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Efficacy of oregano oil as a biocide agent against pathogens *in vitro*, using lux reporter gene technology

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This study aimed to determine the antimicrobial activity and strength of kill of Himalayan oregano oil (HOO) across a range of dilutions typically formulated for potential topical use in healthcare settings, using bioluminescent constructs of bacterial pathogens as real time biosensors for rapid bactericidal monitoring. *Escherichia coli*, *Pseudomonas aeruginosa* and Methicillin sensitive *Staphylococcus aureus,* previously genetically modified by addition of the *luxCDABE* operon to express bioluminescence, were used as reporters of viable metabolically active cells to show a real time *in situ* antimicrobial effect of oregano oil. On the other hand, for genetically non-modified methicillin resistant *S. aureus* and *Candida albicans,* viable count method was used to assess the antimicrobial effect of oregano oil. Minimal inhibitory concentration of Himalayan oregano oil (HOO) and its major components, carvacrol and thymol, were found to be between 0.03 and 0.16%. High positive correlation was obtained between viable count and bioluminescence. These findings suggest that bioluminescence has the capability to replace the plate culture method for evaluating the efficacy of antimicrobial products. HOO may have the potential as a natural potent bactericide in the health care setting, as it has demonstrated bactericidal action towards pathogens in a short time (40-220 s). This is the first *in vitro* method used to assess the antimicrobial activity of oregano oil using lux reporter gene technology as an accurate measure of kill rates which is captured in real-time with high reproducibility and fidelity of responses such that comparisons are accurate.

Key words: Oregano oil, bioluminescence, viable count, lux operon, antimicrobial.

INTRODUCTION

Spread of bacteria causing human infection is a serious problem worldwide especially with the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE), which are frequently associated with extensive hospital outbreaks (Miragaia et al., 2002). Infections caused by

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methicillin-resistant staphylococci (MRS) have become a clinical and therapeutic problem because these organisms are resistant not only to β-lactams but also to many other antimicrobial agents (Nostro et al., 2004). However, the spread of resistant bacteria is not limited to Gram-positive species, since there is now an epidemic emergence of Gram-negative resistant bacteria (Blot et al., 2001; Kasiakou et al., 2005; Falagas and Bliziotis, 2007). The β-lactam antibiotics have traditionally been the main antimicrobial treatment of infections caused by bacteria like Enterobacteriaceae, but the emergence within species (including *Escherichia coli*) producing extended-spectrum β-lactamases (ESBL) has rendered this class of antibiotics largely ineffective. Bacteria containing ESBLs are resistant to cephalosporins e.g. cefuroxime, cefotaxime and ceftazidime, which are the most widely used antibiotics in many hospitals (Dancer, 2001). This is a rapidly developing problem in the UK as well as globally (Woodford et al., 2004).

Hygiene procedures incorporating topical biocidal agents to reduce person to person spread has been a major strategy to contain antibiotic resistance by controlling the carriage and spread of different human pathogens (Boyce and Pittet, 2002). However, antimicrobial resistance is still reported to be increasing (WHO, 2014). This could in part be due to non-compliance with hand hygiene protocols due to inaccessible equipment, lack of time and the deleterious effects of alcohol and soap-based products on the skin (Kampfe and Loffler, 2007). Therefore, alternative strategies or more acceptable and effective hand disinfectant agents are needed. An interesting approach to limit the transmission of resistant pathogens could be the use of the sustainable wild Himalayan oregano essential oil, especially if this could be more acceptable to users while still maintaining the efficacy of current hand disinfectants.

Essential oils are volatile, natural, complex compounds of terpenes (principally carvacrol and thymol) and aromatic compounds (mainly eugenol) and terpenoides (Bakkali et al., 2008). Oregano oil has already been credited with having antimicrobial properties. Esen et al. (2007) relates the antimicrobial ability of the Mediterranean oregano essential oil to the presence of carvacrol and thymol. Recently, it has been found that Himalayan oregano oil also contains carvacrol and thymol. However, the composition of essential oils from a particular species of plant can differ between geogra-phical sources (Cosentino et al., 1999, Bisht et al., 2009), plant age, organ of the plant used and time of the year (Kokkini et al., 1997). Essential oils are usually extracted by steam or hydro distillation of all or part of the plant and contain about 20-60 components of which two or three are found at high concentrations (20-70%) as compared to other components which are present in minor or trace amounts (Bakkali et al., 2008). Essential oils have many applications in medicine, food flavouring and preservations as well as in pharmaceutical industries (Fabian et al., 2006).

In this study, a new application of the wild Indian Himalayan oregano essential oil will be investigated. This essential oil is distilled from the oregano plant (*Oreganum vulgare*); a plant that grows abundantly in the Himalayas. Sustainable production of the oil provides a living for Himalayan communities (Biolaya Organics, 2007). Toxicity studies of oregano oil indicate that it is a very safe product that has not been associated with any serious side effects or negative health outcomes in humans (Meschino, 2005). Generally, no particular resistance or adaptation by bacteria to essential oils has been described. This can be attributed to the oils mode of action affecting several bacterial targets at the same time (Bakkali et al., 2008) and the great number of the oils constituents (Carson et al., 2002). We hoped to improve the quality of care of patients through application of a new hand hygiene regime using HOO which could be more acceptable to users while maintaining the efficacy of current hand hygiene disinfectant.

