The Effect of *Ocimum basilicum* L. oil on the morphology of *E. coli* O157:H7 Analyzed Using Scanning Electron Microscopy

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Abstract

Three growth phases of *E. coli* O157:H7 were identified, i.e. exponential (0 -8 hours), late exponential (8-16 hours) and stationary phase (16-24 hours). The morphology of bacteria treated by basil oil were observed under Scanning Electron Microscopy (SEM). The morphology of normal cells is tightly packed and smooth at the outer surface. Morphological alterations of *E. coli* O157:H7 are shown after treatment with 0.25 mg/ml and 0.5 mg/ml basil oil during exponential, late exponential and stationary phase, respectively. Some treated cells were irregular in shape, deformed, seen like shrunken cells and obviously damaged by basil oil as compared to untreated cells, while most cells were morphologically normal. All of cells were not able to growth on medium, these observations support that basil oil killed *E. coli* O157:H7 primarily by mechanism(s) other than lysis of cells.

Keywords: *E. coli* O157:H7, *Ocimum basilicum* oil, Scanning Electron Microscopy.
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أثر زيت نبات *Ocimum basilicum L.* على الطراز المظهري لبكتريا *E.coli* O157:H7 باستخدام المجهر الإلكتروني الماسح

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الخلاصة

حددت ثلاثة أطوار نمو لـ*E.coli* O157:H7: الطور الأسي (0–8) ساعة، الطور الأسي المتأخر (8–16) ساعة وطور النمو الثابت (16– 24) ساعة. تم ملاحظة الطرز المظهرية للبكتريا المعاملة بزيت نبات الريحان تحت المجهر الإلكتروني الماسح. الخلايا البكتيرية الطبيعية ذات شكل منتظم وذات سطح خارجي منتظم ناعم. شوهدت تغيرات مظهرية في الخلايا البكتيرية المعاملة بزيت الريحان بتركيز 0.25 ملغم/ مل و0.5 ملغم/ مل في الطور الأسي، الطور الأسي المتأخر وطور النمو الثابت. بعض الخلايا المعاملة كانت غير منتظمة الشكل، فاقدة للشكل الطبيعي، منكمشة حيث أنها تضررت بفعل زيت الريحان عند مقارنتها بالخلايا الغير معاملة بالزيت، في حين كانت معظم الخلايا طبيعية الشكل. كل الخلايا المعاملة لم تكن قادرة على النمو على الوسط الزراعي، المشاهدات تؤكد أن زيت الريحان يقتل خلايا *E.coli* O157:H7 وهذه بميكانيكية غير تحلل الخلايا البكتيرية.

الكلمات المفتاحية: *E.coli* O157:H7، زيت نبات الريحان، المجهر الإلكتروني الماسح.

Introduction

The serotype O157:H7 of *Escherichia coli* was first recognized during an outbreak of serious food-borne disease in 1982. This strain was defined as human pathogen and classified into a new category, known as enterohemorrhagic *E. coli* (EHEC) (1). *E. coli* O157:H7 is the most important group of food and waterborne human pathogen, and is now

well recognized as causes of significant morbidity and mortality world-wide. It can infect persons of any age, but children less than age 10 and the elderly have the highest frequency of extraintestinal complications and death (2, 3, 4). There are several reservoirs of *E. coli* O157:H7 such as infected cattle and other animals such as sheep, deer, pigs, dogs, cats, horses and poultry, and insects such as flies and dung beetles. *E. coli* O157:H7 was sporadically isolated from a number of wildlife species inhabiting livestock environments, including rabbits, rats, wild birds and opossums. *E. coli* O157:H7 usually do not cause illness in animals, with a few exceptions such as diarrhea in calves (5). Apart from that, meat and dairy products, fecal-contaminated soil, water, and vegetables are also sources of the pathogen (6). The pathogen is known to possess Shiga toxins, which may cause life-threatening haemolytic uraemic syndrome. The syndrome includes acute diarrhea, hemorrhagic colitis, thrombotic thrombocytopenic purpura. The vehicles of the bacterial transmission are food or water related compounds, especially undercooked ground beef (7). Natural compounds are the desired compound and become a priority for consumers. Essential oil or also known as volatile oil is natural compounds extracted from plant materials (8). The natural functions of essential oil are wide in range. Essential oil shows the property as repellent (9), antioxidants (10), tumor cell reducer (11), anti-hyperglycemic (12), antifungal, carminative, anti depressive or effective for burns and insect bites (13). The Lamiaceae family is one of the families of flowering plants. The genus Ocimum is an important member of this family. This genus has more than 150 species which are distributed in the tropical and subtropical regions of the world. *Ocimum basilicum* L. (basil) is an annual aromatic herb used extensively all over the world in traditional medicine. 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 (14, 15). Some studies were done on basil oil and *E. coli* O157:H7 which include the processed food (16, 17), fresh food (18) or stored food (19). Consumer demands necessitates the use of basil oil as an alternative for synthetic antibiotic to produce environmental friendly products and prevent any adverse effect (10). Although there have been some reports on the antibacterial activities of plant extracts against *E. coli* using SEM (20, 21, 22, 23, 24, 25, 26), the antibacterial activity of basil oil against *E. coli* O157:H7 using SEM has yet to be published. Therefore, this study was undertaken to observe the effects of basil oil on morphological alteration of *E. coli* O157:H7 with the aid of Scanning Electron Microscopy.

