British Microbiology Research Journal
8(6): 624-634, 2015, Article no.BMRJ.2015.156
ISSN: 2231-0886

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Anti-Shiga Toxin Producing *Escherichia coli* O157:H7 Effect of *Ocimum basilicum* L. Essential Oil Analyzed Using Time Kill Assay in a Batch Culture

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/BMRJ/2015/17717
Editor(s):
(1) Lachhman Das Singla, Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, India.
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(3) Regina Helena Pires, Sao Paulo State University, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1092&id=8&aid=9495

Original Research Article

Received 25th March 2015
Accepted 2nd May 2015
Published 29th May 2015

ABSTRACT

Essential Oil extract from *Ocimum basilicum* (Labiatae; basil) was investigated for its in vitro antibacterial properties against an emerging human food-borne pathogen, Shiga Toxin Producing *E. coli* O 157:H7. Agar disc diffusion method and time-kill assay in a batch culture were employed. The results showed that essential oil of basil was effective to inhibit the growth of pathogenic bacteria tested. The diameter of inhibition zone was 15.25±0.58 mm. In a batch culture, four oil concentrations (4, 2, 1, 0.5, 0.25 and 0.125 mg/ml) were initially tested to determine the concentration of basil oil that could inhibit growth of *E. coli* O157:H7. It was found that the oil at 4, 2, 1 and 0.5 mg/ml killed the bacteria after 1 h, while at 0.25 mg/ml the number of bacteria was only reduced. Two oil concentrations, 0.25 mg/ml and 0.5 mg/ml, were chosen to study its effect on *E. coli* O157:H7 at different growth phases, i.e. exponential, late exponential and stationary phase in a batch culture. Oil at both concentrations (0.25 mg/ml and 0.5 mg/ml) could not completely kill the cells at the exponential phase, and upon prolonged incubation, resistant cells emerged. But basil oil at 0.5 mg/ml could completely killed cells at the late exponential and stationary phases suggesting that the oil at 0.25 mg/ml was bacteriostatic, while at 0.5 mg/ml was bactericidal against *E. coli* O157:H7.

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Keywords: Anti- E. coli O157:H7; time kill assay; essential oil; Ocimum basilicum.

1. INTRODUCTION

The toxic side effects of synthetic antibiotics have necessitated a search for new antibiotic agents from plants. Plants contain a variety of secondary metabolites serving as hormones, attractants, repellents, poisons, and other functional agents of the plants, and a large number of them display pharmacological properties that can be and have been used by humans. Identifying novel antimicrobial substances from natural sources is becoming a leading trend in drug discovery [1].

Shiga toxin-producing *Escherichia coli* O157:H7 (known as enterohemorrhagic *E. coli*) is one of the most important foodborne pathogens and considered a serious threat to public health in recent years. It is a serious pathogen causing gastrointestinal tract infection in humans and responsible for disease outbreaks. The pathogen is known to possess Shiga toxins, which may cause acute diarrhoea, hemorrhagic colitis, and life-threatening haemolytic uraemic syndrome [2].

Current therapy for Shiga toxin-producing *Escherichia coli* O157:H7 infection is limited to supportive treatment as antibiotics may increase the risk of systemic complications such as acute renal failure associated with haemolytic uraemic syndrome, perhaps by promoting the release of pre-formed toxin from the periplasm [3].

The genus Ocimum (Lamiaceae) includes more than 150 species which are distributed in the tropical and subtropical regions of the world. *Ocimum basilicum* L. (basil) is an annual herb which grows in several regions around the world. It is one of the most common aromatic herbs used extensively all over the world and in Iraq to add a distinctive aroma and flavour to food. The leaves can be used fresh or dried for use as a spice. Traditionally, basil has been used as a medicinal plant in the treatment of headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunction [4].

The plant is popular in folk medicine in Iraq for its gastroprotective effects, including its use as a digestive and anti-diarrhoeal. The essential oil (also known as volatile oils) of the plant has been reported previously to have antioxidant and antimicrobial activity on a number of Gram-negative, Gram-positive bacteria and fungi [5,6].

Although the antibacterial activity of the essential oil of *O. basilicum* has been well studied, however studies on its antibacterial activity against Shiga toxin producing *E. coli* O157:H7 have not been previously reported. Therefore, the present study aimed at determining the inhibiting or killing potential of *O. basilicum* oil against Shiga toxin producing *E. coli* O157:H7 at different growth phases in a batch culture, using viable plate count, time-kill curve and membrane permeabilization assay.

2. MATERIALS AND METHODS

2.1 Plant Sample

The whole plant of *O. basilicum* was collected at flowering stage. The voucher specimen has been deposited at the herbarium of the Department of Biology, College of Education for Pure Sciences, Anbar University, Iraq.

