# Studies of the antimicrobial activity of Black Seed Oil from *Nigella Sativa*on *Staphylococcus aureus* and *Escherichia coli*

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**Abstract:** It is well known that various plants (whole or some parts) are of definite and useful use for human benefit and well fare. One of these benefits is the ability of many plant seeds, fruits and different parts of exerting antimicrobial activity. The aim of theses uses is the treatment of various infectious diseases. This would be clearly understood that such plant may display a role in inactivating the underlying causes of diseases. These are to include various bacteria and may some of fungi if not extended to include viruses. **Methods:** In this study, commercially available black seed oil was collected from the public market in Libya. The latter was reconstituted with sterile deionised water to various concentrations and then tested for the antimicrobial activity using both Gram positive *Staphylococcus aureus* (ATCC 6538) and Gram negative *Escherichia coli* (ATCC C600). Antimicrobial activity evaluations were performed by the cub-cut agar method and the killing curve (survival curve) method. A further investigation was to understand the mechanism of action of black seed oil and how it inactivates bacterial cells was carried out. All antimicrobial investigation studies were referenced with the efficacy of 5% (v/v) phenol. **Results:** A distinctive zone of inhibition was detected at various concentrations of the black seed oil. This was clearly demonstrated against both *S. aureus* and *E. coli*. Survival curve experiments have demonstrated that *S. aureus* was more susceptible than *E. coli*. This was clearly illustrated in the reduction of log of survivors. This was displayed by 1.7 log kill in the case of *S. aureus* whilst 0.9 log of kill for *E. coli*. Further spectrophotometric experiments displayed a detection of bacterial organelles over a wave length at $A_{350nm}$. This absorbance was followed by the significant reduction in the concentration of theses organelles. **Conclusion:** The black seed oil has shown an antimicrobial activity that able to inactivate both *S. aureus* and *E. coli.* Such activity would be elucidated in its ability to target bacterial cell envelope causing its damage. This might result in bacterial lysis as a result of losing bacterial components and/or target bacterial organelles. Black seed oil, therefore, in addition to its role as a part of food constituents would be of a great benefit for the treatment of various infectious diseases that are of bacterial origin

## Introduction

Many of antimicrobials that are used today are related in terms of natural structure. In many cases, chemically synthesized drugs have obtained their modulated structure from nature and/or modified from herbal sources. With better techniques and knowledge, synthesized compounds from natural plants will most likely also lead to better results in the field of antimicrobial therapy and the control of infectious diseases (1, 2).

Plants are rich in a wide variety of phytochemical products, such as tannins, terpenoids, alkaloid sand flavonoids, which have been found in vitro to have antimicrobial properties (3).

Black seed is an annual herbaceous plant that is believed to be indigenous to the Mediterranean region but has been cultivated into other parts of the world including Arab dessert, Northern Africa and some parts of Asia. Black seed originates from the common fennel flower plant (*Nigella sativa*) of the buttercup (*Ranunculaceae*) family. Nigella sativa and its black seed are known by other names, varying between places. Some named it black caraway others named it black cumin (Kalonji) or even coriander seeds. Nevertheless, this is *Nigella sativa*, which has been known and used from ancient times (3-5) in curing diseases. The Prophet Muhammad (Peace be Upon Him) said in his divine wisdom speech about the Black seed *"Use this Black seed; it has a cure for every disease except death" (SahihBukhari).* Black seed unquestionably has a positive and stabilizing effect on the human body. This would result in supporting the human health and welfare that may go beyond curing simple disorders such as skin diseases and allergies. It would extend to support the immune system that intuitively helps the body in curing and eliminating the underlying cause of many diseases (6, 7), purify the blood, protect against irritants and support bodies during recovery (2, 4, 5). Many studies have displayed the antimicrobial activity of Black seed oil against variety of microorganisms. Such effect was displayed against *E. coli*, *Salmonella*spp, *Shigella* spp. and some strains of Vibrios bacteria. This was also extended to inhibit the proliferation of some fungal growth (8-10). Several studies displayed that the black seed oil contains over 100 valuable nutrients. It contains 21% protein, 38% carbohydrates and 35% plant fats and oils. Among these constituents are the Thymoquinone, Nigellone, and some fixed oils. Other ingredients are to include linoleic acid, oleic acid, calcium, potassium, iron, zinc, magnesium, selenium, vitamin A, vitamin B, vitamin $B_2$, Niacin, and vitamin C (11, 12). These constituents, in addition to others phenolics and flavonoids display a number of medical uses. For examples: treating urinary and respiratory tract infections, relieving cough and digestive problems and possible activity against methicillin resistant *Staphylococcus aureus* (13-15). Its effect extended to display antipa-rasitic action and insect repellent activities, as well as relieve vomiting and diarrhea, and flatulent colic, and to help counteract and relieve from the griping action (10, 16, 17).