Materials and Methods

- **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.
- **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 (27).
- **Culture of *E. coli* O157:H7:** Working culture of *E. coli* O157:H7 was collected from the culture collection of the Laboratory of Food Safety and Quality (Malaysia). The sequences of Stx1 and Stx2 specific genes of *E. coli* O157:H7 used in this study were deposited in Gen-Bank with accession number JX161807 and JX161808, respectively. The culture was kept on nutrient agar slant and stored at 4°C. Prior to use, bacteria were subcultured, at least three times, on a fresh nutrient agar plate. After overnight

incubation at 37°C, several single colonies were transferred to 10 ml of nutrient broth by a sterile inoculation loop and incubated overnight at 37°C with shaking. The bacterial cell suspension was mixed to homogeneity and the turbidity was adjusted to 0.5 McFarland standard (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 37 ºC (28).

- **Determination of standard growth profile of *E. coli* O157:H7:** Growth curve was determined according to the National Committee for Clinical Laboratory Standards guidelines (29). *E. coli* O157:H7 suspension from stock culture was prepared as described earlier. The suspension (0.5 ml) was then mixed with 49.5 ml nutrient broth in a sterile conical flask to achieve a total volume of 50 ml. 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} on microtiter plate. Then 100 µl from each dilution were used for direct cell (total) count using Breed's method and colony count using the colony counting method of Miles and Misra (30). After that, conical flask was incubated in a shaker at 37 ºC for a maximum of 24 h. Total cell count and CFU were then determined at specific time after incubation i.e. after 4, 8, 12, 16, 20 and 24 h.
- **Observation of oil treated bacteria by scanning electron microscope (SEM):** Scanning electron microscopic observations were carried out on *E. coli* O157:H7 untreated (control) or treated with basil oil using a scanning electron microscope (Leo Supra 50 VP Field Emission SEM, Carl-Ziess SMT, oberkochen, Germany) in the Microscopy Unit, School of Biological Sciences, USM. Based on our previous findings from concentration-dependent activity of basil oil against the pathogen (31), oil at 0.25 mg/ml was bacteriostatic while at 0.5 mg/ml was bactericidal. Therefore, in this study, these two concentrations were selected to observe its effects on the pathogen at different growth phases, i.e. exponential, late exponential and stationary phase under SEM. After 30 min treatment with basil oil during exponential, late exponential and stationary phase, cells were withdrawn and centrifuged at 5000 rpm for 5 min. Same procedures were done on 16 h culture of *E. coli* O157:H7, which is normal cell culture without treatment with basil oil. The supernatant was discarded and the pellet was resuspended with appropriate volume of McDowell-Trump fixative which consists of 50 ml of 0.2 M buffer, 11 ml of 37% Formaldehyde, 4 ml of 25% Glutaraldehyde and distilled water to make up to 100 ml. This fixation solution was prepared in 0.1M phosphate buffer pH 7.2 and pellet was suspended for at least 2 h. The sample was then centrifuged again and the supernatant was discarded. Next, the pellet was resuspended with 0.1 M phosphate buffer and the sample was centrifuge at 5000 rpm for 5 min for the second times. The supernatant was discarded and the pellet was resuspended with 0.1M phosphate buffer. Centrifugation was carried again and the pellet was resuspended in 1% osmium tetroxide for 1 h. After that, the sample was centrifuged and the pellet was resuspended with distilled water. This step was repeated twice. Then, the resuspended sample was centrifuged and the pellet gained was dehydrated by resuspending in 50% ethanol for 10 min before centrifugation at 5000 rpm for 5 min. The pellet was then resuspended in 75% ethanol for 10 min, centrifuged before resuspending the pellet in 95% ethanol twice for 10 min each then centrifuging again at 5000 rpm for 5 min. The pellet obtained was resuspended in absolute ethanol for 10