2.2 Isolation of the Volatile Oil

The leaves of the plant were separated and washed. The samples were then dried at 40^{o}C for 24h and ground into powder. The powder was sequentially subjected to hydro-distillation for 4 h, using a Clevenger-type apparatus [7].

2.3 Culture of *E. coli* O157:H7

Working culture of Shiga toxin-producing *E. coli* O157:H7 (Gen-Bank accession number JX161807 and JX161808) was kept on nutrient agar slant and stored at 4ºC. Prior to use, bacteria were subcultured, at least three times, on a fresh nutrient agar plate. After overnight incubation at 37ºC, several single colonies were transferred to 10 ml of nutrient broth by a sterile inoculation loop and incubated overnight at 37ºC with shaking. The bacterial cell suspension was mixed to homogeneity and the turbidity was adjusted to 0.5 McFarland standards (bioMérieux, France) for a final density of approximately 1 x 10^8 CFU/mL. This population size was confirmed by preparing 10-fold serial dilutions of the suspension for colony count. Each dilution (0.1 mL) was spread, in triplicate, on the surface of nutrient agar plate and the total CFU/ml (log_{10}CFU/ml) were determined after 24 h incubation at 37ºC.

2.4 Antibacterial Activity

Agar disc diffusion method was employed for the determination of antibacterial activity, as recommended by Clinical and Laboratory Standards Institute (CLSI) [8]. Briefly, 100 µl of the bacterial suspension was inoculated on nutrient agar and swabbed. The filter discs (6 mm in diameter; AA, Whatmann, UK) were impregnated with 20 µl of essential oil and placed on the agar plates which had been inoculated with *E. coli* O157:H7. Disc without sample were used as a negative control. Chloramphenicol (30 µg/disc) were used as a positive reference. The plates were incubated at 37ºC for 24 h. Antibacterial activity was evaluated by measuring the diameter of the growth inhibition zones in millimeters (including disc diameter of 6 mm) and comparing to the control. This study was carried out in triplicate.

2.5 Determination of Standard Growth Curve of *E. coli* O157:H7

Growth curve was determined according to the Clinical and Laboratory Standards Institute (CLSI) [9]. *E. coli* O157:H7 suspension (0.5 mL) was mixed with 49.5 mL nutrient broth in a sterile conical flask. The suspension was shaken and mixed well. Then, 100 µl of the culture was then subjected to a 10-fold serial dilution from 10^{-1} till 10^{-10} in sterile 0.85% (w/v) sodium chloride solution using microtiter plate. Then 100 µl from each dilution were used for direct cell count using Breed's method and colony count using the colony counting method of Miles and Misra [10]. After that, the conical flask was incubated in a shaker at 37ºC for a maximum of 24 h. Total cell count and CFU were then determined at specific time after incubation i.e. after 4 h, 8 h, 12 h, 16 h, 20 h and 24 h.

2.6 Effects of Basil oil on *E. coli* O157:H7 in a Batch Culture

The inhibiting or killing effect of basil oil against *E. coli* O157:H7 was determined by measuring the reduction in the numbers of CFU/mL at different growth time using time- kill assay. The time-kill assay was carried out by the method described in M26-A guideline published by Clinical and Laboratory Standards Institute (CLSI) [8,9] with little modification.

Six concentrations (4, 2, 1, 0.5, 0.25 and 0.125 mg/ml) of basil oil were used to evaluate its concentration-dependent activity against the pathogen. Nutrient broth, bacterial culture and oil were added, mixed by shaking in a conical flask and incubated. Samples were taken from the culture after 1 h, 2 h, 4 h, 8 h, 12 h and 24 h incubation. After 24 h, colonies formed on nutrient agar were graded quantitatively using colony counting method.

2.7 Effects of Basil oil on *E. coli* O157:H7 at Different Growth Phases in a Batch Culture

Two concentrations (0.5 mg/ml and 0.25 mg/ml) of oil were selected to evaluate its concentration-dependent activity against the pathogen at different growth phases, i.e. exponential, late exponential and stationary phase in a batch culture. After nutrient broth and bacterial suspension were mixed in a flask, initial total number of cells and viable cells or CFU were determined using Breed's method and colony counting method, respectively [8,9,10].

Three sets of experiments were carried out to determine the effect of the oil on *E. coli* O157:H7 in a batch culture. In each experiment, flasks containing nutrient broth were inoculated with 0.1 mL (10^8 CFU/mL) of the organism and mixed. Initial total numbers of cells and viable cells or CFU were determined using Breed's method and colony counting method, respectively [8,9].