The aim of the present study was threefold; to compare the minimal inhibitory concentration (MIC) of different batches of oregano oil containing different carvacrol and thymol percentages, to evaluate the correlation between bioluminescence and conventional viable counting for enumerating bacteria and to establish the bactericidal rate of oregano oil and its major constituents using bioluminescence as a new measure of kill rates, where light output is captured in real-time with high reproducibility and fidelity of responses such that comparisons are accurate. To achieve these aims, representatives of the common UK bacterial pathogens that have been genetically modified with the addition of *the Photorhabdus luminescens luxCDABE* operon (Winson et al., 1998) were used. Bacteria expressing these genes will be bioluminescent. Light output from these bioluminescent bacteria is a highly sensitive reporter of metabolic activity (Marincs, 2000; Alloush et al., 2003), and can therefore, be used to monitor real-time biocide effects of HOO against MSSA, *E. coli* and *Pseudomonas aeruginosa* and to evaluate the biocidal effect of HOO on non-luminescent bacterial species and the yeast *Candida albicans.*

MATERIALS AND METHODS

Bacterial and fungal strains and growth conditions

Bacterial and fungal strains were obtained from the culture collections of the Department of Microbiology at the University of the West of England (UWE) and included *Escherichia coli* Nissle 1917, methicillin sensitive *S. aureus* (MSSA) pAL2, methicillin resistant *S. aureus* (MRSA) SMH 22115 LIewelyn*, P. aeruginosa* PAOI SEI and *C. albicans*. The *E. coli* (Saad et al., 2013), MSSA (Beard et al., 2002) and *P. aeruginosa* (Marques et al., 2005) isolates were previously transformed with a recombinant plasmid, containing the *luxCDABE* gene cassette to express bioluminescence. *E. coli,* MSSA MSSA and *P. aeruginosa* gene cassettes also contained kanamycin, erythromycin and gentamicin resistance genes, respectively, as a means to maintain the plasmid during cultivation by addition of low concentrations of appropriate antibiotic.

Bacterial strains were cultured overnight on nutrient agar, NA, (Oxoid Ltd, Basingstoke, UK) and incubated at 37°C (LTE Scientific incubator IP150-U, UK). *C. albicans* was cultured for 48 h on Sabouraud dextrose agar, SDA (Oxoid Ltd, Basingstoke, UK) at 30°C. Single isolated bacterial colonies from overnight NA culture were sub-cultured into nutrient broth, NB (Oxoid Ltd, Basingstoke, UK) and grown to early logarithmic phase (2, 3 and 2.5 h for *E. coli, S. aureus* and *P. aeruginosa*, respectively) at 37°C in a shaking incubator at 200 rev/min (Orbital incubator SI50, Stuart). Erythromycin, 25 µg/ml (Sigma, Dorset, UK) was included in all culture media containing MSSA with the *luxCDABE* operon, kanamycin, (Sigma, Dorset, UK), was added in all culture media for *E. coli* and gentamicin 10 µg/ml (Sigma, Dorset, UK) was added in all culture media containing *P. aeruginosa.*

Himalayan oregano oil (HOO), Mediterranean oregano oil (MOO), carvacrol and thymol production

HOO and MOO were provided by Biolaya Organics (Kullu, India), purified commercial carvacrol and thymol were purchased from Sigma-Aldrich.

Gas chromatography (GC)

Himalayan oregano oil in this study was analysed using a GC system with a flame ionization detector (FID) and HP5 column (Hewlett Packard, HP 6890 Series) to determine the relative percentage of carvacrol and thymol in the oil. Samples were manually injected using a 1 µl syringe. Each sample was run for 30 min. Carvacrol and thymol in the oil were identified according to the retention time, determined using purified commercial samples of carvacrol and thymol, on the chromatogram. The percentages of carvacrol and thymol were calculated from the slope of the best fit line of the resulting correlation between the percentage concentration and the area under the curve of the chromatogram of each component.

Agar incorporation assay

The minimal inhibitory concentration (MIC), defined as the lowest concentration of oregano oil, its components or MOO that inhibit the visible growth of bacteria after overnight incubation, were determined by the nutrient agar dilution method outlined by Andrews (2001). Selective nutrient agar (Oxoid Ltd, Basingstoke, UK) media was modified by the addition of Kanamycin or Erythromycin as appropriate. Oregano oil, carvacrol, thymol and MOO were added to the molten agar at 56°C as kanamycin and erythromycin are heat stable at this temperature (Traub and Leonhard, 1995). The final concentrations of essential oils in the medium ranged from 0.01 to 0.1% v/v. This solution was vigorously shaken while hot to allow mixing and even distribution of the oil. The inoculum was prepared from an 18 h bacterial culture. Four spots, each corresponding to 10 µl (10^4 CFU/spot) of the bacteria, was used to inoculate the prepared agar plates and incubated at 37°C for 18 to 24 h.

***In vitro* susceptibility testing of bacteria and yeast against oregano oil and its components**

E. coli, MSSA and *P. aeruginosa* that have been previously genetically modified with addition of *luxCDABE* operon to express bioluminescence were used as reporters of viable metabolically active cells, to show *in vitro* real time *in situ* antimicrobial effects of oregano oil and its components. A cell suspension density of 10^6 CFU/well was used by appropriate dilution of culture in phosphate buffered saline, PBS. At time zero, test agent (oregano oil or components) or positive control (0.03% bleach) were added and light detection from reaction wells was immediately measured (within 5 seconds) using the Sirius Berthhold Detection System (Pfarzheim, Germany). Quantitative bioluminescence in relative light units (RLU) was measured every minute over a period of 30 min, against each of the bioluminescent target species, using the single kinetic assay provided with the FB12 Sirius software detection system. For non-modified MRSA and *C. albicans*, a conventional viable count method was used to assess the antimicrobial effect of oregano oil. All experiments were replicated a minimum of three times. Results were plotted on a graph showing the rate of change of bioluminescence or viable count against time.