min and centrifuging at 5000 rpm for 5 min. Finally the pellet was resuspended in hexamethyldisilazane (HMDS) for 10 min and centrifuging under same condition. HMDS was then discarded from the tube and the pellet was dried in a dessicator at room temperature. The dried cells were mounted onto an SEM specimen stub with a double-sided sticky tape. Then, the specimen was coated with gold and viewed under SEM.

Results and Discussion

- **Standard growth profile of *E. coli* O157:H7:** Colony Forming Unit determined after every 4 hours were used to determine the standard growth profile of *E. coli* O157:H7 over a period 24 hours (Fig. 1). This is because of the sensitivity and reliable of this technique, even a single cell can be detected by colony formed. As expected the total count was higher than the CFU throughout the incubation period of 24 hours. Difference between total count and CFU increased gradually from approximately 1-log at the exponential phase to 2-log (highest difference recorded) at the early stationary phase. Then the differences between the two counts dropped gradually while CFU remained unchanged during the stationary phase. At 24 hours which is the end of incubation, total count was only 0.5-log higher than CFU. From CFU determination, three growth phases in a batch culture were clearly defined, i.e. exponential (0-8 h.), late exponential (8-16 h) and stationary phase (16 -24 h). In subsequent studied, standard growth profile based on CFU was used.
- **Effects of basil oil on the morphology of *E. coli* O157:H7 at different growth phases:** The morphology of bacteria treated by basil oil were observed under SEM. Fig. 2 shows normal *E. coli* O157:H7 cells not treated with oil. The morphology of normal cells is tightly packed and smooth at the outer surface. Morphological alterations of *E. coli* O157:H7 are shown in Fig. 3, 4 and 5 after treatment with 0.25 mg/ml basil oil during exponential, late exponential and stationary phase, respectively. Fig. 5, 6 and 7 show *E. coli* O157:H7 cells that were treated with 0.5 mg/ml basil oil at different growth phases, i.e. exponential, late exponential and stationary phase, respectively. Some cells were wrinkled or lysed. However most of the cells appeared normal morphologically, but they were presumed dead because they were unable to form colonies.