In the first experiment, three flasks of each selected concentration (0.5 and 0.25 mg/ml) were used to determine the effects of basil oil at on *E. coli* O157:H7 at exponential phase. Total count and viable count were determined at 4 and 5 h. Then Basil oil was added immediately to a desired final concentration of 0.5 and 0.25 mg/ml. Total count and viable count were then determined at 6, 7, 9, 12, 16, 20 and 24 h.

In the second experiment, three flasks of each selected concentration were also used for the late exponential phase experiment. Total and viable count were determined at 4, 8 and 10 h, after which basil oil was added immediately into conical flasks to a desired final concentration of 0.5 and 0.25 mg/ml. Total and viable count were determined at 11, 13, 16, 20 and 24 h.

Similarly, the effect of the oil on the bacteria at stationary phase was also determined employing three flasks of each selected concentration as well. Total and viable count were determined at 4, 8, 12, 16 and 17 h. Basil oil was then added immediately to a desired final concentration of

0.5 and 0.25 mg/ml. Total and viable count were determined at 18, 19, 21 and 24 h.

All experiments included two untreated control samples (without oil) *viz.* one control consisted of nutrient broth alone. Killing curves were constructed by plotting $\log_{10}$ CFU/ml versus time and the assessment of bacterial growth reduction was analyzed by comparing viable cell counts ($\log_{10}$ CFU/ml) between the control and treatments at specific time and to the initial inoculum. Bactericidal activity was defined as a reduction of 99.9% ($\geq$ 3 $\log_{10}$) of the CFU/ml in the original inoculum. Bacteriostatic activity was defined as maintenance of the original inoculum concentration or a reduction of less than 99.9% ($<$ 3 $\log_{10}$) of the CFU/ml in the original inoculum [11,12].

Two controls (A negative control containing basil oil and nutrient broth without the bacterial and a positive control containing bacterial suspension cultured in nutrient broth only) were included to ensure the validity of these experimental results and to rule out any broth contamination.

2.8 Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows, version 17, SPSS Inc., Chicago, IL). Statistical significance was determined by Chi square test. A *p*-value of less than 0.001 ($p < 0.001$) was considered statistically significant.

3. RESULTS

3.1 Antibacterial Activity

The inhibitory effect of basil oil was tested against *E. coli* O157:H7 using disc diffusion assay. The oil at a concentration of 15 mg/disc showed antibacterial activity against *E. coli* O157:H7 and the diameter of inhibition zone was 15.25±0.58 mm while chloramphenicol (positive control) at 30 µg per disc produced a big zone of inhibition (16.33±0.83).

According to Prabuseenivasan et al. [13], any compound giving an inhibition zone diameter of > 7 mm in a disc diffusion assay is considered as having antibacterial properties. Based on this criterion, it can be concluded that basil oil possessed antibacterial activities against *E. coli* O157:H7.

3.2 Standard Growth Profile of *E. coli* O157:H7

The standard growth profile of *E. coli* O157:H7 in a batch culture was determined at regular intervals (4 h, 8 h, 12 h, 16 h, 20 h and 24 h) using colony counting method. The pathogen grew logarithmically and its growth curve in nutrient broth at 37ºC showed a sigmoidal form (Fig. 1). Four growth phases were clearly defined, i.e. lag phase (0-4 h), exponential phase (4-8 h), late exponential phase (8-16 h) and stationary phase (16-24 h).

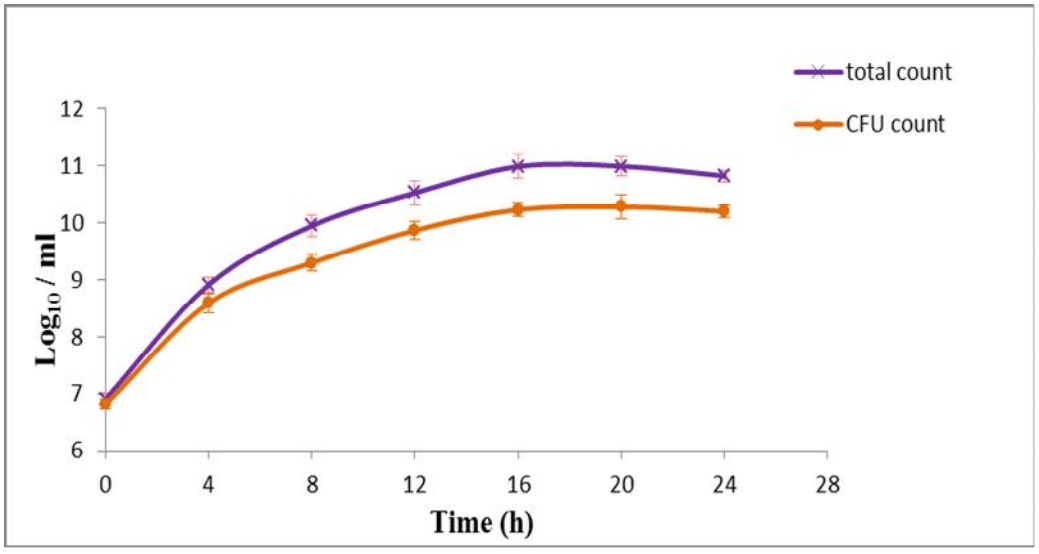

Fig. 1. Standard growth profile of *E. coli* O157:H7 in batch culture. Data are presented as the mean value of three replications ± SD

