

The background of the cover is a detailed, colorful illustration of various microorganisms. It features several large, rod-shaped bacteria with textured surfaces, some with flagella. Interspersed among them are numerous spherical viruses, many of which are covered in sharp, radiating spikes. The color palette is dominated by warm tones of orange, yellow, and red, with some cooler blue and purple accents, creating a vibrant and scientific atmosphere.

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Laboratory of Conservation and Utilization for Bio-resources

Key Laboratory for Microbial Resources of the Ministry of Education,

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School of Life Science,

Yunnan University, Kunming,

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221005 India

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Dr. Somboon Tanasupawat

Department of Biochemistry and Microbiology, Faculty

of Pharmaceutical Sciences, Chulalongkorn University,

Bangkok 10330 Thailand

Dr. Vivekananda Mandal

*Post Graduate Department of Botany,
Darjeeling Government College,
Darjeeling – 734101.
India*

Dr. Shihua Wang

*College of Life Sciences,
Fujian Agriculture and Forestry University
China*

Dr. Victor Manuel Fernandes Galhano

*CITAB-Centre for Research and Technology of Agro-
Environment and Biological Sciences, Integrative
Biology and Quality Research Group,
University of Trás-os-Montes and Alto Douro,
Apartado 1013, 5001-801 Vila Real
Portugal*

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*Institute for Surgical Pathology,
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*Department of Ecosystem Biology, Faculty Of Science,
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*CNR-Istituto di Scienze delle Produzioni Alimentari
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*Chung Shan Medical University
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*Department of Clinical Microbiology and Immunology,
College of Medical Laboratory,
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China*

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*School of Biotechnology,
Banaras Hindu University, Varanasi
India*

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Hospital, Taipei, Taiwan*

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*Aquatic Animal Health Research Unit, USDA, ARS
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Laboratory Service
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South Africa*

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Wroclaw Teaching Hospital,
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ARTICLES

- Efficacy of *Acinetobacter baumannii* bacteriophage cocktail on *Acinetobacter baumannii* growth** **2159**
Thawatchai Kitti, Rapee Thummeepak, Udomluk Leungtongkam, Duangkamol Kunthalert, and Sutthirat Sitthisak
- Antibacterial and hemolytic activities of *Mimosa tenuiflora* (Willd) Poir. (Mimosoidea)** **2166**
Sônia Carmem de Morais Leite, Cássio Ilan Soares Medeiros, Paula Christianne Gomes Gouveia Souto Maia, Maria Iranilda Silva Magalhães, Francisco Orlando Rafael Freitas, Hilzeth de Luna Freire Pessôa, Tiago Bezerra de Sá Sousa Nogueira, Alanna Michely Batista de Morais, Vandezita Dantas de Medeiros Mazzaro, Vanessa Passos Brustein and Geraldo Gonçalves de Almeida Filho
- Efficacy of oregano oil as a biocide agent against pathogens in vitro, using lux reporter gene technology** **2172**
Nahla Omer ELtai, Vyvyan Salisbury and John Greenman

Full Length Research Paper

Efficacy of *Acinetobacter baumannii* bacteriophage cocktail on *Acinetobacter baumannii* growth

Thawatchai Kitti¹, Rapee Thummeepak², Udomluk Leungtongkam², Duangkamol Kunthalert², and Sutthirat Sitthisak^{2,3*}

¹Faculty of Oriental Medicine, Chiang Rai College, Chiang Rai, 65000, Thailand.

²Department of Microbiology and Parasitology, Faculty of Medical Sciences, Naresuan University, Phitsanulok, 65000, Thailand.

³Centre of Excellence in Medical Biotechnology, Faculty of Medical Science, Naresuan University, Phitsanulok, 65000, Thailand.

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Acinetobacter baumannii is an opportunistic pathogen which is a major cause of nosocomial infection. Bacteriophages are bacterial viruses that are used as alternative agents in the treatment of multidrug-resistant bacterial infections. In this research, our purpose was to investigate the efficacy of a cocktail of five bacteriophages. Ten (10) bacteriophage isolates were determined for the host range analysis and five bacteriophages, ØABP-02, ØABP-19, ØABP-29, ØABP-39 and ØABP-44 that showed broad host range (36.9-64.6 %) were selected for the preparation of the bacteriophage cocktail. Transmission electron microscopy revealed ØABP-02 and ØABP-44 belonged to family *Myoviridae* and ØABP-19, ØABP-29, ØABP-39 belonged to family *Podoviridae*. The bacteriophage cocktail was tested for its efficacy on growth inhibition against 44 *A. baumannii* clinical isolates using the colorimetric microtiter plate method. The results of the growth inhibition assay of the bacteriophage cocktail showed that the growth inhibition against *A. baumannii* ranged from 45.1-96%. High efficacy of the bacteriophage cocktail was found against the *A. baumannii* strains that can be infected by the five bacteriophages (>77%). Our study demonstrates high efficacy of the bacteriophage cocktail on inhibiting the growth of *A. baumannii*. The bacteriophage cocktail is a valuable alternative agent for controlling the multi- drug resistant *A. baumannii* in hospitals.

Key words: Bacteriophage, *Acinetobacter baumannii*, growth inhibition, host range.

INTRODUCTION

A. baumannii are opportunistic pathogens that cause a variety of infections such as respiratory tract infections, urinary tract infections, skin infections and bacteremia. *A.*

baumannii have developed antibiotic resistance which is a serious problem in the treatment of these infections. The infections of multi-drug resistant *A. baumannii* (MDR-

*Corresponding author. E-mail: sutthirats@nu.ac.th. Tel: 66-55-964626, 66-84-5734203. Fax: 66-55-964770.

AB) and extensively drug-resistant *A. baumannii* (XDR-AB) are of particular concern in patients in intensive care and burns units which cause high mortality rates (Alp et al., 2012; Park et al., 2013). In recent decades, the prevalence of MDR-AB and XDR-AB has increased worldwide. MDR-AB and XDR-AB infected patients must stay longer in hospital than patients without such drug resistant infections. This leads to a higher cost of hospitalization. Bacteriophage therapy has been claimed to be a potential candidate for the treatment of MDR-AB and XDR-AB. Bacteriophages are viruses that specifically infect bacteria and kill the host cell when the progeny of the phage particles leave the cells. So far, more than 10 *A. baumannii* bacteriophages have been isolated and investigated for their biological properties (Chang et al., 2012; Jin et al., 2012; Kitti et al., 2014; Lee et al., 2011; Lin et al., 2010; Merabishvili et al., 2014; Popova et al., 2012; Shen et al., 2012; Thawal et al., 2012; Yang et al., 2010; Yele et al., 2012). These bacteriophages have been examined for being potential candidates in phage therapy. The host range of bacteriophages is significant for use as antibacterial agents. However, narrow host specificity of *A. baumannii* bacteriophages, ranging from 13-59%, have been reported from different geographical isolates (Jin et al., 2012; Merabishvili et al., 2014; Thawal et al., 2012). Consequently, to assure that bacteriophages possess a broad host range activity that includes the target bacteria, multiple phage types possessing a diversity of host ranges are often combined into mixtures called 'phage cocktails' (Chan et al., 2013). Phage cocktails have been applied to improve the chances of success in bacterial killing in the treatment of *Pseudomonas aeruginosa* and *E. coli* O157:H7 (Chan et al., 2013). Due to the narrow host range of *A. baumannii* bacteriophages, the objective of our study was to screen new bacteriophages with broad host range activity and determine the efficacy of a *A. baumannii* bacteriophage cocktail in inhibiting the growth of *A. baumannii*.

MATERIALS AND METHODS

Bacterial strains

Eleven MDR-AB isolates obtained from Buddhachinaraj hospital, Phitsanulok, Thailand were used as host bacteria for bacteriophage isolation (Niomsup et al., 2009). Sixty-five clinical isolates of *A. baumannii* obtained from Sawan Pracharak Hospital, Nakorn Sawan, Thailand were used for growth inhibition assay. Bacteria were cultivated in Luria - Bertani broth (LB) or Luria - Bertani Agar (LBA). Confirmation of the *Acinetobacter* species was based on biochemical tests and detection of the 16S rRNA gene.

Isolation of bacteriophage

All bacteriophages were isolated from wastewater treatment plants from two hospitals in Phitsanulok Province. Samples were collected and centrifuged to remove debris. The supernatant was filtered. Then, 5 ml of the filtered supernatants were mixed with 5 ml of double strength broth containing overnight culture *A.*

baumannii. After 48 h, the culture was centrifuged, and the supernatant was used for the detection of lytic bacteriophages by a double-layer method. Phage enrichment and purification were performed as described by Kitti et al. (2014).