Correlation between bioluminescence and the conventional viable count method

The optical densities of bacterial suspensions in early logarithmic phase of growth were all adjusted to a McFarland standard of 0.5. The suspension was then serially diluted (10 fold dilution) up to 10^{-7} in NB containing the appropriate antibiotic. The Miles and Misra (or surface viable count) method (Miles et al., 1938) and automatic spiral platter (Whitley automatic Spiral Plater; Scientific Wasp) were used in conjunction with bioluminescence to evaluate the correlation between light output and bacterial viability for the serial dilutions prepared. Bioluminescence (RLU) was measured by transferring 1 ml from each serial dilution in a borosilicate glass tube (Fisherbrand, Loughborough, UK) and this tube was inserted into the Sirius Berthold Detection System, (Pfarzheim, Germany). Data were plotted and expressed as $\log_{10}$ RLU/s and CFU/ml

Biocidal effect of oregano essential oil and its components

A spot of 20 µl (10^5 CFU) of the bioluminescent bacteria (in log phase) was exposed to a known concentration of oregano oil for a period of time equivalent to the time taken to completely inhibit production of light (as determined by *in vitro* real-time susceptibility testing). The inoculum was then streaked onto a NA plate without antimicrobial agent, incubated at 37°C for 18-24 h and examined for growth of bioluminescent bacteria. Bioluminescence was visualized under a low light Anadore IXON D4-897 EMCCD camera (Figure 7a).

Statistical analysis

All tests were replicated a minimum of three times. Inactivation was expressed by reduction in $\log_{10}$ counts or RLU after every treatment. The error bars in the figures indicate the mean ± standard deviation from the data obtained from at least three independent experiments. The activity of a biocide (D-value) was calculated using the formula $D = (t_2\text{-}t_1)$ (log N_1-Log N_2) where N_1 and N_2 are survivors at times t_2-t_1 (Adams and Moss, 2007). The kill rates (k) were obtained from the slopes of the kill curves measured by regression analysis. T-test, R^2 and P value were calculated using Microsoft excel.

RESULTS

Minimal inhibitory concentrations (MICs)

Table 1 show that the MICs for oregano oil and its components were found to be between 0.03 (v/v) and 7% (v/v). The lowest MIC value against all the organisms

Table 1. MICs of different batches of Himalayan oregano oil, Mediterranean oregano oil, carvacrol and thymol against *E. coli*, MSSA, MRSA and *P. aeruginosa*.

Organism	Oregano oil and its components					
	HOO Aug. 2010*	HOO Oct. 2009*	HOO Jul.2009*	MOO	Carvacrol	Thymol
E. coli Nissle,	0.03% (v/v)	0.06% (v/v)	0.06% (v/v)	7.00% (v/v)	0.04% (v/v)	0.16% (v/v)
S. aureus RN2440 pUNK1 (MSSA)	0.03% (v/v)	0.06% (v/v)	0.06% (v/v)	7.00% (v/v)	0.04% (v/v)	0.16% (v/v)
S. aureus SMH 22115 LIewelyn (MRSA)	0.03% (v/v)	0.06% (v/v)	0.06% (v/v)	7.00% (v/v)	0.04% (v/v)	0.16% (v/v)
P. aeruginosa PAOI SEI	0.03% (v/v)	0.06% (v/v)	0.06% (v/v)	7.00% (v/v)	0.04% (v/v)	0.16% (v/v)

Table 2. Percentage of carvacrol and thymol in different batches of oregano oil as determined by GC.

Component	Essential oil			
	HOO August 2010	HOO October 2009	HOO July2009	MOO
Carvacrol	1.96%	0.55%	1.86%	7.00%
Thymol	36.93%	24.55%	26.67%	0.41%

tested was reported for the HOO batch from August 2010, while the highest value was reported for impure Mediterranean oregano oil (MOO). The MICs for one batch was the same for each bacterial species tested.

Gas chromatography analysis of HOO and MOO

Analysis of HOO and MOO by GC showed that the different batches of oregano oil employed contain different percentages of carvacrol and thymol (Table 2), whilst MOO had less thymol and more carvacrol than HOO. The retention time of carvacrol and thymol were obtained by comparing to analyse purified commercial samples of carvacrol and thymol. A large difference in the relative percentage of these two components was found between pure HOO oregano oil and impure MOO, as shown in Figure 1.

Correlation between bioluminescence and the conventional viable count method

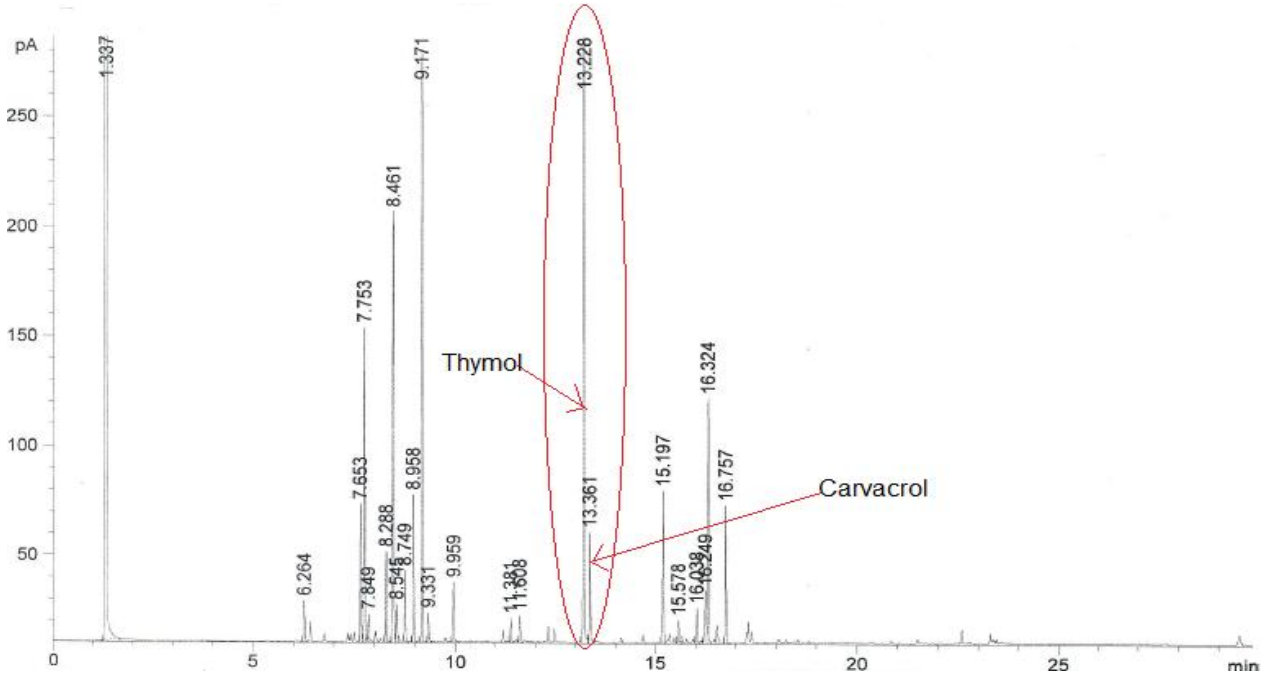
Results as shown in Figure 2 (a, b and c) support a positive correlation between bioluminescence (RLU/s) and viable count (CFU/ml) during logarithmic phase of growth (R^2 = 0.988; 0.9426 and 0.9004 P<0.0001) for *E. coli, P. aeruginosa* and MSSA, respectively.