3.3 Effects of Basil Oil on the Growth of *E. coli* O157:H7 in a Batch Culture

The test was initially carried out to determine the concentration of basil oil that could inhibit growth of *E. coli* O157:H7 in a batch culture. The result, represented quantitatively, is presented in Table 1.

The oil concentrations at 4, 2, 1 and 0.5 mg/ml completely killed the organism within one hour of exposure. No bacterial colonies were formed when the samples were taken from the flask after 1 h incubation. At lower concentrations of 0.25 mg/ml and 0.125 mg/ml, some bacteria were killed after 1 h, but upon further incubation there was an indication of growth. At the end of the 24 h incubation, high numbers of colonies were recorded. The positive control showed high bacterial growth over the incubation period. Based on this result 0.5 mg/ml and 0.25 mg/ml were chosen for the next study.

3.4 Effects of Basil Oil on *E. coli* O157:H7 at the Different Growth Phases

The results from section 3.3 suggested that basil oil at 0.5 mg/ml killed the bacteria after 1 h, while at 0.25 mg/ml was only reduce the number of bacteria. Therefore, both concentrations of oil were used in this subsequent experiment to observe its effects on bacterial growth at different phases in a batch culture.

3.4.1 Effects of basil oil on *E. coli O157*:H7 at the exponential phase of development

On addition of 0.25 mg/ml of the oil to an exponential phase culture of the bacteria (at exactly 5 h/after 5 h), total cell counts and CFU decreased rapidly. A significant differences (P < 0.0001) was found between the treatments and controls. At 12 h (7 h after addition of the oil), CFU were undetectable (Fig. 2). Thereafter, both total counts and CFU increased and remained unchanged until the end of the incubation period at 24 h. Final CFU in the treated culture was 10^8/ml, which was 0.5-log lower than that of control.

Meanwhile for the concentration of 0.5 mg/ml of the oil, number, number of cells and CFU dropped was more drastic and the disappearance of CFU occur at a much more earlier period, (9 h i.e 4 h after the introduction of the oil) (Fig. 3). A significant differences (P < 0.0001) was found between the treatments and controls. After this period, both total counts and CFU then increased until end of the incubation period i.e. 24 h as it occur in the previous experiment with 0.25 mg/ml. Final CFU in the treated culture was 10^6/ml, that was 4-log lower than that of control which was 10^{10}/ml. Overall, final population of CFU in cultures treated with oil at 0.5 mg/ml final concentration was 2.5-log lower than in cultures treated with oil at 0.25 mg/ml.

3.4.2 Effects of basil oil on *E. coli* O157:H7 at the late exponential phase

The oil at 0.25 mg/ml concentration was added after 10 h, at which time the bacterial culture was in the late exponential phase. Results showed that CFU and total count dropped rapidly after 11 h (1 h after introduction of oil), and remained unchanged until the end of the incubation period at 24 h (Fig. 4). Meanwhile upon injection of 0.5 mg/ml of the oil, number of cells and CFU decreased rapidly. At 11 h (1 h after of addition of oil), CFU were undetectable and remained undetected until 24 h incubation (Fig. 5). A significant differences (P < 0.0001) was found between the treatments and controls.

Table 1. Effects of basil oil on the growth of *E. coli* O157:H7

Time (h) / Concentration (mg/ml)	0	1	2	4	8	12	24
4	+++++	-	-	-	-	-	-
2	+++++	-	-	-	-	-	-
1	+++++	-	-	-	-	-	-
0.5	+++++	-	-	-	-	-	-
0.25	+++++	+	+	++	++++	+++++	+++++
0.125	+++++	+++	+++	++++	+++++	+++++	+++++
Control	+++++	+++++	+++++	+++++	+++++	+++++	+++++