Host range analysis

Host range analysis of the ten bacteriophages was determined by spot tests. Hundred microliter of overnight bacterial cultures were added to 2.5 ml of soft agar, mixed gently and poured into an agar plate. Subsequently, 5 µl aliquots of phage suspension (1.0×10^8 PFU) were spotted on the lawn of bacteria. Plates were dried and incubated at 37°C for 7 h. The clearance zone indicating lysis at the spot of phage inoculation implied that the host was sensitive.

Morphology of bacteriophages

Five bacteriophages which showed high lytic activity were used to determine the morphology. Phage suspension (10^{12} PFU/ml) was dropped onto the surface of a formvar-coated grid and negatively stained with 0.5% uranyl acetate for 3-5 min. After drying, the preparations were observed in a transmission electron microscope (Philips, Oregon, USA).

Bacteriophage enrichment and preparation of bacteriophage cocktail

A. baumannii were grown in 100 ml LB broth until OD₆₀₀ reached 0.4. Phages were added at a multiplicity of infection (MOI) of 0.5 and incubated at 37°C until complete lysis. Then, 2-3 ml chloroform was added and bacterial debris was pelleted by centrifugation at 4000 g for 10 min. Each phage was enriched three times and the phage concentration was determined using the plaque assay method (Kitti et al., 2014). The phage stocks were stored at $4 \pm 1^\circ\text{C}$. Bacteriophage titer was determined before a growth inhibition assay was performed. The titer of each bacteriophage was determined and diluted into 10^6 PFU/ml in SM buffer. Each bacteriophage was mixed (1:1:1:1:1) to prepare the bacteriophage cocktail.

Growth inhibition assay

A bacterial growth inhibition assay was performed as described by Knezevic and Petrovic (2008) using the cocktail of five *A. baumannii* bacteriophages (Knezevic and Petrovic, 2014). Briefly, *A. baumannii* were cultured in LB broth incubated at 37°C with shaking at 150 rpm / min for 24 h.

The overnight cultures were diluted (1:100) into LB broth and incubated at 37°C with shaking at 150 rpm/ min until OD₆₅₀ reached 0.4 then 50 µl (approximately 2×10^8 CFU/ml) were inoculated in 50 µl double strength LB broth into 96-well microtiter plates wells flat bottom (Nunc, USA). One hundred microliters of the five bacteriophage cocktail was added to each well. Then 50 µl of 0.1% filter sterilized TTC (Hi-media) was added. The plate was incubated at 37°C in the dark for 24 h. The absorbance at OD₅₄₀ was measured by a Micro-plate Reader. The experiment was replicated twice with triplicate samples. The percentage inhibition of the bacteriophage cocktail against all *A. baumannii* was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{the absorbance of controls} - \text{the absorbance of treated wells}}{\text{the absorbance of controls}} \times 100$$

Table 1. Characteristics and host range of bacteriophages used in this study.

Phage Isolates	Host bacteria	Plaque size (mm)	Host range 1* N = 11 (%)	Host range 2** N = 65 (%)
ØABP-01	A1589	5-8, Clear	11 (100)	6 (9.23)
ØABP-02	A1389	6-8, Clear with turbid	5 (45.45)	32 (49.23)
ØABP-04	A1522	3-5, Clear with turbid	6 (54.54)	16 (24.61)
ØABP-05	A1521	3-5, Clear with turbid	6 (54.54)	0 (0)
ØABP-07	A1386	3-5, Clear	4 (36.36)	3 (4.62)
ØABP-19	A1589	5-7, Clear	8 (72.72)	41 (63.07)
ØABP-24	A1244	3-5, Clear	2 (18.18)	3 (4.62)
ØABP-29	A1589	5-7, Clear	8 (72.72)	42 (64.61)
ØABP-39	A1511	1-2, Clear	5 (45.45)	33 (50.8)
ØABP-44	A1244	4-6, Clear	3 (27.27)	24 (36.92)

Host range was determined using spot test analysis. Host range1*: Host range was determined using 11 MDR-AB isolates obtained from Buddhachinaraj hospital, Phitsanulok, Thailand. Host range2**: Host range was determined using clinical *A. baumannii* isolates from Sawan Pracharak Hospital, Nakorn Sawan, Thailand.

RESULTS

Isolation of lytic phage

Fifty-four isolates of *A. baumannii* bacteriophages were collected from two treatment plants. Ten isolates with high lytic activity on a broad range of 11 *A. baumannii* were selected for further characterization. These are designated as ØABP-01, ØABP-02, ØABP-04, ØABP-05, ØABP-07, ØABP-19, ØABP-24, ØABP-29, ØABP-39 and ØABP-44 (Table 1).

Host range analysis

Ten (10) *A. baumannii* bacteriophages isolated from hospitals in Phitsanulok Province were used to investigate the ability to infect 65 *A. baumannii* isolated from a hospital in Nakorn Sawan Province. Five bacteriophages that showed a broad host range were ØABP-02(49.23%), ØABP-19 (63.07%), ØABP-29(64.61%), ØABP-39(50.8 %) and ØABP-44(36.9 %) (Table 1). Five that showed low specific host ranged from 0-25.75% were ØABP-01, ØABP-04, ØABP-05, ØABP-07 and ØABP-24 (Table 1). ØABP-02, ØABP-19, ØABP-29, ØABP-39 and ØABP-44 were selected in this study for the preparation of the bacteriophage cocktail.

Bacteriophage susceptibility type of *A. baumannii*

We classified 65 *A. baumannii* by spot tests using five bacteriophages, into 12 groups; A-L (Table 2).

Among the bacterial strains tested, 21 were bacteriophage non-susceptible strains (group A) and were also non multi-drug resistant strains. Fourteen *A. baumannii* were susceptible to infection by 5

bacteriophages. All of them were multi-drug resistant bacteria and belonged to group L (Table 2).

Phage morphology

To classify ØABP-02, ØABP-19, ØABP-29, ØABP-39 and ØABP-44 into morphology specific groups, phage particles were examined with an electron microscope. Our data shows ØABP-19, ØABP-29 and ØABP-39 have an icosahedral head (70,110, 70 nm) with a short tail, belonging to the *Podoviridae* family (Figure 1a, b and c). ØABP-02 and ØABP-44 showed a contractile tail (60, 11 nm) and an icosahedral head (80, 44 nm) (Figure 1d and e), a morphology characteristic of the family *Myoviridae*. All five bacteriophages were tailed bacteriophages and identified as members of order *Caudovirales*.

Effect of bacteriophage cocktail on *A. baumannii* growth

We used the colorimetric microtiter plate method to determine the efficacy of the five bacteriophages in inhibiting *A. baumannii* growth. The ratio of bacteriophage to infected target host was preliminarily determined using one bacteriophage. We found that the amount of bacteriophage and *A. baumannii* that showed good inhibition effects was 10^6 PFU/ml and 10^8 CFU/ml (data not shown). The effects of the bacteriophage cocktail on bacterial growth was tested using 44 *A. baumannii*. The results of the growth inhibition assay using the bacteriophage cocktail are presented in Table 2 and Figure 2. The bacteriophage cocktail exhibiting the growth inhibition of *A. baumannii* ranged from 45.1-96% (Figure 2). *A. baumannii* that can be infected with the five bacteriophages (group L) showed the percentage growth

Table 2. Percent inhibition of phage cocktail and *A. baumannii* bacteriophage susceptibility pattern.

Typing	Phage susceptibility pattern	MDR (%)	Non-MDR (%)	% inhibition of phage cocktail
A	Non-susceptible	0(0)	21 (32.3)	-
B	ØABP-19	1(1.5)	0 (0)	47.8±3.6
C	ØABP-29/19	4(6.2)	0 (0)	45.1±8 - 80.7±3.3
D	ØABP-29/39	1 (1.5)	0 (0)	77.6±2.98
E	ØABP-2/44	1(1.5)	0 (0)	91.2±1.58
F	ØABP-2/19/29	2(3.1)	0 (0)	73.8±3.3 - 92.4±1.2
G	ØABP-19/29/39	1(1.5)	0 (0)	57.2±2.7
H	ØABP-2/19/29/39	10(15.5)	0 (0)	45.4±2.8 - 95.0±0.7
I	ØABP-2/19/29/44	2(3.1)	0 (0)	73.8±2.9 - 84.6±3.0
J	ØABP-2/29/39/44	1 (1.5)	0 (0)	91.6±5.37
K	ØABP-19/29/39/44	6 (9.2)	0 (0)	86.8±5.7 - 94.5±0.2
L	ØABP- 2/19/29/39/44	15 (23.1)	0 (0)	77.4±3.2 - 95.7±0.4