In vitro susceptibility testing of oregano oil against bacteria and yeast

As illustrated in Figures 3 and 4, HOO reduced the bioluminescence emitted by the genetically transformed *E. coli, P. aeruginosa* and *S. aureus* during the 30 min. The highest rate of bioluminescence reduction (90%) occurred during the first minute of contact between bacteria and HOO and 99% reduction of bioluminescence in 15 min. The biocidal activity of oregano oil is described by means of the decimal reduction time (D-value), which is defined as the time in seconds required to reduce the population by one log fold at a fixed biocide concentration, and the kill rate was summarized in Table 3. Both the kill rate and D- value for *C. albicans* were found to be much lower than those for the bacterial strains tested. Figure 3 supports a positive correlation between reduction in bioluminescence and CFU/ml of *E. coli* Nissle. exposed to 0.03% as no significant difference between the two methods was observed (T test P=0.8485). Oregano oil (0.03%) also demonstrated biocidal effect against *C. albicans* where an 80% reduction of CFU occurred in 15 min (Figure 5).

Experiments on shelf storage at room temperature in the dark of HOO from the same batch as the fresh HOO used for this study showed oregano oil to be very stable over a period of two months in terms of both its biological inhibitory

(a)



(b)

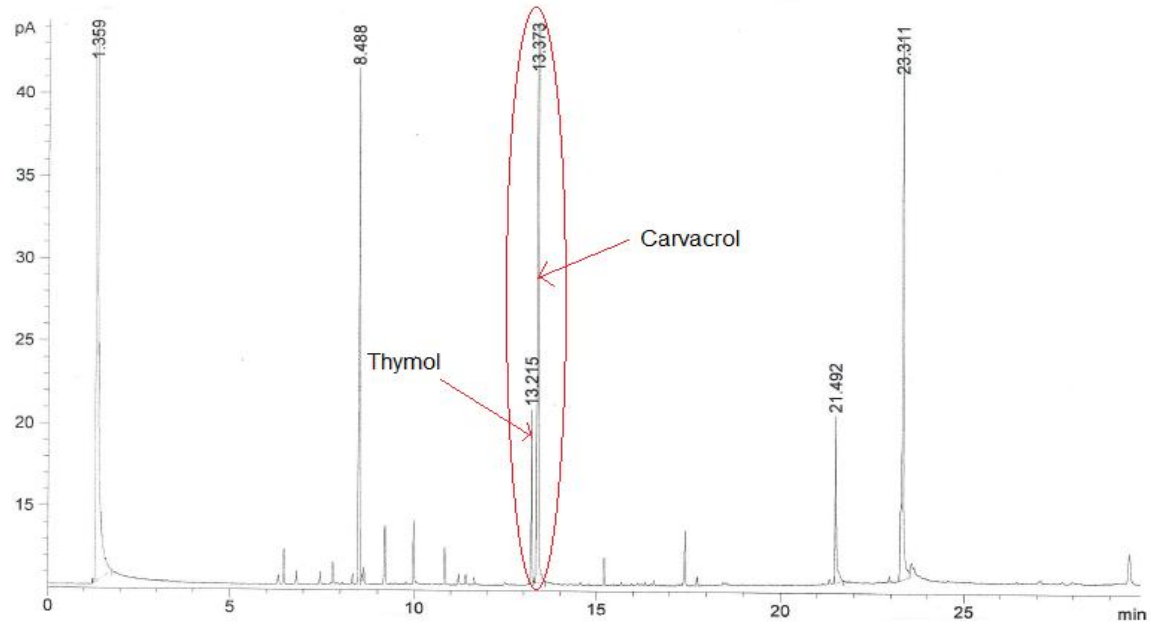


Figure 1. Chromatogram showing the relative prescence of thymol and carvacrol in Himalayan Oregano oil (a) and Mediterranean Oregano oil (b) pA: height in picoamperes; numbers on the peaks are the retention time in minutes (time spent on the column, in minutes, for the component to be analysed).