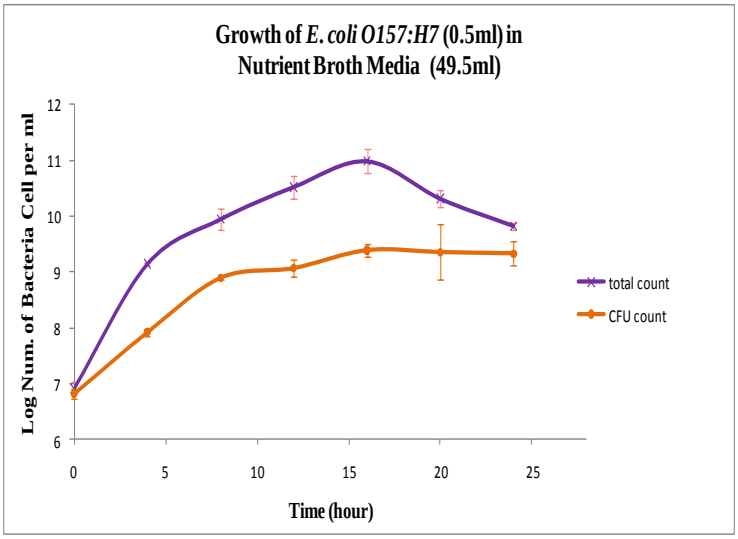

Fig. (1) Standard growth profile of *E. coli* O157:H7

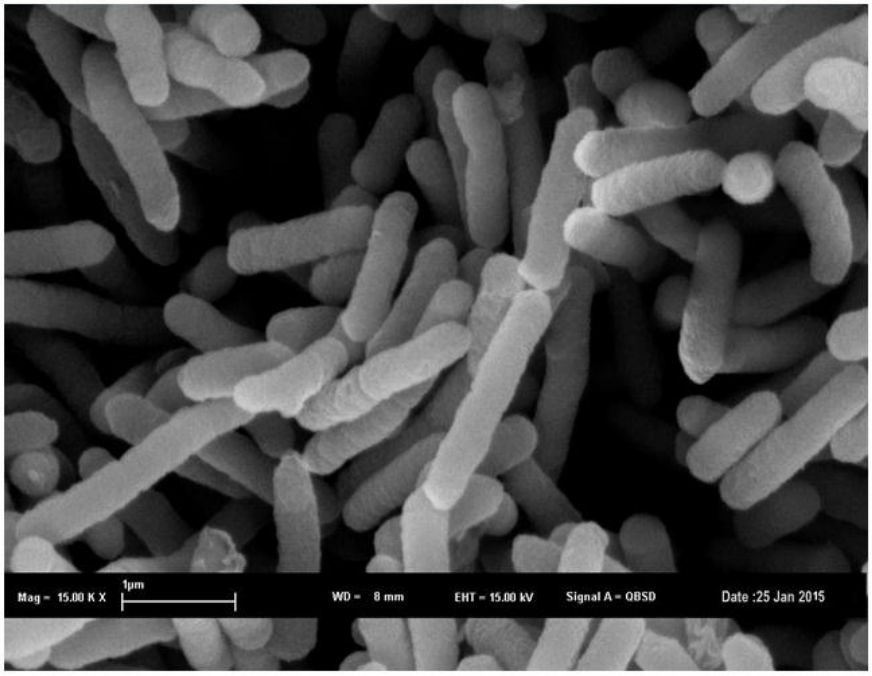

Fig. (2) SEM micrograph of *E. coli* O157:H7 (Normal cells) not treated with oil

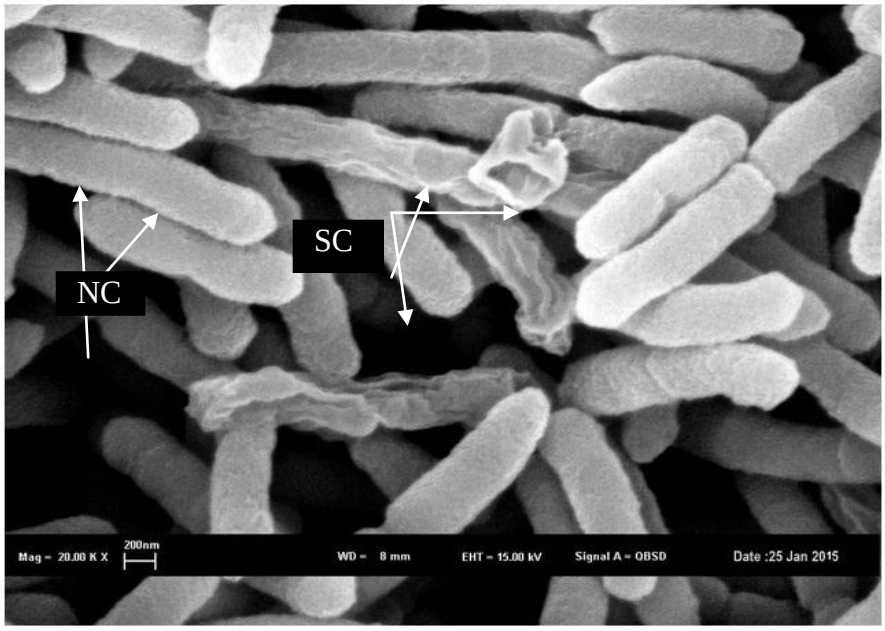

Fig. (3) SEM micrograph of *E. coli* O157:H7 treated with 0.25 mg/ml basil oil at exponential phase. NC: Normal cells; SC: Shrunken cells