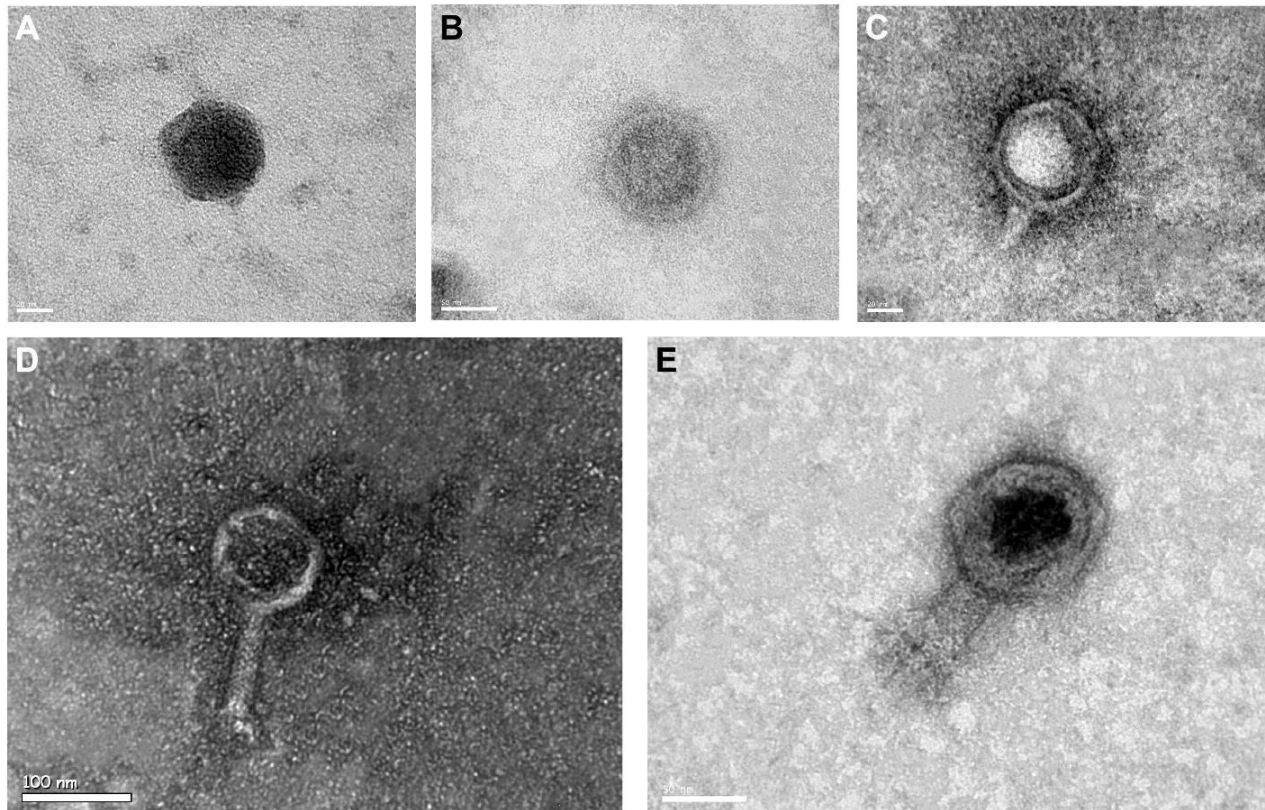


Figure 1. The Transmission Electron Micrograph of the *Podoviridae* (A,B,C) and *Myoviridae* (D,E) family of bacteriophages. ØABP-19(A), ØABP-29(B) and ØABP-39(C) belonging to the *Podoviridae* family. ØABP-02 (D) ØABP- 44 (E) belonging to the *Myoviridae* family.

inhibition higher than 77% (Figure 2). No growth inhibition was found in the non MDR-AB strains which are bacteriophage non susceptible strains (group A).

DISCUSSION

The bacteria–phage interactions, host range and dosage

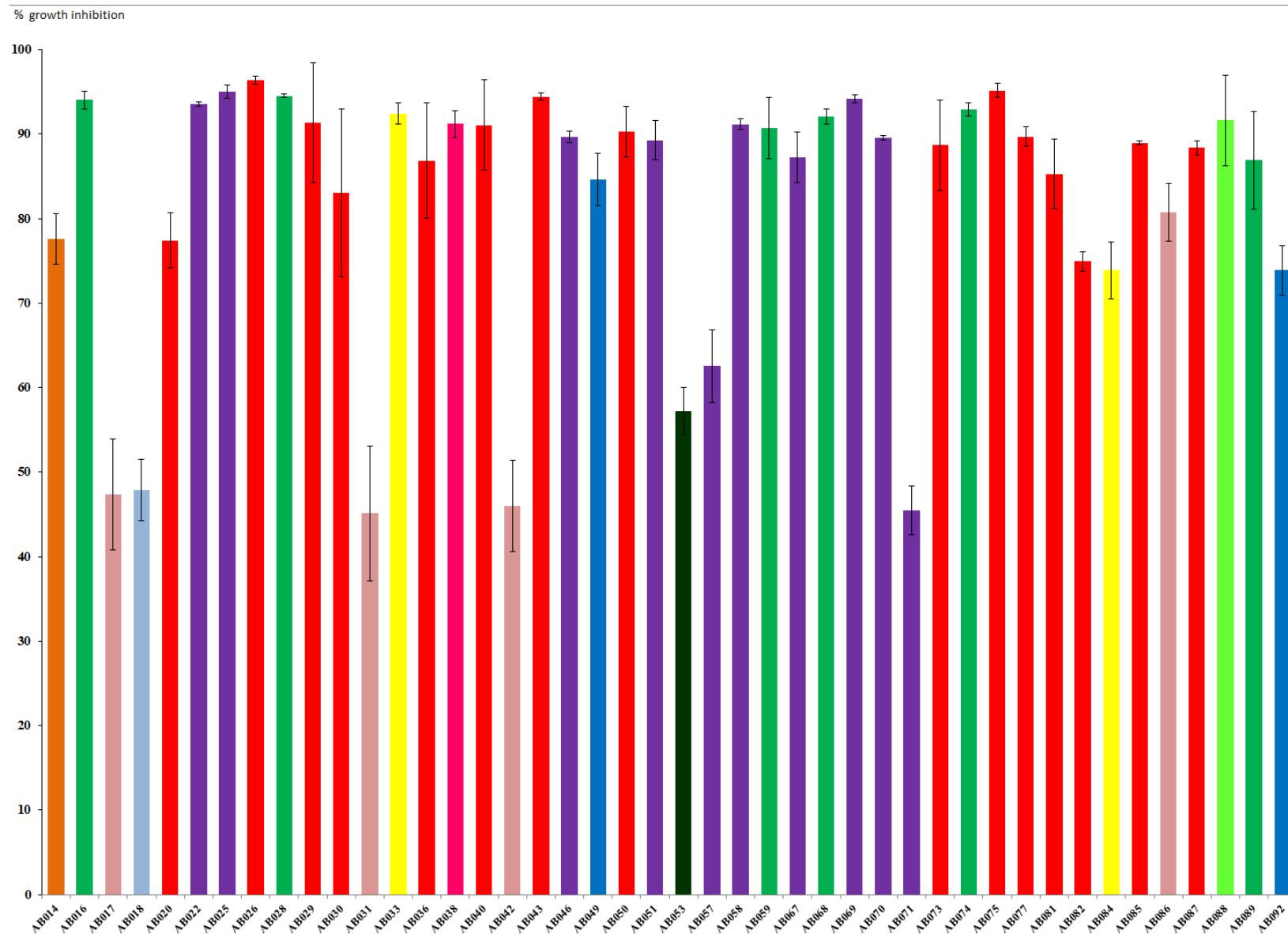


Figure 2. *In vitro* efficacies of the bacteriophage cocktail against different *A. baumannii* bacteriophage susceptibility group. Each bar represents the percent growth inhibition against *A. baumannii* strains. The color of the bar represents the bacteriophage susceptibility group; group B, light blue; group C, brown group D, orange; group E, pink; group F, yellow; group G, black; group H, violet; group I, dark blue; Group J, light green; group K, dark green; group L, red.

are important information to the successful development of phages for therapy (Levin and Bull, 2004; Hyman and Abedon, 2010).