To the best of our knowledge, no previous studies were reported about the antimicrobial activity of black seed oil in Libya at least. Thus this study was aimed to evaluate the antimicrobial activity of the commercially black seed oil and understanding its possible effect on challenged bacteria.

## Materials and methods

*Chemicals and Preparations:* Black seed oil at different concentrations (v/v) of 10%, 30%, 50%, 70% and 90% were prepared into phosphate buffer saline (PBS) (0.01 M) where the pH is adjusted to 7.0 and kept at cold room. Solubility was performed first

into 0.02% DMSO (18) then volume completed by PBS to desired concentration. Nutrient broth and Nutrient agar media used to grow bacterial strains. All dehydrated media were obtained from BDH (Poole, U.K.). All reagents were of the highest grade of purity (Aristar®) of the BDH suppliers.

*Microorganism and Culture Maintenance: Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC C600) strains were maintained in long term preserve system. This was prepared into the lab using 10% (v/v) glycerol in nutrient broth media. The stock was dispensed into 1 ml aliquots and stored at - 80$^{o}$ C till required. Fresh cultures were prepared from frozen stocks prior to every experimental procedure. This was performed by streaking, using sterile loop, freshly prepared nutrient agar plates which were then incubated at 37° C for 18hrs, from which a colony was used to run the experiments.

*Antimicrobial Susceptibility Experiments:* All antimicrobial susceptibility experiments were conducted by transferring 5ml of the tested strains grown at late exponential phases (O.D. of $A_{470nm}$ A0.9 and 1.2, respectively) for *S. aureus* and *E. coli* strains. This will give bacterial suspension of 1x10$^{8}$ cfu per ml. Absorbance was measured using the spectrophotometer (PU 8675 Vis spectrophotometer), Philips, Germany. The Black seed oil was added, to the bacterial suspension, to give final concentrations (v/v) of 10%, 30%, 50%, 70% and 90%. A sample of volume 1 ml was taken every 1 hr intervals up to 6 hrs and then at 24 hrs where the serial dilutions were performed and the viable count calculated using the spread plate technique. Viable count was performed by transferring 100 µl of each sample taken onto predried nutrient agar plates where then incubated for 24 hrs and counted for viable cfu per ml. Data were expressed as the log of survivors against exposure time. These were used to set up the survival curve (dose response curve). All experiments were performed in triplicates and the mean was used throughout this study. Experiments were also designed to understand the effect of the Black seed oil on which part of the bacterial cell. This was performed by collecting samples of the exposed populations every 1hr up to 6 hrs and then at 24 hrs to measure the absorbance at the wave length 350 nm. Data were expressed as the absorbance reading $A_{350nm}$ against the exposure time. This will indicate, based on the absorbance reading, which part of the bacterial cell was targeted by the Black seed oil. In preliminary evaluation of the antimicrobial activity of the Black seed oil, cup cut agar method was performed. A 5% (v/v) phenol was used and data were compared to that of Black seed oil.

## Results

***Antimicrobial Susceptibility Experiments***

The cup-cut agar method was used as a preliminary step for the qualitative evaluation of the antimicrobial activity of the Black seed oil at the concentrations of 10%, 30%, 50% and 70% (v/v). In the case of *E. coli*, activity was not clearly observed. Black seed oil concentrations were, therefore, elevated to 90%, whereas a clear zone of inhibition on *E. coli* was then determined. On the other hand, inactivating activity of black seed oil

was clearly observed at 10%, 30%, 50% and 70% (v/v) on *S. aureus*. The zone of inhibition was related to the extract concentration (Table 1).

It should be mentioned that the *S. aureus* showed a remarkable susceptibility over that shown by the *E. coli*. This was clearly noticed on the diameter of the zone of inhibition. In general both bacteria were susceptible to the effect of the Black seed oil at different concentrations, where the *E. coli* showed susceptibility only at the 90% concentration (Table 1).