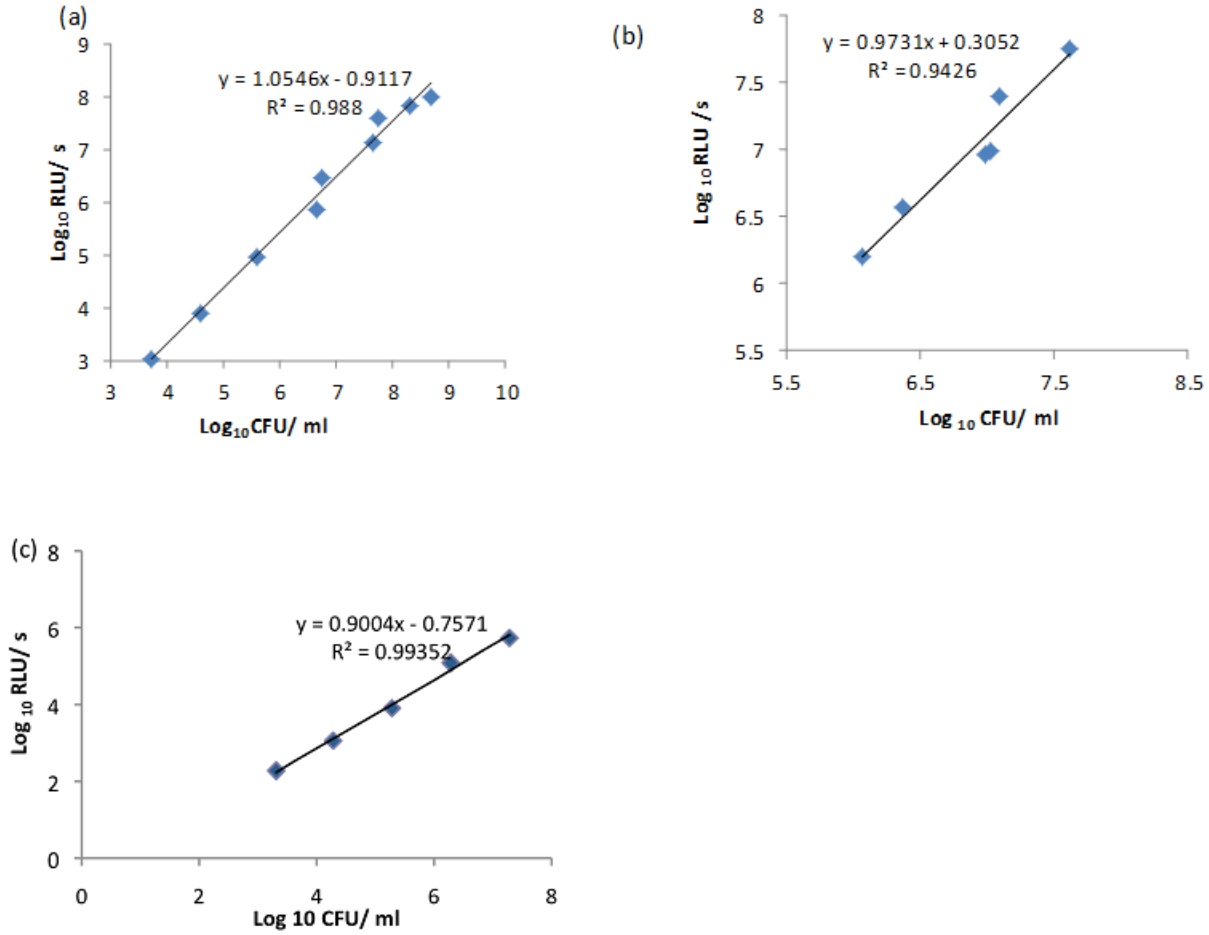


Figure 2a, b and c. Relationships between viable count (log_{10} CFU/ml) and bioluminescence (log_{10} RLU/s) of lux recombinant *E. coli* (a), *P. aeruginosa* (b) and MSSA (c).

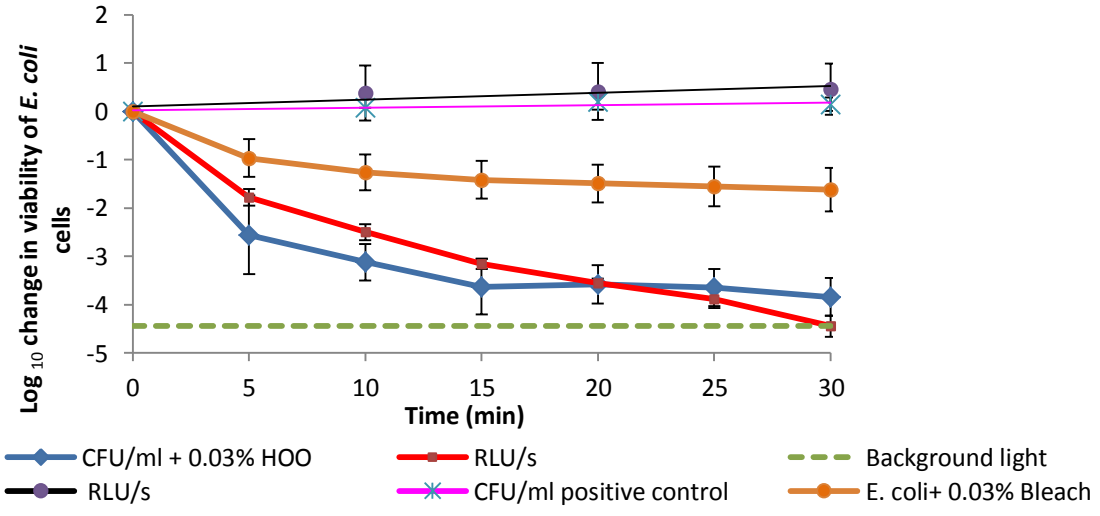


Figure 3. Reduction in bioluminescence and CFU/ml of *E. coli* Nissle exposed to 0.03% Himalayan oregano oil. Number of treated cells = 10^6/well. T test P= 0.8485. Error bars show the mean and ± one standard deviation of 3 replicates.