+: Bacterial growth,-: No growth

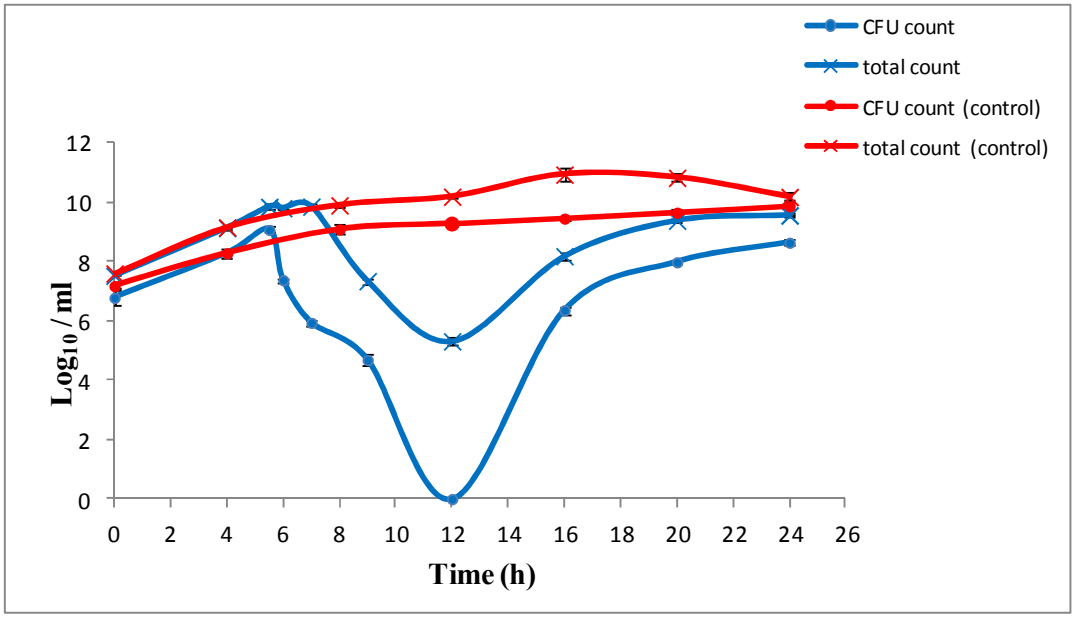

Fig. 2. Effects of 0.25 mg/ml basil oil on *E. coli* O157:H7 growth at the exponential phase. Data are presented as the mean value of three replications ± SD. Values are significantly different ($p < 0.001$) based on Chi square test

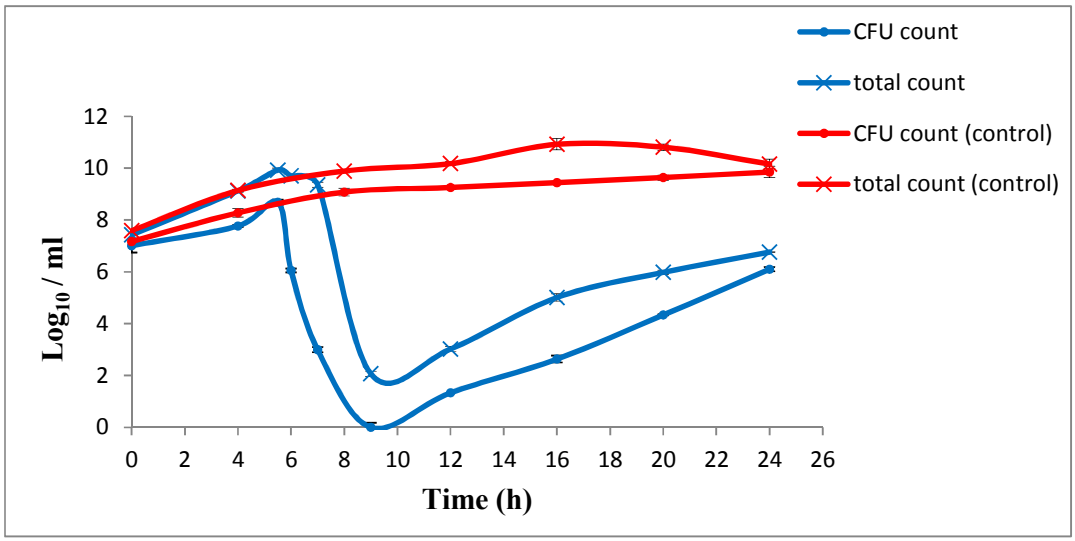

Fig. 3. Effects of 0.5 mg/ml basil oil on *E. coli* O157:H7 growth at the exponential phase. Data are presented as the mean value of three replications ± SD. Values are significantly different ($p < 0.001$) based on Chi square test

3.4.3 Effects of oil on *E. coli* O157:H7 at the stationary phase

After 17 h 0.25 mg/ml of the oil was introduced to the bacteria culture, at which time culture was in the stationary phase. Results showed that CFU and total count dropped rapidly, and remained unchanged until the end of the incubation period at 24 h (Fig. 6). Meanwhile both total counts and CFU were undetected in cells treated with 0.5 mg/ml and remained undetected until 24 h incubation (Fig. 7). A significant differences

(P < 0.0001) was found between the treatments and controls.