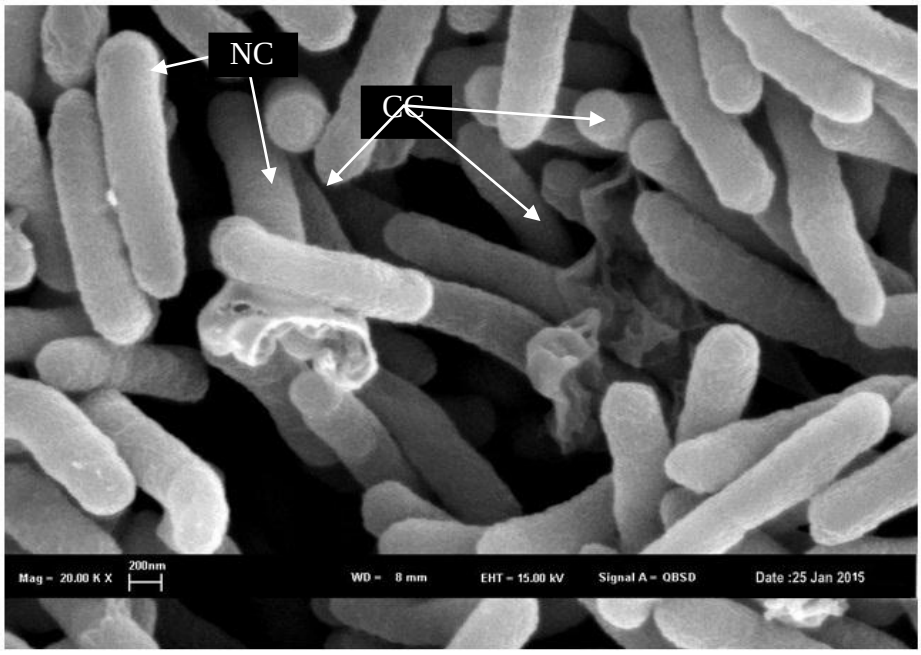

Fig. (4) SEM micrograph of *E. coli* O157:H7 treated with 0.25 mg/ml basil oil at the late exponential phase. NC: Normal cells, CC: collapsed cells

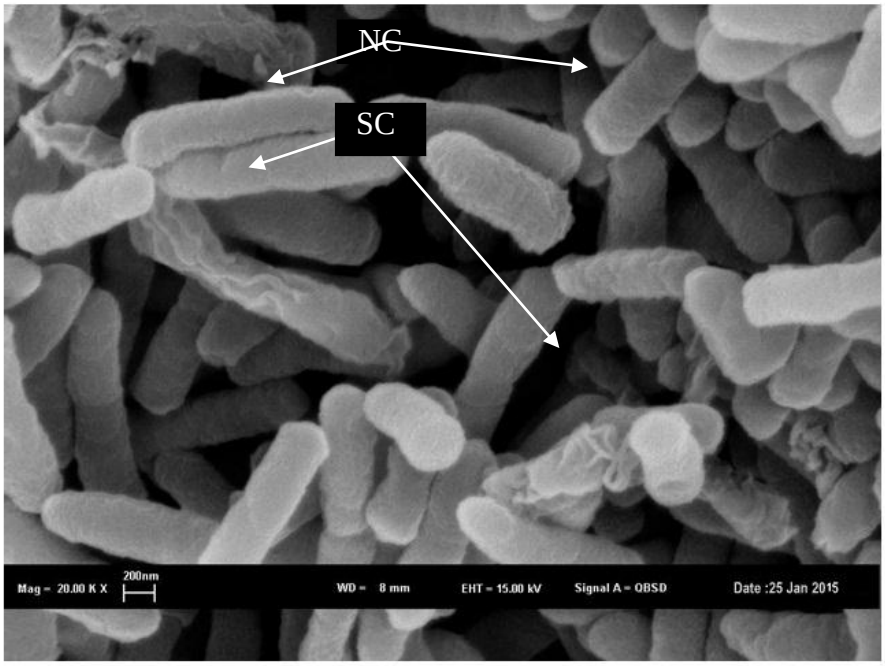

Fig. (5) SEM micrograph of *E. coli* O157:H7 treated with 0.25 mg/ml basil oil at the stationary phase. NC: Normal cells, SC: Shrunken cells