In this study, we determined the host range of ten bacteriophages to identify five potential bacteriophages as candidates for developing phage cocktails. Our results classified 65 *A. baumannii* into 12 bacteriophage susceptibility groups (Table 2). The phage-susceptibility test provides a specific method for differentiating the MDR-AB from non MDR-AB. *A. baumannii* strains in group L (14 isolates) were infected with the five bacteriophages, whereas *A. baumannii* strains in group A (21 isolates) were non-bacteriophage susceptible strains. All of the strains in group A are non-MDR-AB. Bacteria have evolved several adaptive immunity mechanisms to resist bacteriophage infection. These include adsorption resistance which results in reduced interaction between phage and bacterium (Hyman and Abedon, 2010). The variety of receptor sites on the bacterial surface are involved in phage adsorption and penetration into the host cell (Rakhuba et al., 2013). In addition, the differences in the cell wall polysaccharide structure receptor on the bacterial surface are major factors in bacteriophage sensitivity (Ainsworth et al., 2014). The diversification of restriction–modification (RM) systems include phage-genome uptake blocks, superinfection immunity, restriction modification, and the generation of anti-phage sequences in CRISPR loci are also immunity mechanisms to resist bacteriophage infection after phage adsorption (Hyman and Abedon, 2010; Bikard et al., 2013). A previous study showed that ØABP-01 exhibited high lytic activity and a broad host range (100%) when using *A. baumannii* host isolated from the same hospital as the bacteriophage isolated (Kitti et al., 2014). Our data indicates that ØABP-01 showed narrow host range activity (9.23%) against *A. baumannii* host isolated from a different hospital as the bacteriophage isolate. This can be explained by the difference in the immunity mechanism of bacteriophage infection resistance among geographic differences *A. baumannii* clones.

Bacteriophages that have a broad host range are valuable candidates for further study of the efficacy of *A. baumannii* bacteriophage cocktail. Among the five bacteriophages in our study, two families of bacteriophages belonging to *Podoviridae* and *Myoviridae* were observed under an electron microscope. This reflects similar previous findings that most of the *A. baumannii* bacteriophages identified so far belong to *Podoviridae* and *Myoviridae* (Jin et al., 2012; Lee et al., 2011; Lin et al., 2010; Popova et al., 2012; Thawal et al., 2012). They are classified in *Caudovirales* which includes 96% of bacteriophages isolates identified to date (Ackermann, 2009).

Most studies in *A. baumannii* bacteriophages assessing the efficacy of bacteriophages used one bacteriophage at a time. Our studies used the colorimetric microtiter plate method to determine the efficacy of bacteriophage

cocktail in bacterial growth inhibition. This test is economically cheaper and less time consuming than the conventional method. The different MOI of bacteria and bacteriophages are involved in the efficacy of inhibition in this test. We found the MOI 0.01 of bacteriophages and *A. baumannii* showed good inhibition effects. This MOI was used in the growth inhibition system. The bacteriophage cocktail showed high percent growth inhibition (> 70%) against the *A. baumannii* groups I, J, K and L. These strains are susceptible to at least 4 or 5 bacteriophages. However, a low percent of growth inhibition was found against some of the groups of *A. baumannii* (B, C, G and H). The occurrence of bacteriophage resistant mutant isolates may explain this phenomenon. Mutation can occur during overnight incubation of bacteria with bacteriophages in a microplate system (Fridholm et al., 2005). Isolation of bacteriophages that infected the resistant mutant isolates are needed and further investigated for fulfilling the limitation of bacteriophages in the treatment of MDR-AB.

Conclusion

In conclusion, this study showed the high efficacy of the bacteriophage cocktail against most phage susceptible *A. baumannii*. All of them were multi-drug resistant bacteria. The bacteriophage cocktail was found to be potential agent against multi-drug resistance bacteria and have some future important in phage therapy.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Antibacterial and hemolytic activities of *Mimosa tenuiflora* (Willd) Poir. (Mimosoidea)

Sônia Carmem de Moraes Leite^{1†}, Cássio Ilan Soares Medeiros^{1,2,*}, Paula Christianne Gomes Gouveia Souto Maia³, Maria Iranilda Silva Magalhães⁴, Francisco Orlando Rafael Freitas⁵, Hilzeth de Luna Freire Pessôa⁶, Tiago Bezerra de Sá Sousa Nogueira², Alanna Michely Batista de Moraes⁷, Vandezita Dantas de Medeiros Mazzaro⁸, Vanessa Passos Brustein⁹ and Geraldo Gonçalves de Almeida Filho^{1,2}

¹Department of Microbiology Research, Integrated College of Patos, Patos, Brazil.

²Health Sciences Center, Federal University of Paraíba, João Pessoa, Brazil.

³Materials Science and Engineering, Federal University of Campina Grande, Campina Grande, Brazil.

⁴Department of Health Sciences, Cruzeiro of Sul University, São Paulo, Brazil.

⁵Department of Morphology, Federal University of Pernambuco, Recife, Brazil.

⁶Department of Cell and Molecular Biology, Federal University of Paraíba, João Pessoa, Brazil.

⁷Department of Animal Sciences, Federal University of Campina Grande, Campina Grande, Brazil.

⁸Department of Medicine, Faculty of Medical Sciences of Santa Casa de São Paulo, São Paulo, Brazil.

⁹Department of Fundamental Chemistry, Federal University of Pernambuco, Recife, Brazil.

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Mimosa tenuiflora is a shrub-sized plant native of the Northeast region of Brazil where it is popularly known as “jurema preta” and is widely used in folk medicine, especially the stem bark extract mixtures. Due to its high content of tannins and flavonoids, it is considered to have anti-inflammatory and antimicrobial activity. The antimicrobial activity of the ethanolic extracts of *M. tenuiflora* (EEMt) was determined by the minimal inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) by means of the broth microdilution technique. The MIC corresponded to the last dilution in which the presence of bacteria was not verified. In order to obtain the MBC, the spread-plating in Mueller Hinton agar (MHA) of the corresponding MIC, MIC×2 and MIC×4 dilutions was performed. The EEMt presented antibacterial activity against *S. aureus* (ATCC 25.925) and *P. aeruginosa* (ATCC 25.619) where the MIC and the MBC were 128 and 256 µg/mL, respectively; concentrations inferior to the cytotoxic concentrations for human erythrocytes (A, B and O). For *S. aureus* (ATCC 25.213), the MIC and the MBC were 512 and 1024 µg/mL, respectively. As for the *E. coli* ATCC 8859 and *E. coli* ATCC 2536 they were 1024 and >1024 µg/mL, respectively. It was concluded that the EEMt has a good antibacterial activity, presenting a low toxicity and a better activity against Gram positive strains. However it exhibited a good activity against the *P. aeruginosa* strain which is a Gram negative microorganism of clinical importance.

Key words: *Mimosa tenuiflora*, ethanolic extract, antimicrobial activity, hemolytic activity.

INTRODUCTION

Currently, with the emergence of bacterial strains, resistant and multiresistant to the majority of the available

antimicrobial agents, there has been a renewal of the interest in the research for new alternative antimicrobial

agents (Zhu et al., 2015; Eun-Jeong et al., 2015; Saiprasad et al., 2015). The search and use of medicinal plants with bioactive properties is an age-old practice, present in several phytotherapy treaties and pharmacopoeias of the great civilizations (Ronghui et al., 2014). The consumer has also valued the availability of more natural and healthier pharmaceutical products which may bring health benefits. These factors have contributed to increase the interest in the research of natural products which present biological activities such as the antimicrobial activity (Manuel et al., 2010; Militello et al., 2011).

M. tenuiflora (Willd.) Poir. is a species of Mimosoideae, a botanic sub-family of the Fabaceae family, and it is commonly found in the Northeast of Brazil, characteristic of the 'Caatinga' vegetation, where some of these plants are known as "jurema preta" (Diego et al., 2013; Ana et al., 2014; Silva et al., 2015). It is a shrub-sized tree disseminated in the States of Piauí, Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe and Bahia. The Mimosoideae has the average height of 5 to 7 m, and are composed of approximately 82 genus with 3.271 species distributed worldwide mainly in tropical, sub-tropical and temperate regions (Maria et al., 2010; Juarez et al., 2013; Cleilton et al., 2014).

The 'jurema preta' as well as other species *Mimosa* genus, has been used by indigenous tribes of the Brazilian Northeast's culture since long before the Portuguese colonization. After the colonization it was also used by Afro-Brazilians. The plant was used to make hallucinogenic drinks due to the presence of a psychoactive alkaloid called N, N- dimetiltryptamine (DMT), which is inactive when administered orally (Fernanda et al., 2010; Reinaldo et al., 2012; Alain et al., 2013; Alan and Maria, 2013).

In folk medicine, the stem barks of *M. tenuiflora* (Willd.) Poir. is used in the treatment of several diseases and pathologies, such as burns and external and internal inflammations, probably due to its elevated content of tannins and flavonoids, which is believed to have antimicrobial activity (Rafael et al., 2008; Camargo-Ricalde, 2000). Studies carried out in Mexico and in Brazil evaluated the antimicrobial properties of the stem bark of *M. tenuiflora* and demonstrated a great inhibitory action of the ethanolic and hydroalcoholic extracts, against Gram-positive, Gram-negative bacteria and dermatophyte fungi. Studies of the stem bark of the 'jurema preta' confirmed its pharmacological properties and showed an exceptional antimicrobial activity of the ethanolic extract against bacterial strains of *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus* spp.