4. DISCUSSION

In the third world and developing countries, and even in developed nations, Bacterial food-borne agents are the major causes of intestinal infectious disease [14]. Throughout the 1980s and until today, infections with *E. coli* O157:H7 are an important public health problem. More recently there has been a growing concern about *E. coli* O157:H7 because this bacterial pathogen constitute the greatest burden of food-borne illness for which etiology is known [3].

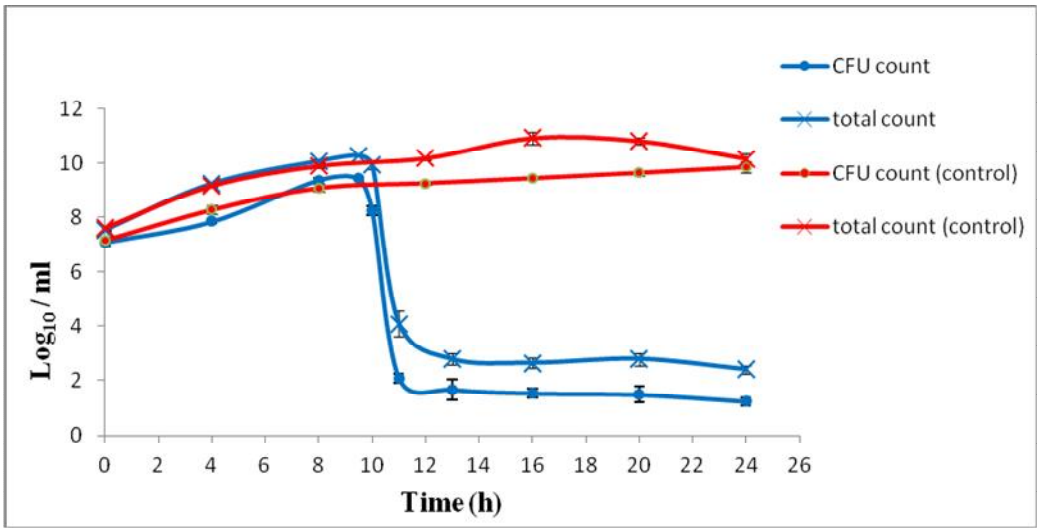

Fig. 4. Effects of 0.25 mg/ml basil oil on *E. coli* O157:H7 growth at the late exponential phase. Data are presented as the mean value of three replications ± SD. Values are significantly different (p < 0.001) based on Chi square test

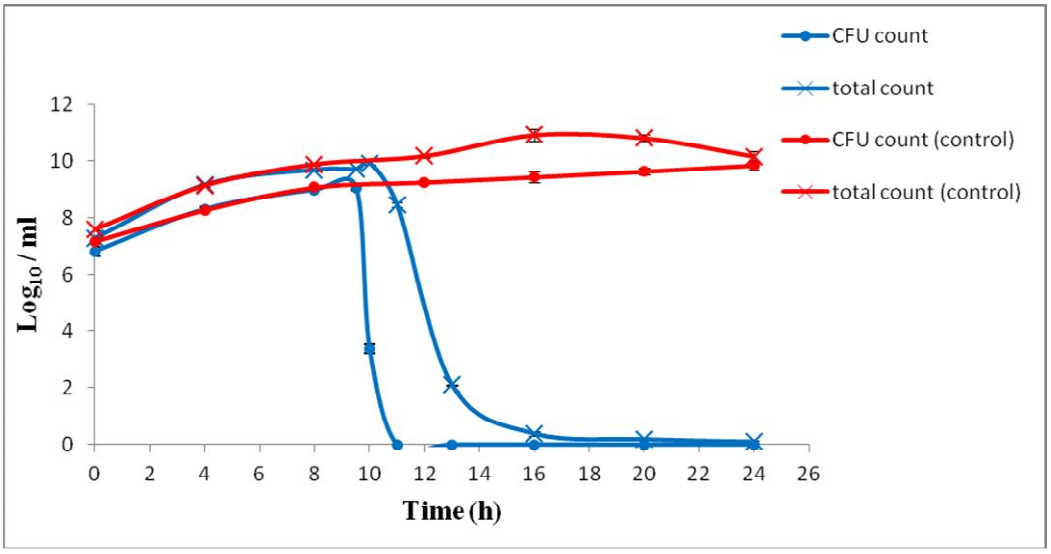

Fig. 5. Effects of 0.5 mg/ml basil oil on *E. coli* O157:H7 growth at the late exponential phase. Data are presented as the mean value of three replications ± SD. Values are significantly different (p < 0.001) based on Chi square test