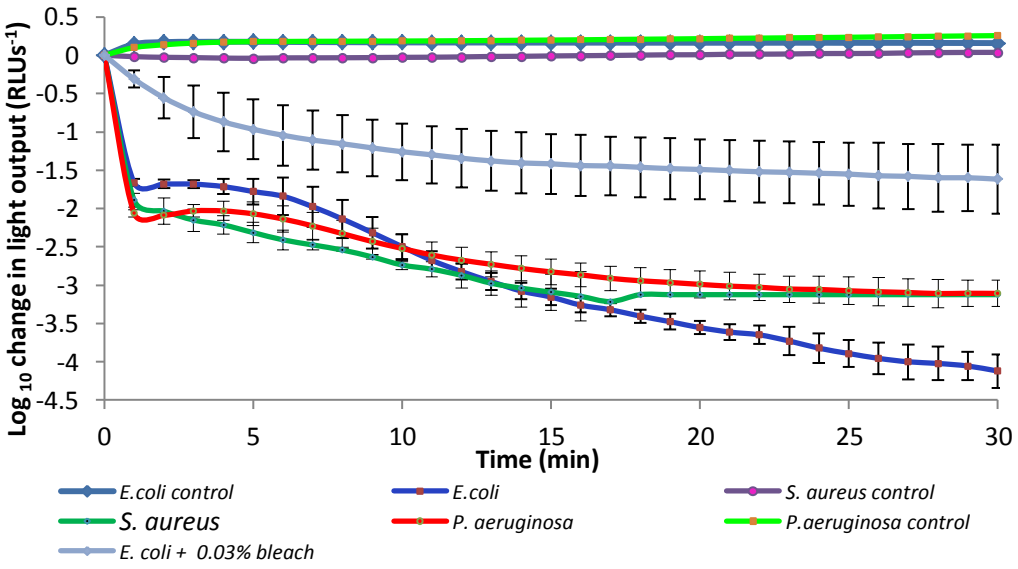


Figure 4. Reduction in bioluminescence from *E. coli*, *S. aureus* and *P. aeruginosa* exposed to 0.03% Himalayan oregano oil. Number of treated bacterial cells 10^6/well. Error bars show mean and ± one standard deviation; number of replicate = 3.

Table 3. Comparison of kill rates, calculated D-values for the first ten seconds and after 300 s (with zero to 2 s time lag between addition of compounds).

Species	K (mean kill rate/sec) First 10 seconds	K (mean kill rate/sec) After 300 second	D-value (s) First 10 seconds	D-value (s) After 300 seconds
E. coli	0.0325 ± 0.0062	0.0015± 0.0002	3.670± 0.350	3527± 4660
S. aureus	0.0327 ± 0.0154	0.0008± 0.0004	2.950± 0.350	2159± 2.830
P. aeruginosa	0.0360 ± 0.0000	0.0012± 0.0006	2.600± 1.500	2146± 109.6
C. albicans	0.0009 ± 0.0000	0.0003± 0.0001	365.8± 76.67	1144± 324

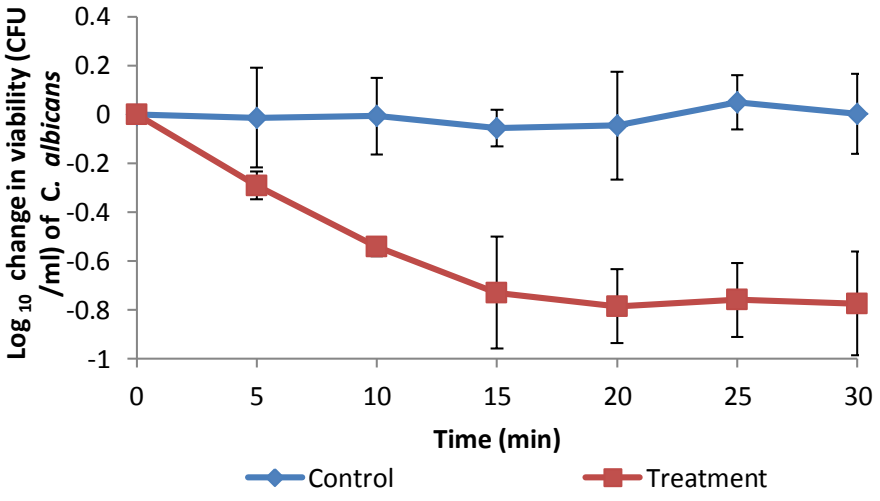


Figure 5. Reduction in CFU/ml of *C. albicans* exposed to 0.03% Himalayan oregano oil. Number of treated cells 10^6/well. Error bars show mean and ± one standard deviation; number of replicate = 3.

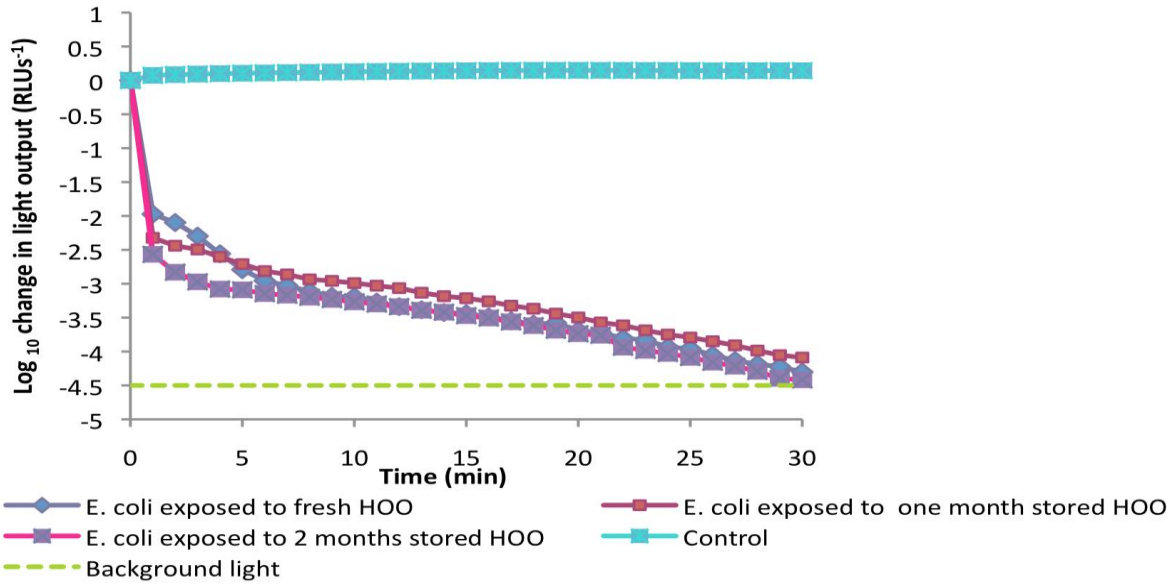


Figure 6. Reduction in bioluminescence from *E. coli* exposed to 0.1% Himalayan oregano oil stored over time. Number of treated cells 10^6 /well.

activity and chemical components (Figure 6). For this experiment, a high concentration of (0.1%) HOO was used in expectation of decay with time; however, the material was too stable to determine its activity decay with time.