(Itácio et al., 2010; Marcelo et al., 2012; Edilson et al., 2011; Reinaldo et al., 2014).

Considering the broad potential of application of the unrefined extracts of the stem barks of the mentioned plant species, as well as the fact that a plant species may present a variable chemical composition, and, therefore, also a variable biological activity according to the geographical localization, the aim of the present work was to evaluate the "in vitro" antibacterial activity of *Mimosa tenuiflora* (Willd) Poir. by means of the microdilution in plates technique and therefore the determination of the MIC and MBC as well as to determine the hemolytic activity.

MATERIALS AND METHODS

Samples of plants and substances

The collection of the 'jurema-preta' was carried out in the rural area of the municipality of Santa Terezinha, interior of the 'sertão' region of the State of Pernambuco, located in an area denominated 'Alto Pajeú'. The plants were identified and stored in the Microbiology Research Laboratory (MRL) Of the Integral Faculties of Patos-PB in exsiccate codified as: *M. tenuiflora*-2735-LPM. The parts of the plants used in the studies were the stem barks. The following substances used in this work were commercially obtained: dimethylsulfoxide (DMSO) and Tween-80 were, respectively, bought from Labsynth Products for Laboratories Ltd. (Diadema, SP, Brazil) and Vetec Fine Chemicals Ltd. (Duque de Caxias, RJ, Brazil), respectively.

Ethanolic extract of *Mimosa tenuiflora* (EEMt)

A quantity of 250 g of the stem bark of *M. tenuiflora* was dried at room temperature and kept away from light; at night it was submitted to artificial drying in a kiln with temperature not superior to 35°C and posteriorly pulverized (Correa Junior et al., 1994; Furlan, 1998). The powdered material was extracted by maceration using 1 L of ethanol (EtOH) at 95% as a solvent, at room temperature, and homogenized, and then it was left to rest for 72 h at room temperature. After that, the extracts were filtered and concentrated in vacuum in a rotary evaporator at an average temperature of 35°C (Beatriz et al., 2006). For the tests, the material of the dry extract was dissolved in DMSO at 0.5%. The DMSO was chosen due to its lower toxicity when compared to the ethanol.

Culture medium

To test the biological activity of the ethanolic extracts, Brain Heart Infusion broth (BHI-Difco) and Müller-Hinton agar (MHA-Difco) were acquired from Difco Laboratories (Detroit, MI, USA). They were prepared and used according to the manufacturer's instructions.

*Corresponding author. E-mail: cassioism@hotmail.com. Tel: +55 (83) 8820-8635.

†These authors contributed equally to this work.

Bacterial strains

The tests were performed with 5 standard bacterial strains: *S. aureus* 25,213 and 25,925, *E. coli* 2536 and 8859 and *P. aeruginosa* 25,619 obtained from the American Type Culture Collection (ATCC) that originated from the Department of Molecular Biology of the Federal University of Paraiba (DMB, UFPB). The strains were maintained in MHA at 37 and 4°C until they were used.

Preparation of inoculum

The suspensions were prepared of recent bacterial cultures, plated on MHA, and incubated at 37°C for 24 h in a microbiological incubator. After the incubation, about 4-5 bacterial colonies were transferred with a sterilized microbiological strap to test tubes containing 5 mL 0.9% saline solution (Farmax-Distribuidor Ltd., Amaral, Divinópolis, MG, Brazil). The resulting suspensions were stirred for 15 s with the aid of a vortex (Fanem Ltd., Guarulhos, SP, Brazil).

The turbidity of the final inoculum was standardized using a suspension of barium sulphate and sulfuric acid at 1% (tube 0.5 in the McFarland standard). The final concentration obtained was of around $1-5 \times 10^6$ Colony Forming Units per Milliliters (CFU/mL). The confirmation of the final concentration was carried out by microorganism counting in a Neubauer chamber (Clelland and Squires, 1991; NCCLS, 2008).

Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The determination of the MIC of the ethanolic extracts against the five strains used in the biological tests was done by the broth microdilution method (Clelland and Squires, 1991; NCCLS, 2008). One hundred microliters (100 μ L) of BHI broth were transferred to the wells of a U-shaped bottom 96-well microdilution plate (Alamar, Diadema, SP, Brazil). After that, 100 μ L of the EEMt was inoculated in the wells of the first horizontal line of the plate. Dilutions were duplicated in series, where an aliquot of 100 μ L was removed from the most concentrated well into the following well, and originated concentrations of 1024-1 μ g/mL. Finally, 10 μ L of the inoculum of the bacterial suspensions was added to each well of the plate, in which each column represents a bacterial strand. At the same time, positive and negative controls were made for the bacterial viability. The plate was incubated at 37°C during 24 h in a microbiological incubator. After the adequate incubation time, the presence (or absence) of growth was visually observed. The formation of cellular aggregates or "buttons", as well as the turbidity of the means in the plate wells was considered. The MIC was defined as the lowest concentration which produced a visible inhibition of bacterial growth faced with the unrefined ethanolic extract of *M. tenuiflora* (Willd.) Poir.

To determine the MBC, we subcultivated aliquots of 1 μ L of the MIC and two immediately anterior (MIC \times 2 and MIC \times 4) of the contents of the wells of microdilution plates in Petri dishes containing MHA. After 24 h of incubation at 37°C, a reading was performed to evaluate the MBC, which was considered the lowest concentration which impeded the formation of up to three CFU. The concentrations immediately superior to MIC were sufficient to demonstrate the bactericidal effect of the natural products, seen as the bacteriostatic effect that was determined by the absence of growth in the wells of the microdilution plates (Glauco et al., 2008; Ernst et al., 1996; Espinel-Ingroff et al., 2002; Patrícia et al., 2010).

The biological activity tests were carried out in duplicate, and the results were expressed as the arithmetical average of MIC and the MBC.

Determination of the hemolytic activity

The human erythrocytes were obtained from samples to be disposed from the Clinical Hematology Unit of the Clinical Analysis Teaching Laboratory (BIOLAB) of the Integral Faculty of Patos / FIP.

Aliquots of human blood (type A, B and O) were mixed with NaCl at 0.9% at a ratio of 01:30, under slow and constant stirring. After that, the samples were centrifuged (FANEM) at 3000 rpm for 5 min in order to obtain the erythrocytes. This procedure was repeated twice and the sediment of the last centrifugation was resuspended in 0.9% of NaCl 0.5% up to a final concentration of 0.5%. The fraction of the EEMt was added to a 2 ml of erythrocyte suspension at various concentrations (1, 10, 100, 1000 and 2000 μ g) in different preparations for a final volume of 2.5 mL. The erythrocyte suspension was the negative control (0% of hemolysis) and the erythrocyte suspension plus 50 mL of Triton X-100 (SIGMA) at 1% was the positive control (100% of hemolysis). The samples were incubated for 1 h at room temperature under slow and constant stirring (100 rpm). After this time, they were centrifuged at 3000 rpm during 5 min and the hemolysis was quantified by spectrophotometry at 540 nm (Beckman DU model – 640, USA) (Mebs et al., 1985; Dresch et al., 2005). The tests were carried out in triplicate. The results were expressed as a percentage which represents the arithmetical average of three measurements.

RESULTS AND DISCUSSION

Antibacterial activity

The results of the antibacterial activity of the EEMt fractions were determined using the MIC and the MBC of the broth microdilution. The EEMt was capable of inhibiting 99.9% of the growth of the used strains, showing specific MIC and MBC for each of the tested strains (Table 1). The ethanolic extracts of some *Mimosa* species have been mentioned in literature because of their antibacterial activity against *S. epidermidis*, *E. coli*, *P. aeruginosa* and activity against *Candida albicans*. The tannins probably are the majority components with antimicrobial activity (Lozoya et al., 1989; Meckes-Lozoya et al., 1990). Studies carried out by research groups in Mexico report the biological activity of the EEMt and the existence of components such as steroids, terpenoids, alkaloids, flavonoids, tannins and others phenolic components (Rivera-Arce et al., 2007).

Generally the Gram positive bacteria are more sensitive to the antibiotics than Gram negative ones. This is expected, as the Gram negative bacteria have an already known external structural membrane which provides a type of barrier to the penetration of numerous molecules which could cause cellular damage, and the periplasmic space contains enzymes capable of hydrolyzing strange substances introduced from the exterior (Madigan and Martinko, 2004; Fabíola et al., 2002).