**Table 1:** Activity of the Black seed oil on *S. aureus* and *E .coli* expressed as zone of inhibition in mm.

| Bacteria | Concentration (v/v) | | | | |
|---|---|---|---|---|---|
| | 10 % | 30 % | 50 % | 70 % | 90% |
| | Zone of Inhibition in mm | | | | |
| *S. aureus** | 18 | 24 | 25 | 38 | Not applied |
| *E. coli*** | No | No | No | No | 13 |

* 5% phenol showed Zone of inhibition (mm) = 26
** 5% phenol showed Zone of inhibition (mm) = 25
All experiments performed in triplicate and repeated three times, where the mean used to plot the data

As the Black seed oil has proved to display a significant antimicrobial activity, it is very important therefore to reference this activity to one of the most potent agents. In this context the phenol 5% (v/v) was the reference antimicrobial agent used (Table 1). In the case of *S. aureus*, the zone of inhibition of 70% oil represented about 1.5 fold of the phenol one. This was 38mm for the extract and 26mm for the phenol. The zone of inhibition for 50%, 30% and 10% concentrations were 25 mm, 24 mm and 18 mm, respectively. In the case of *E. coli*, its susceptibility was also observed. This was 13 mm for the oil (90%) compared to that of the 5% phenol (25 mm). This represents about half fold of the phenol activity (Table 1). No activity was detected for the concentrations 10, 30, 50 and 70%.

This study was also designed to evaluate the antimicrobial activity of Black seed oil quantitatively by determine the remaining of survival cells after exposure to different concentrations over period of times. Results in Figures 1 and 2 showed antibacterial activity of the Black seed oil 70% and 90% towards *S. aureus* and *E. coli*, respectively.

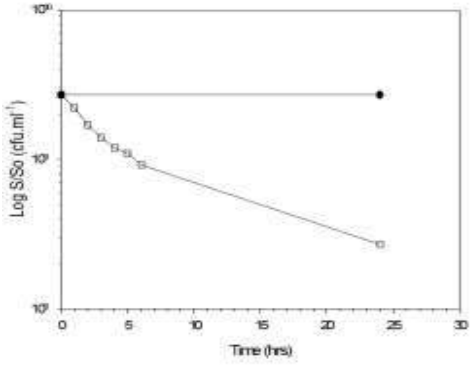


**Figure 1:** Effect of Black seed oil on *S. aureus*. ●control, ○ 70% black seed oil.

In the case of *S. aureus*, the level of bacterial kill was clearly demonstrated. This was about 1.7 log reduction compared to the control over 24 hrs exposure (Fig. 1). The level of reduction in the cfu per ml was from $1.76 \times 10^9$ to $4.6 \times 10^7$ cfu per ml. This displayed that about 97% of the population were killed. Similarly, *E. coli* populations were also susceptible but with lesser extent. This was demonstrated in the rate of bacterial survivals over 24 hrs exposure to 90% of the agent concentration. The reduction in the *E. coli* survivals was measured by about 0.9 log reductions (Fig. 2).

This indicated that about 80% of the population was killed and 20% survived. It is therefore clear that the *S. aureus* population is more susceptible to the effect of the black seed oil than that of the *E. coli* one. It is very important to notice that the bacterial population used to investigate the antibacterial activity of the Black seed oil was collected specifically at the late exponential phase for both *E. coli* and *S. aureus* (Figs. 3 and 4). This was intended to avoid both the exponential phase that characterized by its susceptibility to the effect of antimicrobials and the stationary phase where the bacteria are already started to die. This will impact that the obtained results was mostly reflect the action of the oil minimizing the incidence of biological and physiological variations of the bacteria.

Further investigation in this study was aimed to understand the possible antibacterial action of the Black seed oil on the bacterial cell. An experiment of measuring the absorbance of challenged bacterial populations at low wave length ranged at $A_{350nm}$ was designed. This was aimed to measure the absorbance of bacterial structure when small bacterial organelles such as RNA, DNA and ribosomes are suspended and released into the medium.