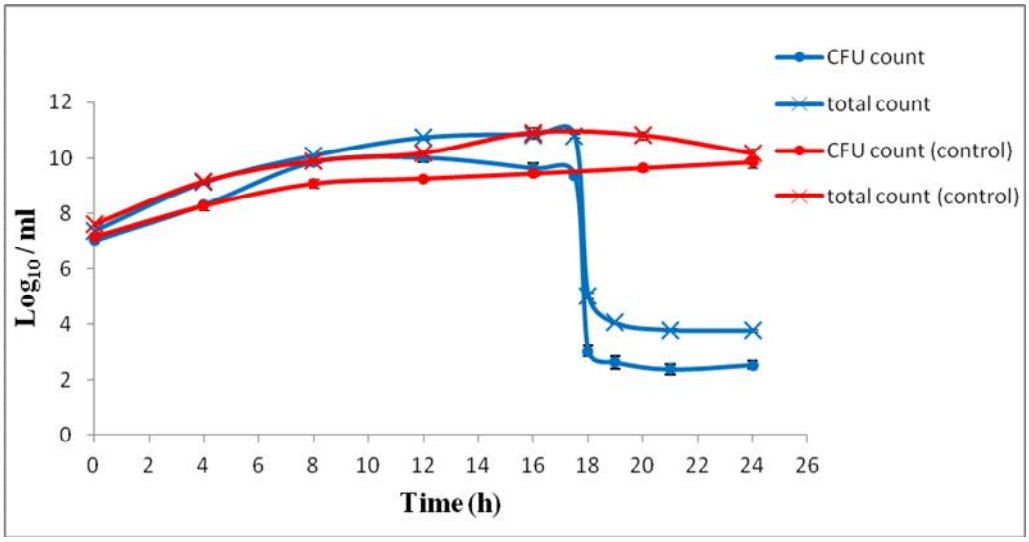

Fig. 6. Effects of 0.25 mg/ml basil oil on *E. coli* O157:H7 growth at the stationary phase. Data are presented as the mean value of three replications ± SD. Values are significantly different ($p < 0.001$) based on Chi square test

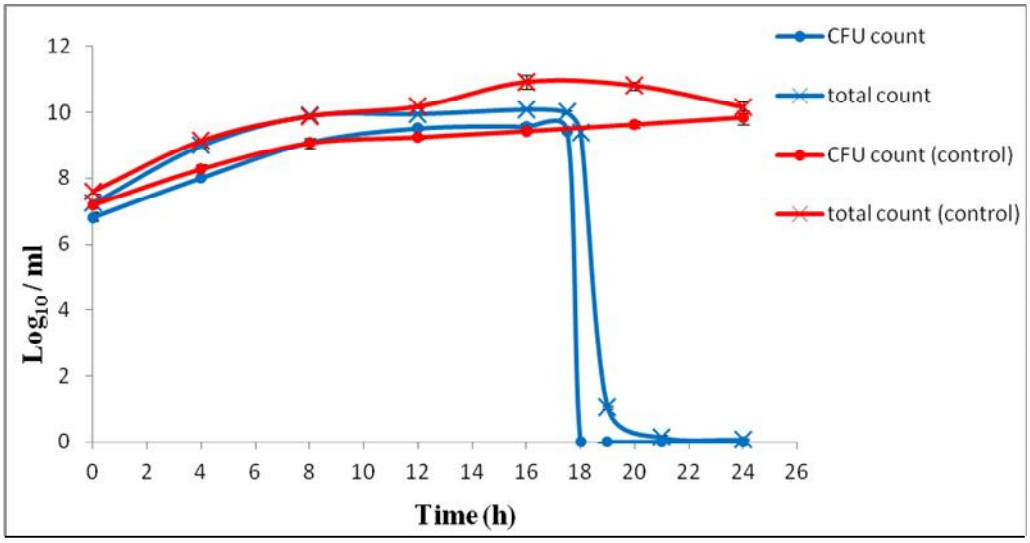

Fig. 7. Effects of 0.5 mg/ml basil oil on *E. coli* O157:H7 growth at the stationary phase. Data are presented as the mean value of three replications ± SD. Values are significantly different ($p < 0.001$) based on Chi square test

Ocimum basilicum L. (basil) is one of the most common plants used traditionally all over the world and in Iraq to treat many diseases. The essential oil from the plant has been reported previously to have antimicrobial activity on a number of Gram-negative and positive bacteria and fungi, some of which are food spoilage microorganisms [15,16,17,18].

From disc diffusion assay, basil oil exhibited antibacterial activity against *E. coli* O157:H7. It has been reported that the antibacterial activities of the chemical composition of *O. basilicum* oil could be due to the presence of several compounds, like linalool, eugenol, methyl eugenol, thymol, and bornyl acetate. In addition, some essential oil components interfere with the lipids of cell membranes, causing leakage of

intracellular materials, and ultimately the cell lysis [19]. An important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable [20]. Leakage of ions and other cell contents can then occur [21,22]. Some essential oils, such as oregano (*Origanum vulgare*) and thyme (*Thymus vulgris*), have been reported to posses significant bacteriostatic and bacteriocidal properties towards *E.coli O157:H7* [23].