Evaluation of the hemolytic activity on human erythrocytes

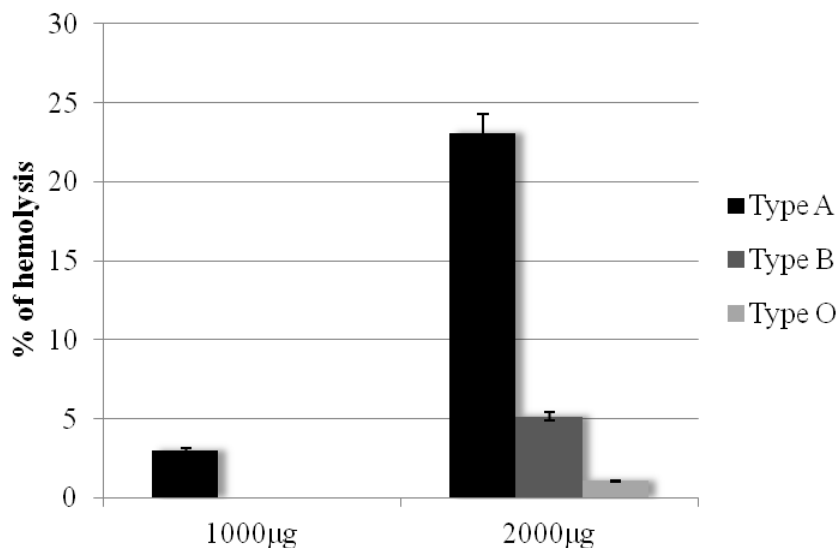
The EEMt fractions did not present any hemolytic activity

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the EEMt Fraction against Gram positive and Gram negative bacterial strains.

Bacterial strains	Fractions EEMt	
	MIC($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
<i>S. aureus</i> ATCC 25.925	128	256
<i>S. aureus</i> ATCC 25.213	512	1024
<i>E. coli</i> ATCC 8859	1024	>1024
<i>E. coli</i> ATCC 2536	1024	>1024
<i>P. aeruginosa</i> ATCC 25.619	128	256

Table 2. Hemolytic effect of the *eemt* on human erythrocytes.

Erythrocytes	Hemolytic activity (%)	
	EEMt fraction	
	1000 μg	2000 μg
A	3.0	23.1
B	0.0	5.17
O	0.0	1.08

**Figure 1.** Hemolytic effect of the EEMt on human erythrocytes. At the concentration of 1000 μg , EEMt presented respectively 3.0, 0.0 and 0.0% haemolysis and at the concentration of 2000 μg , EEMt presented 23.1, 5.17 and 1.08% hemolysis on the human erythrocytes (A, B and O), respectively.

up to the concentration of 1000 μg and in 2000 μg presented low toxicity as reported by Mekces-Lozoya et al. (1990) (Table 2 and Figure 1). The triterpenic saponins are considered to be substances probably responsible for this activity, causing the rupture of the erythrocytes membranes (Banerji et al., 1981). Thereby, the extract fractions showed to have a good antibacterial potential against Gram positive and Gram negative bacteria and

low toxicity for the human erythrocytes cells.

Conclusions

Based on these results, the present study demonstrated that the EEMt has a good antibacterial activity, with better activity against Gram positive strains; but, however, it

had a good activity against the *P. aeruginosa* strain which is a Gram negative microorganism and which may be isolated in several infections, above all in systemic cases and which commonly presents an elevated profile of resistance to many antibiotics of long-standing use. The low toxicity to the human host of the tested product may be promising and could encourage new research about the phytochemical, toxicological and pharmacological aspects, in order to support its possible rational use in the antimicrobial therapy, especially as anti *S. aureus* and anti *P. aeruginosa*.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

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

Nahla Omer ELtai*, Vyvyan Salisbury and John Greenman

Department of Applied Sciences, School of Health and Life Sciences, University of the West of England, Coldharbour Lane, Bristol, BS16 1QY, UK.

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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

*Corresponding author. E-mail: nahlaa@hotmail.com.

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 geographical 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 *Photobacterium 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 Llewelyn, *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 (10⁴ 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 10⁶ 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 Berthold 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 log₁₀ RLU/s and CFU/ml

Biocidal effect of oregano essential oil and its components

A spot of 20 µl (10⁵ 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 log₁₀ 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 $D = (t_2 - t_1) (\log N_1 - \log N_2)$ 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, R² 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
<i>E. coli</i> 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)
<i>S. aureus</i> 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)
<i>S. aureus</i> SMH 22115 Llewelyn (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)
<i>P. aeruginosa</i> 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.

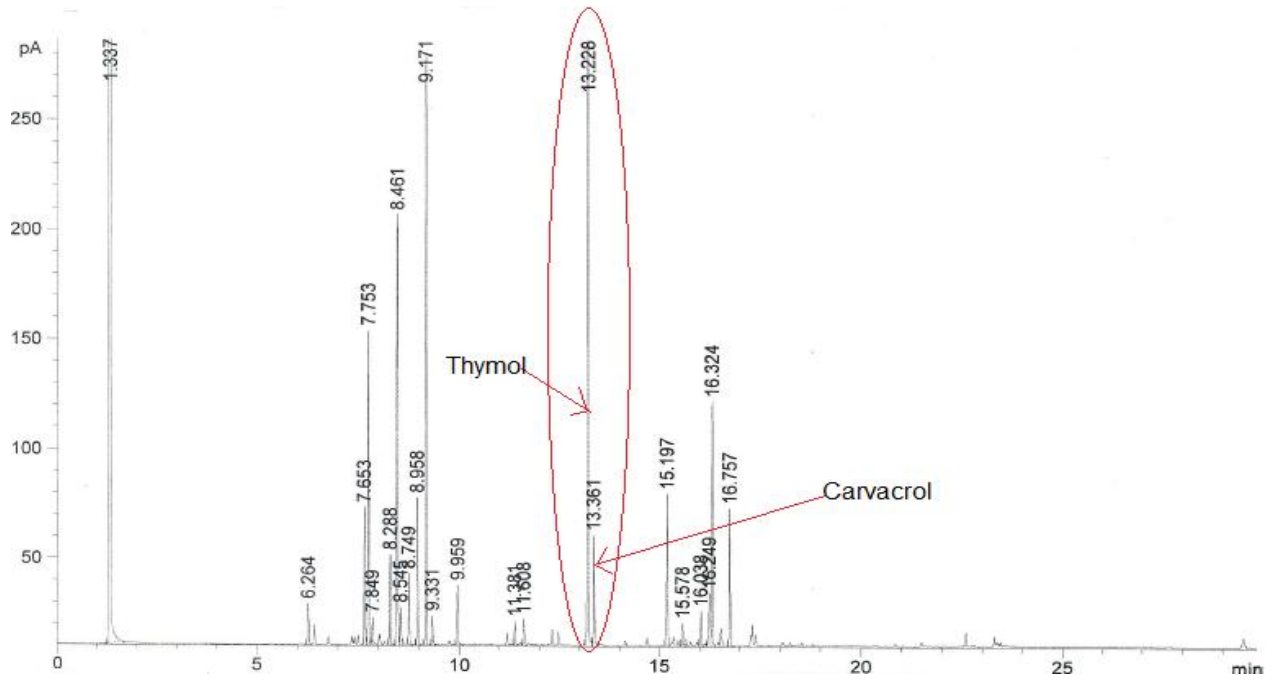
In vitro susceptibility testing of oregano oil against bacteria and yeast

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)

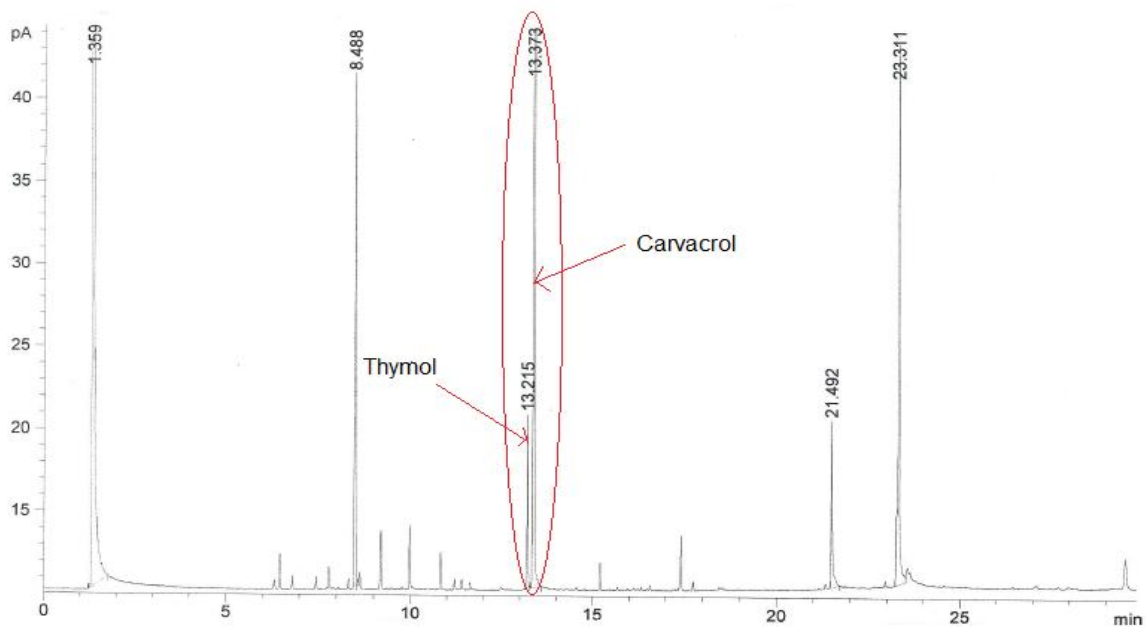


Figure 1. Chromatogram showing the relative presence of thymol and carvacrol in Himalayan Oregano oil (a) and Mediterranean Oregano oil (b) pA: height in picoamperes; numbers on the peaks are the retention time in minutes (time spent on the column, in minutes, for the component to be analysed).