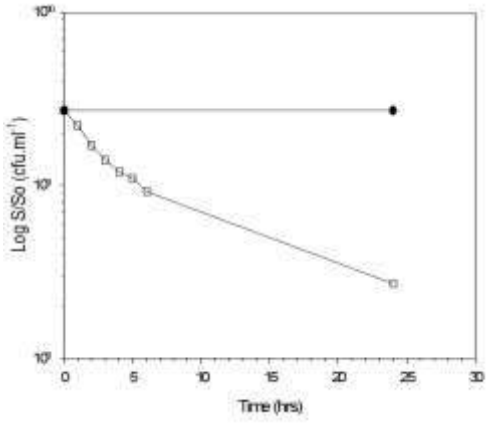


**Figure 2:** Effect of Black seed oil on E. coli. ●control, ▫ 90% black seed oil.

In such instances, if reading was detected at such low wave lengths, this would therefore indicate the possibility of antibacterial agents has an effect on the bacterial envelope and bacterial organelles were released and absorbance was detected.

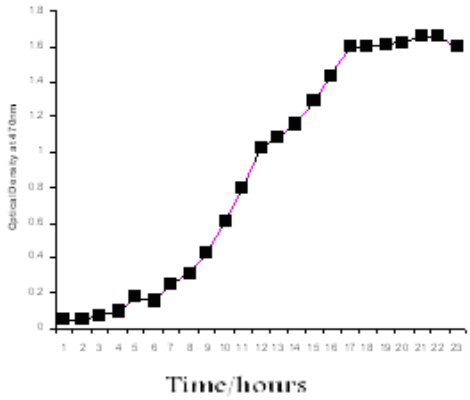


**Figure 3:** Growth curve of *S. aureus*.

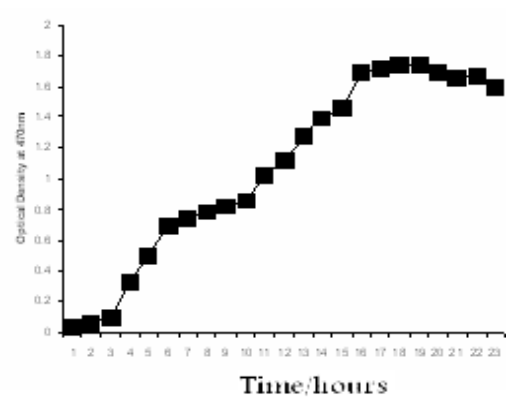


**Figure 4**: Growth curve of *E. coli*

In this study, detections of light absorbance at $A_{350nm}$ were succeeded. This would intuitively displayed that black seed oil managed to distort and interfere with the cell envelop for both *S. aureus* and *E. coli* to various extents. All detections were explained in Figure 5 over 24 hrs exposure. All samples were collected alongside with the viable count sampling for colony forming unit measurements. Figure 5 has showed that the absorbance was relatively high at the first 3 hrs exposure compared to those at next exposures up to 6hrs whilst, on the other hand, it was higher than that at 24 hrs exposure. The degree of variation was higher in the case of *S. aureus* than that of the *E. coli* by about 10 folds (Figure 5). This would clearly display that Gram positive bacteria are more susceptible to be inactivated by black seed oil, which would be explained in the variation in their cell envelope structure. These data are in consistence with the data collected above (Figs. 1, 2).

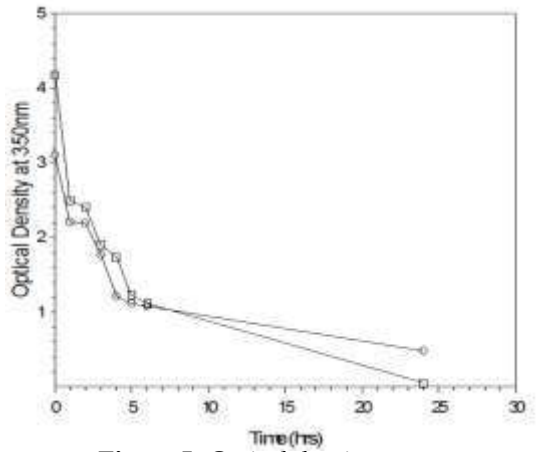


**Figure 5:** Optical density measurements for challenged *S. aureus*(○) and *E. coli*(▫) populations to70% and 90% black seed oil respectively at $A_{350nm}$ over 24hrs exposure

## Discussion

When comparing the activity of the Black seed oil to the most powerful antimicrobial and biocide, data shown that the oil potency was variable based on the type of bacteria. This was about 1.5 and 0.5 folds for the *S. aureus* and *E. coli* respectively, of that of phenol. This would indicate that such potency could be the reason behind its acceptability for community and public use and, in treating various cases of bacterial infections. Similarly, the ability of the black seed oil for observed inactivation of the population over 24 hrs of exposure is another evidence of its antibacterial activity. This was clearly demonstrated in the survival curve method. The survivors fractions recovered on the plates were 2% and 20 % for both *S. aureus* and *E. coli* respectively. In this respect, the phenol 5% v/v had eradicated the whole population upon exposure.