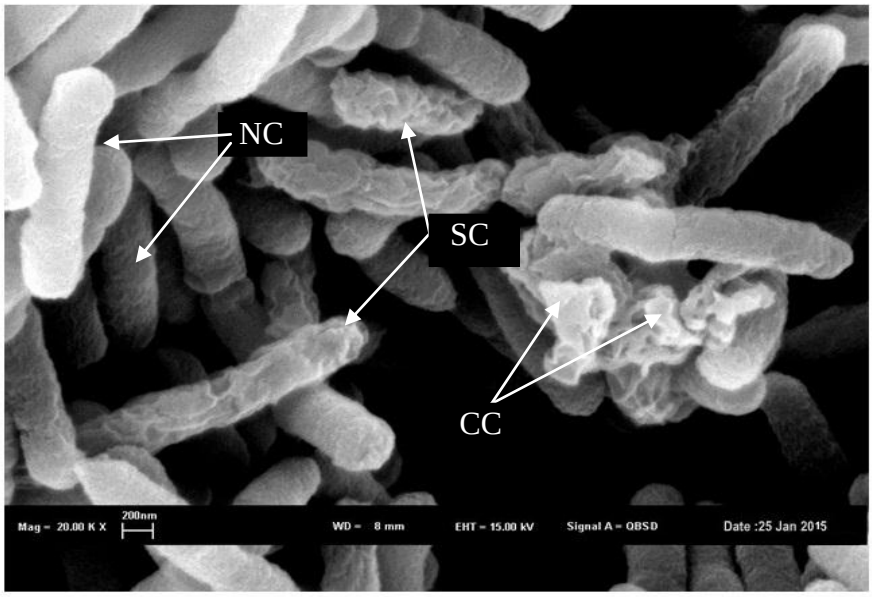

Fig. (6) SEM micrograph of *E. coli* O157:H7 treated with 0.5 mg/ml basil oil at the exponential phase. NC: Normal cells, SC: Shrunken cells, CC: collapsed cells

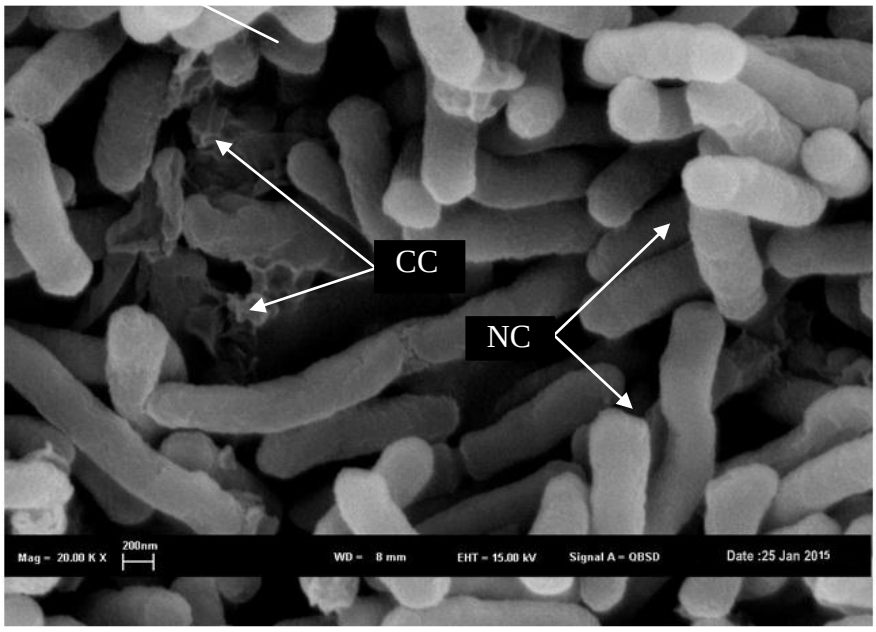

Fig. (7) SEM micrograph of *E. coli* O157:H7 treated with 0.5 mg/ml basil oil at the late exponential phase. NC: Normal cells, CC: collapsed cells