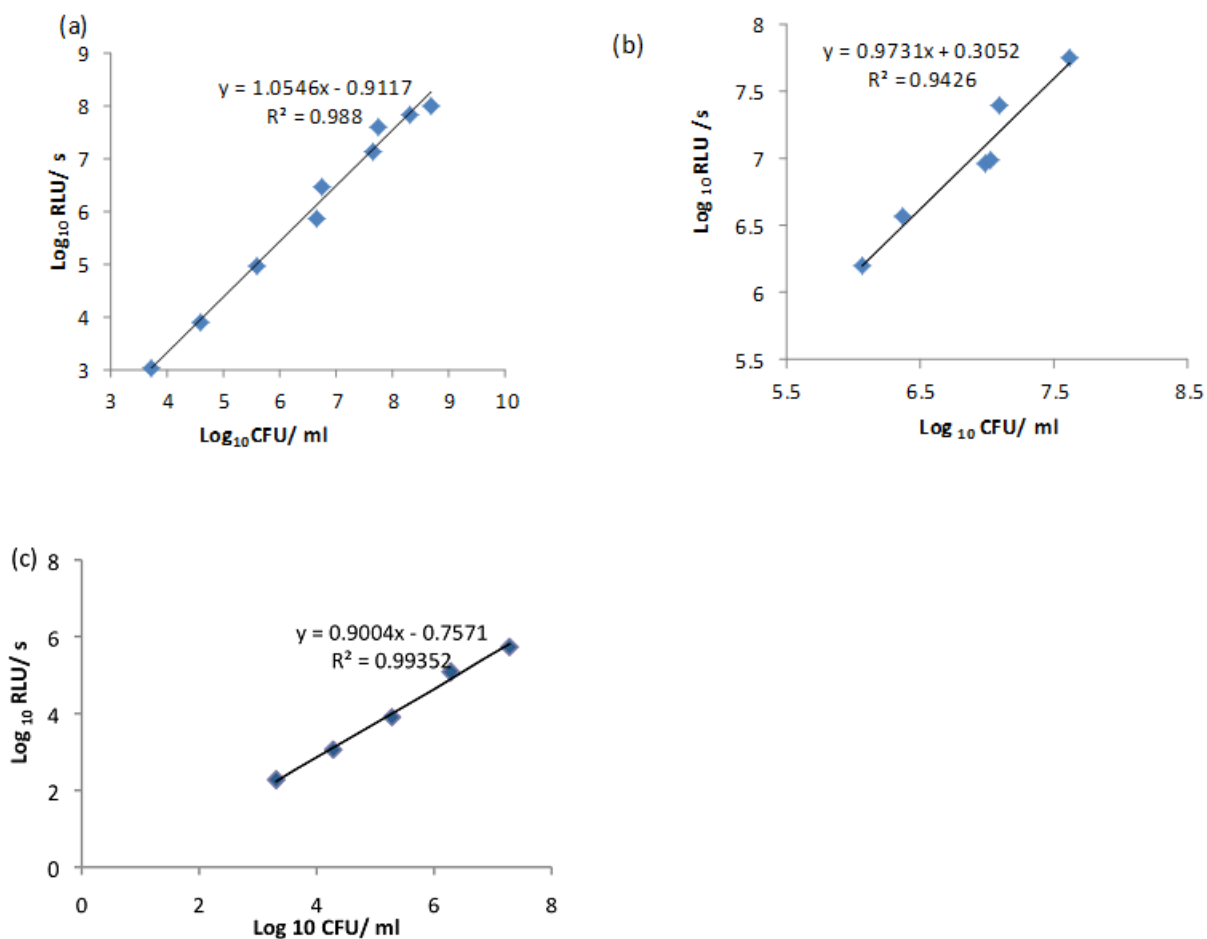


Figure 2a, b and c. Relationships between viable count (\log_{10} CFU/ml) and bioluminescence (\log_{10} RLU/s) of lux recombinant *E. coli* (a), *P. aeruginosa* (b) and MSSA (c).

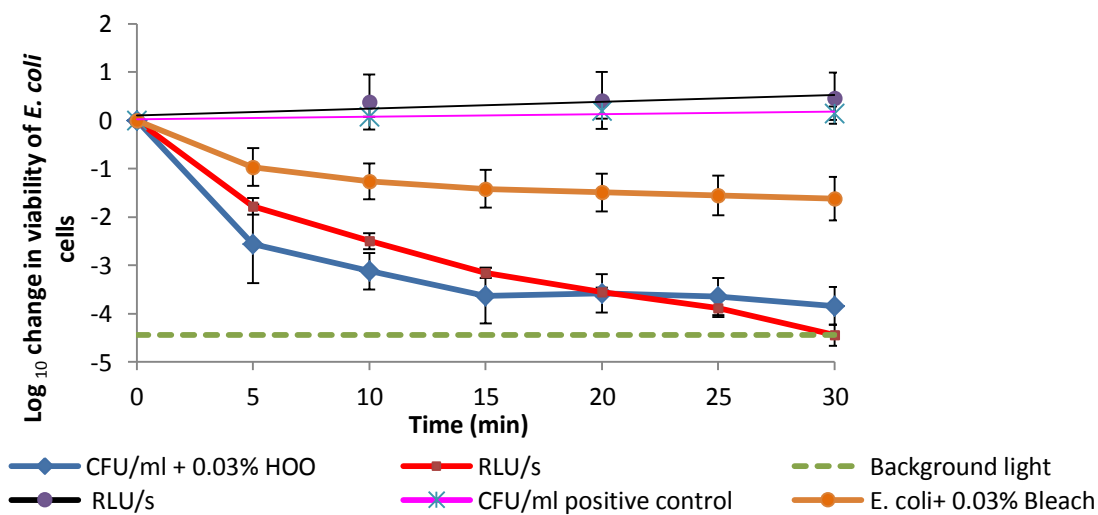


Figure 3. Reduction in bioluminescence and CFU/ml of *E. coli* Nissle exposed to 0.03% Himalayan oregano oil. Number of treated cells = 10^6 /well. T test $P = 0.8485$. Error bars show the mean and \pm one standard deviation of 3 replicates.

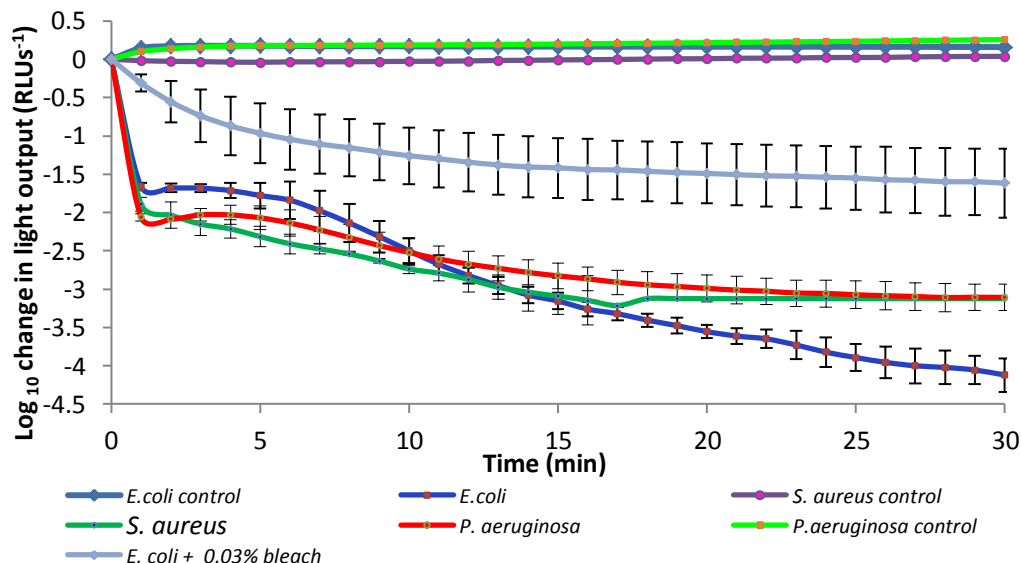


Figure 4. Reduction in bioluminescence from *E. coli*, *S. aureus* and *P. aeruginosa* exposed to 0.03% Himalayan oregano oil. Number of treated bacterial cells 10^6 /well. Error bars show mean and \pm one standard deviation; number of replicate = 3.