Biocide effect of oregano essential oil and its components

Both *E. coli* and MSSA were left in contact with the oregano oil (at 10xMIC) on culture plate for a time period ranging from 20 s to 30 min. Bacteria were then removed from the plate and sub-cultured in oregano oil free media, incubated at 37°C for 18-24 h to detect survival and recovery. Figure 7a shows the bioluminescence and the reduction of number of colonies of the *E. coli,* when left in contact with HOO for a period ranging from 20 to 190s and sub-cultured in oregano oil free media at 37°C for 18-24 h. At 190s exposure, the growth was completely inhibited. Figure 7b captures the same image under normal light.

DISCUSSION

The MICs values for different batches of HOO were found to be between (0.03-0.06% v/v) against MSSA, MRSA, *E. coli* and *P. aeruginosa.* The slight differences revealed between the MICs, had also been determined in previous studies (Nostro et al., 2004; Fabian et al., 2006) and may be explained by the composition of the oils tested, in particular the carvacrol and thymol content which are believed to be the principal inhibitory components in the oil (Lambert et al., 2001). The results for thymol and carvacrol were similar to those published by Nostro et al. (2004) which indicated that carvacrol is a more potent antimicrobial agent than thymol. On the other hand, the MIC for impure MOO (7% v/v) in this study, was found to be significantly higher than 0.06-0.125% v/v published by Nostro et al. (2004). This can be explained by the results of the GC which showed a very low amount of thymol (0.41%) and 7% of carvacrol in the present study as compared to 24.7 and 14%, respectively found by Nostro et al. (2004). These findings therefore suggest that the total amount of carvacrol and thymol are very important when predicting the antimicrobial properties of the oil. Among the batches of HOO investigated, the lowest MIC was found for the batch August 2010 (0.03%) as compared to 0.06% of the July 2009 and October 2009 batches. This also can be explained by the relatively high amount of carvacrol and thymol found in August 2010 batch as compared to other batches. This discrepancy in thymol, carvacrol contents could be due to the harvest time (Ozken, et al., 2010), geographic location (Bishat et al., 2009) or the drying method of the plant (Figiel et al., 2010). The data in Table 1 showed no difference between the susceptibility of MRSS and MSSA in the oregano oil and its components. This result correlates well with the data published by Nostro et al. (2004) which also showed no significant difference between the susceptibility of 9 MRSS strains and 15 MSSA strains to MOO.

In vitro, real-time death kinetics was carried out using HOO (batch August 2010), against lux recombinant *E. coli*, *P. aeruginosa* and MSSA. Figures 3 and 4 showed a vast reduction in bioluminescence (90%) emitted by the three genetically modified strains in the first minute of the contact of the antimicrobial agent at the MIC. This finding

