A. baylyi ADP1_recA_lux

A. baylyi ADP1 is typical of water and soil bacteria occurring in the natural environment, and so *A. baylyi* ADP1_*recA_lux* bioreporter cells are ideal for assessing bacterial response to AIT- and CNAD- treatment in the context of controlling biofouling (Tiensing et al., 2001). By exploiting bacteria's inherent SOS stress response to DNA damage, the *A. baylyi* ADP1_*recA_lux* biosensor assay revealed that bacterial exposure to AIT and CNAD did not activate *recA*, indicating that neither compounds compromised the integrity of bacterial genomes. Because *recA* homologs exist in all species, it can be deduced that these natural compounds are unlikely to be genotoxic or mutagenic to multicellular organisms as well. In contrast, EtBr, a known mutagen that deforms DNA and thus was chosen as positive control for this study, and NaOCl, a conventional biocide frequently used in water disinfection, both induced the transcription of *recA*, indicating that both chemicals were DNA damaging toxicants. NaOCl has been shown to exhibit genotoxicity in microbes (Buschini et al., 2004), and based on the results of this current study, NaOCl may also have similar genetically deleterious effects on human cells since the same *recA*-related response to DNA damage is present in mammalian cells as well. As an antimicrobial agent, it is advantageous that the compound be specifically lethal against target microbes but safe for higher organisms, so that damage and toxicity towards multicellular life forms are minimized.

A gradual increase of bioluminescence was observed between 15 min and 185 min in *A. baylyi* ADP1_*recA_lux* cells treated with CNAD at all three concentrations tested (Fig. 5), but the intensity of light emitted never surpassed the baseline expression of bioluminescence. This suggests that cellular activity was initially inhibited upon exposure to CNAD, but the bioreporter cells were able to recover to some degree, as indicated by the steady increase of bioluminescence toward the baseline. Although having increased over time, the light output never significantly exceeded 100% of the control ($P \leq 0.05$). Therefore, it can be inferred that CNAD did not activate *recA* at any point, suggesting that DNA damage did not occur as a result of CNAD treatment. A steady decline in bioluminescence followed thereafter without any spikes in light output above the baseline, which again is indicative of gradual cell inhibition or death via mechanisms independent of DNA disruption.

4.4. Transcriptional studies of recA

The same RNA extraction protocol was followed for all samples and controls at all time points, thus RNA yield depended on gene transcription levels that resulted from exposure to the various compounds tested in this study. RT-PCR was carried out using the same volume (1 μl) of extracted RNA for all samples. Therefore, the amount of RT-PCR product was directly proportional to the amount of *recA* mRNA present in the initial 1 μl of template, which is why this method was appropriate for the quantification of *recA* transcript levels upon various treatments.

Temporal *recA* expression profiles obtained from NanoDrop® quantification of RT-PCR products (Fig. 5) were similar to the bioluminescence profiles from the *A. baylyi* ADP1_*recA_lux* biosensor study (Fig. 3a and b). This corroborates the genotoxicity-sensing mechanism of the *A. baylyi* ADP1_*recA_lux* biosensor, where the intensity of light produced by the bioreporter cells is directly proportional to their level of *recA* expression, which itself is directly related to the degree of DNA damage. Since there was no rise in *recA* transcript abundance at any point during treatment with 0.2 mg/L AIT and 2 mg/L CNAD, this suggests that these two compounds did not cause DNA damage. In contrast, 2 μM EtBr and 0.001% NaOCl caused increased *recA* expression, which indicated that both were genotoxic.

Development of resistance in bacteria often occurs through spontaneous or induced mutation(s) after exposure to the toxicant. It is relatively easier for a compound to confer resistance if its mechanism of inhibition is DNA disruption because cells may overcome the damage by inducing the standard SOS response to DNA damage, which involves error-prone DNA repair and mutagenesis. Because the inhibitory mechanism of AIT and CNAD seems to be related to structural and metabolic injury rather than to DNA damage, the chance of resistance development against these two natural compounds is likely to be lower than that of genotoxic toxicants that target DNA. Based on current findings of this *recA*-dependent biosensor assay, AIT and CNAD would be good alternatives to widely-used but genotoxic antimicrobial agents such as NaOCl.

5. Conclusion

In summary, the results of this study demonstrated that major constituent compounds of plant EOs, especially AIT and CNAD, have strong antimicrobial activity against four bacterial species. The *E. coli* HB101_pUCD607_*lux* biosensor assay demonstrated that the mechanism of action of AIT and CNAD did not simply involve membrane partitioning and damage by an oily substance, but also included other process(es) that injure and compromised cellular metabolism and energy production. A second complementary biosensor assay, which employed the genotoxicity-sensing strain *A. baylyi* ADP1_*recA_lux*,

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revealed that AIT and CNAD did not activate the essential DNA repair gene *recA*, which is evidence that it is unlikely that these two natural compounds cause damage to bacterial and higher genomes, and resistance development via mutagenesis is reduced.

6. Uncited reference

Turgis et al., 2009

Acknowledgments

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