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

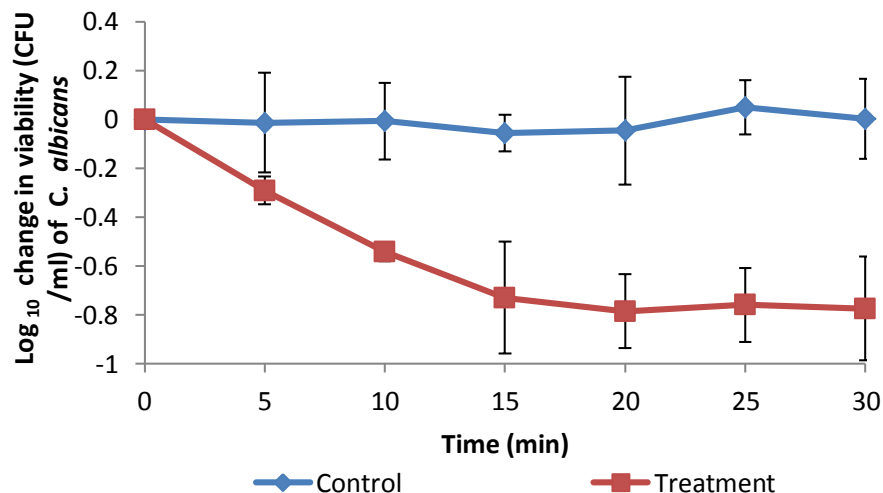


Figure 5. Reduction in CFU/ml of *C. albicans* exposed to 0.03% Himalayan oregano oil. Number of treated cells 10^6 /well. Error bars show mean and \pm one standard deviation; number of replicate = 3.

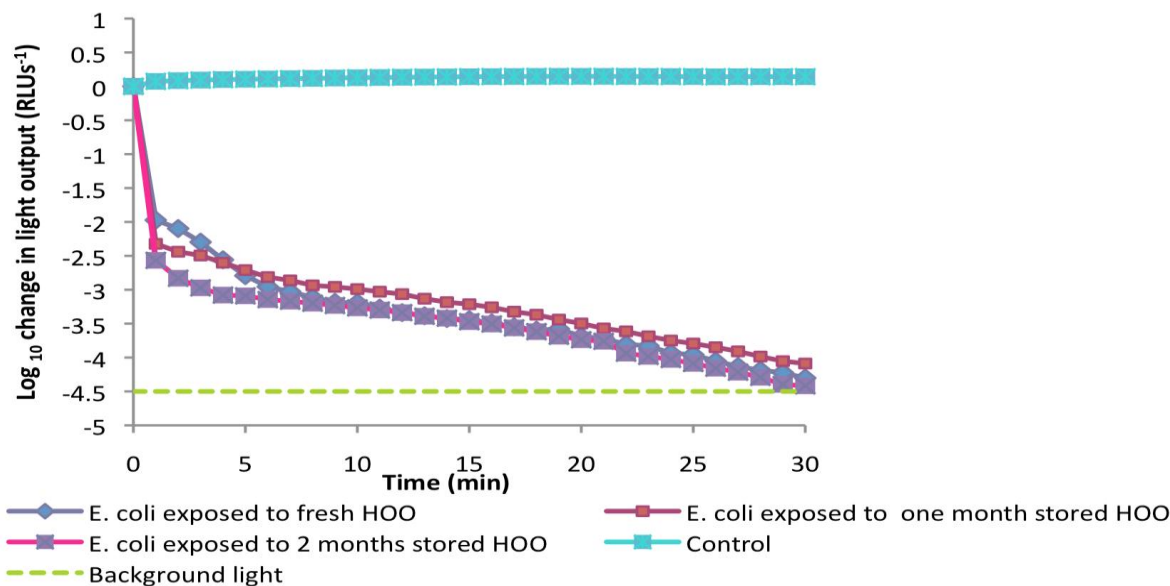


Figure 6. Reduction in bioluminescence from *E. coli* exposed to 0.1% Himalayan oregano oil stored over time. Number of treated cells 10^6 /well.

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

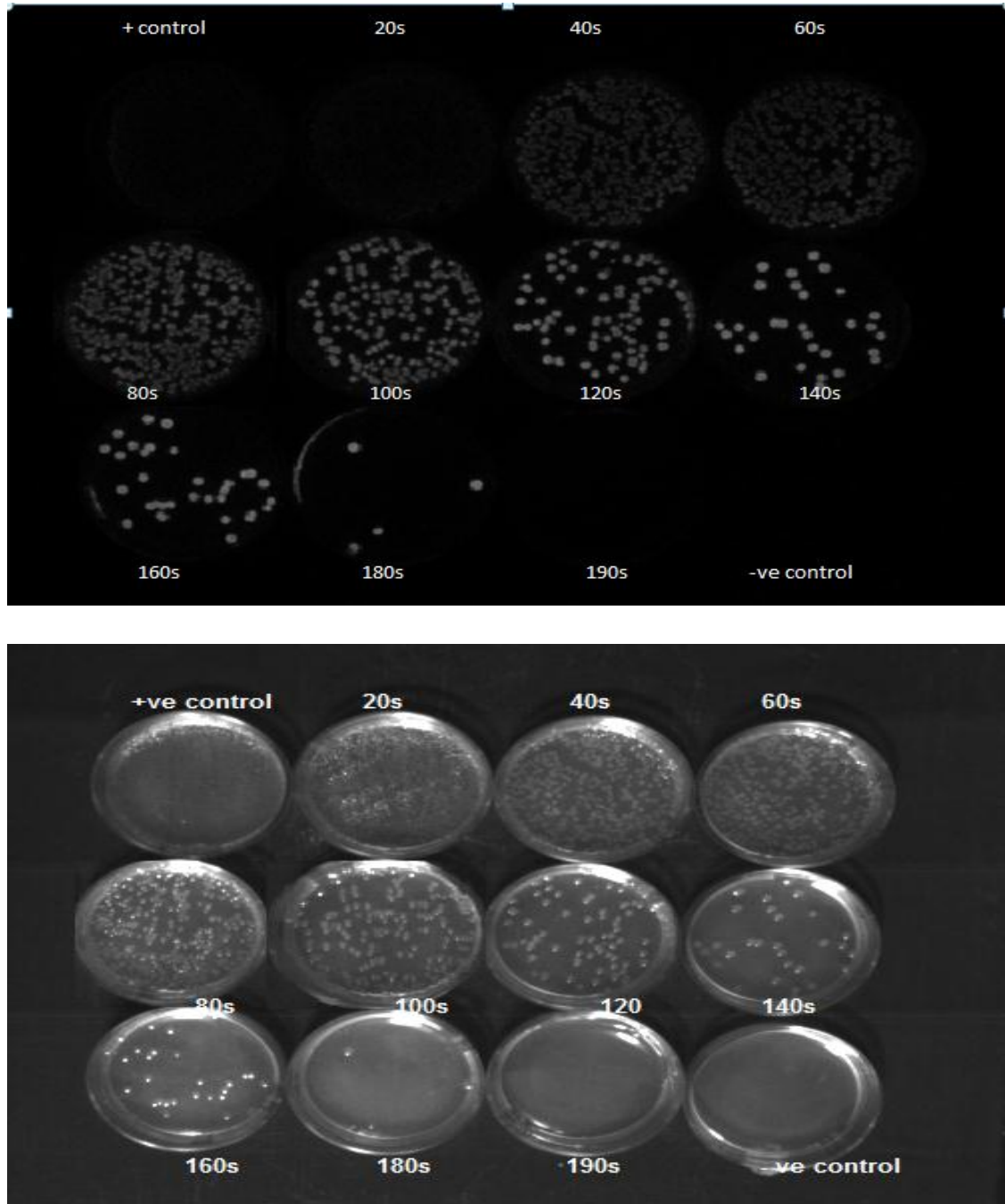


Figure 7. *E. coli lux+* after exposure to 10x MIC HOO for different periods of time. (a) bioluminescent colonies; (b) colonies under normal light (not bioluminescence). + control is positive for lux without exposure to HOO.

concur 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 $\mu\text{g mL}^{-1}$ 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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