Based on the CFU growth profile (Fig. 1), the growth of *E. coli* O157:H7 in the absence of the oil (control) was promoted and its population increased exponentially until it reached a maximum size (9.8 $\log_{10}$) at 16 h and the maximum population size remain unchanged in stationary phase. For pathogenic bacteria, disease symptoms often develop during the exponential or logarithmic phase because the bacterial population has reached a high enough level to cause tissue damage. If toxins are produced, these poisons may cause destruction of tissues and become apparent [24]. Therefore, antibacterial activity is important to be investigated or be applied during these phases.

In the study of the effects of basil oil on the growth of *E. coli* O157:H7 at different growth phases, no CFU was detected at 7 h after 0.25 mg/ml oil was introduced to the culture at the exponential phase (Fig. 2). For the 0.5 mg/ml oil concentration, the numbers of cell detected through viable plate count decreased and no colony was formed after 4 h, which is shorter period compared to 0.25 mg/ml oil (Fig. 3). Inability to observe CFU may due to the death of bacteria or 'injured' bacteria. While *E. coli* O157:H7 dealt with the stress imposed by basil oil, physiological changes happened and the cells became injured. These 'injured cells' have difficulties to form colony on growth media without the recovery or repair processes [25].

The colonies were able to form after 7 hours of oil introduction for 0.25 mg/ml and 4 hours for 0.5 mg/ml oil concentration. The bacteria were observed after that and continued to grow. In this case, basil oil is considered as a stress agent towards *E. coli* O157:H7. In order to survive under stress condition, bacteria with identical genetic population may react differently to overcome the stress and so called heterogeneity [26]. When bacteria are exposed to stress, the bacteria may be negatively affected or may adapt to the stress. The stress might reduce the growth rate and induce an adaptive stress response. Heterogeneity can result in bifurcation into distinct subpopulations and this phenomena is called bistability [27].

The subpopulation showed the ability to tolerate the antibacterial stress of the oil. The tolerance mechanisms could be by altering the concentration of saturated and unsaturated fatty acids and thus change the membrane fluidity. The mechanisms also could be production of protective proteins, transformation into dormant state or adaptive mutations of bacteria [28]. After the bacterial success to tolerate the stress imposed, the bacteria continued to growth and undergo another growth cycle.

Other than emergence of mutations, some bacteria can also resist the action of the antibacterial agent. This study showed that bacteria are able to resume growth when the antibacterial agents are removed; the cells which are able to survive are called persisters [27]. The emergence of persisters can be explained by their antibiotic tolerance, post- antibiotic effect or viable but non-culturable bacterial state [29].

With lower concentration of antibacterial agent, the tolerance of bacteria towards the oil become higher, hence, the growth rate could resumed with higher rate. However, when the oil was added during the lag exponential phase of the bacterial growth, no bacteria were detected in 1 h after the addition of 0.5 mg/ml concentration.The total death could be due to the stress imposed, natural growth inhibiting factor of bacteria or cells' physiology. Combination of these three factors caused cell mortality faster in stationary phase compared to late exponential phase. The results showed that the antibacterial activity and growth phases are interrelated. By comparing between growth phases affected by basil oil, the effects of oil on exponential phase was observed to take longer time to suppress or cause death to the bacteria, then followed by lag exponential phase and stationary phase. The explanation of this phenomena is closely related to the bacteria growth rate throughout the growth cycle. The bacterial growth rate started to increase initially and decrease steadily until it become zero at stationary phase and negative at death phase. If the oil affects the bacteria during the high growth rate, the bacteria are able to reproduce actively and deal with the antibacterial agent, either by being resistant or mutate. In contrasts, if the

bacteria growth rate is decreasing or reach null, the populations of bacteria are affected effectively by the stress imposed.

5. CONCLUSION

Therefore these studies showed that the oil at 0.25 mg/ml was only bacteriostatic, while at 0.5 mg/ml the oil was bactericidal, in all the growth phases studied. The activity of the fraction is dependent on concentration and physiological state of the organism. The inhibition mechanisms are faster in late exponential phase and stationary phase. Therefore, the antibacterial properties of the oil were more effective towards the older *E. coli* O157:H7 compared to bacterial cells which are in actively dividing step.

Thus, the essential oil from *O. basilicum* was active against an emerging human pathogen, *E. coli* O157:H7 and could be used as potential antibacterial agent, as well as food preservatives against food spoilage microorganisms.

COMPETING INTERESTS

Author has declared that no competing interests exist

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