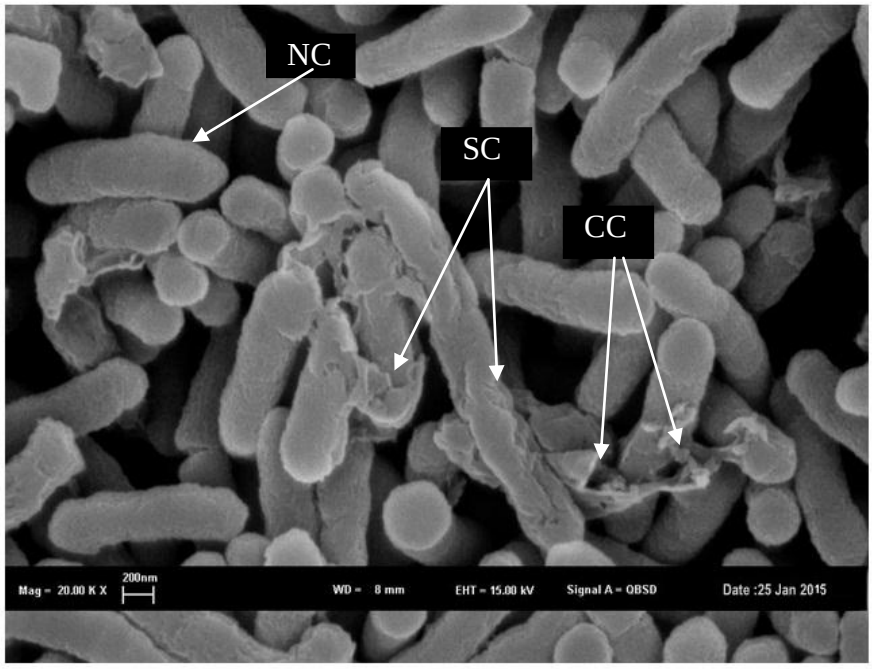

Fig. (8) SEM micrograph of *E. coli* O157:H7 after treatment with 0.5 mg/ml basil oil at the stationary phase. NC: Normal cells, SC: Shrunken cells, CC: collapsed cells

Observation by SEM was carried out to enable the observation of morphological changes of *E. coli* O157:H7 induced by basil oil. From the results shown in Fig. 3 to 7, the cells were obviously damaged by basil oil. Antibacterial or antibiotics are able to interfere in different ways to the metabolic pathways of bacteria. As a consequence, the structure of the cell wall changes directly or indirectly. Alterations in the shape of the bacteria happen subsequently. Hence, the cells become wrinkled or even burst. The bactericidal mechanism can be the damaging factor, such as retardation in DNA synthesis, folate metabolism, protein synthesis, or peptidoglycan synthesis (32). Besides damaged cells, some bacterial cells showed normal structure and viable through microscopic count but the growth of bacteria on nutrient agar plate was undetectable. Therefore, there is evidence that basil killed *E. coli* O157:H7 without lysing the cells. This supports earlier observation in batch culture studies that total counts were higher when CFU were undetectable. When the bacteria is treated with antibacterial agent, the substrate might adhere to the cells and hinder the development or growth of bacteria cells (33). The basil oil might adheres to bacteria cells and hinder it from forming colony on nutrient agar plate. Another explanation for that is related to bactericidal mechanism. The death of majority cells is not caused by the destruction of membrane cell. This evidence further proves that the mechanism(s) killing *E. coli* O157:H7 did not solely depend on the lysis of cells but rather by other mechanism(s). However, the exact mechanism(s) is undetermined in this project. Essential oils are volatile products of plant secondary metabolism and made from a very complex mixture of volatile molecules. Plant essential oils containing aldehydes or phenols as major constituents have the highest antibacterial activity (34). For the antibacterial study of essential oil, oregano (*Origanum vulgare*) and thyme (*Thymus vulgris*) essential oils showed significant

bacteriostatic and bacteriocidal properties towards *E.coli* O157:H7. These oils can be used to prevent bacterial growth especially in food industry (28). The results published on the chemical composition of *O. basilicum* oil reveal that its antibacterial activity could be due to the presence of several compounds, like linalool, eugenol, methyl eugenol, thymol, and bornyl acetate (35). 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 (36) Leakage of ions and other cell contents can then occur (37, 38) .Some essential oils, such as oregano (*Origanum vulgare*) and thyme (*Thymus vulgris*), showed significant bacteriostatic and bacteriocidal properties towards *E.coli O157:H7* (28).

Conclusion

In conclusion, basil oil was active against older cells of *E. coli* O157:H7, that is, those cells that were in the late exponential and stationary phases. By SEM, morphological alterations were detected among cells treated with basil oil. The oil was able to kill *E. coli* O157:H7 cells not solely by lysing the cells but rather by other mechanism(s) not known from this study.

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