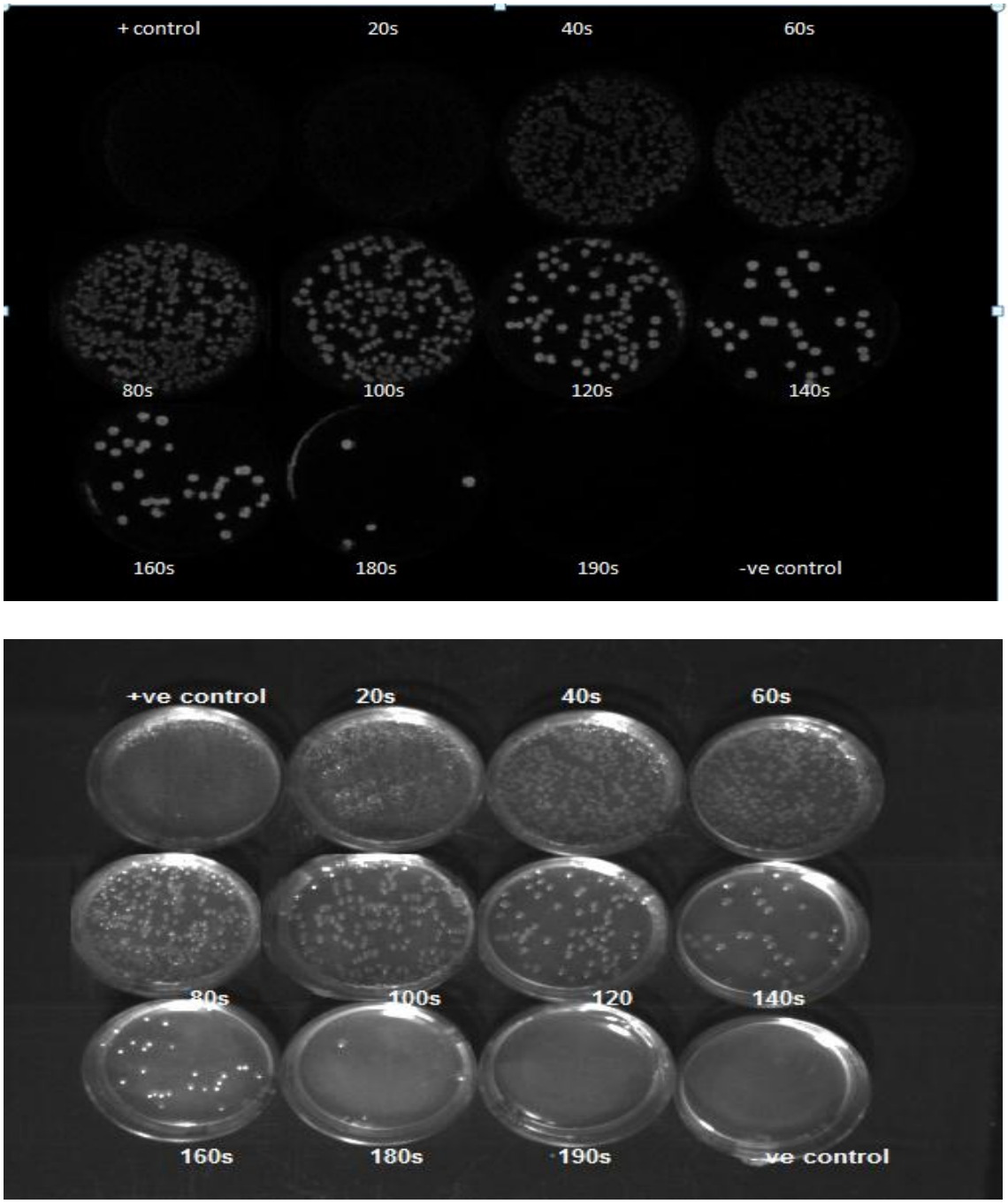


Figure 7. *E. coli* lux+ after exposure to 10x MIC HOO for different periods of time. (a) bioluminescent colonies; (b) colonies under normal light (not bioluminescence). + control is positive for lux without exposure to HOO.

concurs with the results obtained by Souza et al. (2010) which indicates that loss of potassium ions occurred immediately after addition of the essential oil at 0.6 and 1.2 microLmL^{-1} and followed up to 120 min. This may be explained by the extremely lipophilic nature of carvacrol, thymol and the other numerous hydrocarbons that oregano oil contains. These lipophilic compounds encounter a bacterial lipid membrane, passively diffuse and accumulate in the hydrophobic core of the membrane affecting the bacterial cell membrane functionally and structurally causing loss of membrane integrity which is vital for the survival of the bacteria (Sikkema et al., 1995). After this reduction in the first minute, the rate of loss of bioluminescence reduces briefly, and it reaches 99% reduction in less than 15 min. On the other hand, *C. albicans* showed kill rate of 0.0009 s^{-1}

(Table 3) which, is on average 37.5- fold lower as compared to the studied bacteria. However, an overall 80% reduction in 15 min of C. *albicans* indicates that HOO can also be used as an antifungal agent. Results for biocidal effect after sub-culture in antimicrobial free media (Figure 7a and b) showed that the positive control lacked bioluminescence due to loss of plasmid and/or substrate starvation on a confluent plate after 18 h at 37°C. The plates of 140 to 180 s where there are fewer colonies are brighter than those at 40 and 60 s, which support this. Complete inhibition of light was related to complete death of the bacteria tested with exception of MOO, which showed some recovery of *E. coli* when left for a period that shows complete inhibition of light. This may be due to reduction in light of bacteria to a boundary beyond the detection limit of the luminometer or it may be that the bacteria can remain metabolically inactive for a period of time and recover again in the absence of the killing agent. The lux genes are on a plasmid, but it is unlikely to be lost in the presence of the selective antibiotic (antibiotic resistant genes are on the same plasmid). These results showed that HOO has exhibited a bactericidal effect towards *E. coli* within 3 min and 40 s. These results showed that bioluminescence emitted by *E. coli* containing *lux* genes correlate not only with the level of metabolic activity of the bacteria during early log phase, but also with the bacterial cell viability. De Barros et al. (2009) demonstrated that oregano oil causes a biocidal effect on *S. aureus* using the viable count method. However, to the best of our knowledge, no study has been reported in the literature using bioluminescent reporters to analyse the antimicrobial properties of oregano oil and its components. Beard et al. (2002) demonstrated that expression of bioluminescence had a negligible effect upon both growth rate and MIC, which indicates that the effect of HOO should be relevant to the wild-type strains.

We cannot conclude that HOO is more effective than MOO according to results of this study, since the starting formulation is different (HOO is neat oil while olive oil is added to the MOO, the only preparation of MOO that is available for this study) but we can conclude that total carvacrol and thymol content of oregano oil is important when predicting the antimicrobial properties.

Shelf storage experiments over a period of two months showed that oregano oil is stable in terms of both its biological inhibitory and chemical components; too stable to determine its bio-inhibitory activity decay with time (or half-life) (Figure 6).

HOO may have the potential as a natural potent bactericidal agent in the health care setting as it has demonstrated bactericidal action towards significant representative members of the common UK bacterial pathogens including MRSA and fungal pathogens, and also could be used as a surface disinfectant. Thus, HOO could make its way from the traditional flavouring use, to the medical field by its potentiality to be a useful addition to the current repertoire of hospital disinfectants. As our results support a high positive correlation between bioluminescence (RLU/s) and viable count (CFU/ml) during logarithmic phase of growth (R^2 =0.988; 0.9426 and 0.9004 P<0.0001), we can conclude that bioluminescence technique, by measurement of light output, provides a rapid accurate means of collecting data of kill rates, which are captured in real-time with high reproducibility and fidelity of responses such that comparisons are accurate on the sustainable bactericidal action of oregano oil. As compared to the viable count method, it has the potential to replace plate culture methods for rapid and convenient evaluation of the efficacy of biocidal product. The main inventive step on this study, involved the application of bioluminescent constructs of bacterial pathogens as target biosensor species of antimicrobial action, measuring rates of kill with fast sample times in a highly reproducible system and by comparison with control and bleach as a positive control killing agent.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Abbreviations: pA, Height in picoamperes; **HOO,** Himalayan oregano oil; **MOO,** Mediterranean oregano oil; **MIC,** minimum inhibitory concentration; **RLU,** relative light unit; **CFU,** colony forming unit.

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