This was demonstrated at zero time of exposure where the plates showed no recovered colonies (data not shown). Such inactivating activity of the Black seed oil was demonstrated by dramatic decrease in the number of survivors. In such instances, the black seed oil would clearly play a very important role in inactivating bacterial populations. This would be elucidated in understanding its mechanism of action. The authors suggest that the oil was able to interfere with bacterial envelope function. Doing so will lead to the inability of bacterial cell to control molecule movements which might lead to bacterial lysis and burst. This would eventually lead to cell death.

In this context, preliminary information about understanding the possible mechanism of action of the black seed oil was obtained. This was achieved by conducting an experiment of measuring the absorbance of challenged bacterial populations at low wave length ($A_{250 - 350nm}$). This will detect the bacterial cell organelles if they are released into the medium (15, 19-21).

Findings of optical density measurements for challenged *S. aureus* and *E. coli* populations to 70% and 90% black seed oil respectively at $A_{350nm}$ over 24hrs exposure had confirmed these findings where detection of absorbance $A_{350nm}$ was achieved. Such readings would laid the principle of bacterial cell envelope is the primary target for black seed oil. In one hand, *S. aureus* was more susceptible with very low absorbance reading at 24hrs exposure. On the other hand, *E. coli* susceptibility was significantly less with a higher absorbance (10 folds) at 24 hrs exposure. In both cases, non treated control samples showed no absorbance detections at $A_{350nm}$ confirming these findings. In this manner, the bacterial physiology of the cell envelope of Gram negative that differ from that of Gram positive bacteria in having the layer of outer membrane would contribute for such variation in susceptibility (21) and therefore in absorbance detection. In details, the outer membrane layer of the Gram negative bacteria would retard the access of the black seeds molecules (considering molecule size) to a certain extent. This would result in delaying the onset of bacterial kill and therefore the magnitude of organelles detection (22-24). Conversely, the absence of the outer membrane in the case of Gram positive bacteria eliminates such factor (25, 26) where the black seed oil molecules found their way to interfere with bacterial wall and therefore enable early detection of the cell organelles. Interestingly, further reduction in the reading of absorbance $A_{350nm}$ was significantly detected over the exposure time. Such dramatic decrease for both bacteria, considering the magnitude in reduction, would indeed, extend the postulation for the ability of black seed oil to further target the bacterial organelles in its action. This would be expected either directly by inactivating such organelles or by indirectly through the role of bacterial lysozymes (autolysin enzymes) (27-29). Bacterial lysozymes are normally produced when bacteria are exposed to stress agent and the recovery is a futile attempt (30, 31). Therefore the black seed oil would further have the ability in activating the release of such enzymes explaining the ultimate and rapid inactivation of bacteria.

Previous studies postulated that Black seed oil might have an effect in the prevention and treatment of severe bacterial infections, especially those that are difficult to treat and/or are antibiotic resistant. They postulated their mechanism of action on *S. aureus* and *E. coli* is to disintegrate the cell envelope releasing associated bacterial material from the cells to the surrounding medium. Such effect might be attributed to a number of the components of the black seed oil (32) that are related to either the disruptive ability of the bacterial envelope and/or inducing the release of some autolytic enzymes as postulated above. Such postulations would deserve further investigation and implying that the interference with the bacterial permeability barrier is not the sole mechanism of action. In this context, a possible identification of the oil constituents and performing molecular inve-

stigation is strongly suggested. According to the present study, it can be concluded that Black seed oil had proven to show significant antibacterial activity against various bacterial types and through different inhibitory mechanisms. This confirms the satisfactory use of the Black seed oil at least help in treating various underlying causes of different infectious diseases.

*Acknowledgments:* The authors would like to thank all people who supported and helped in performing this research. We would like to thank: research assistants Asma-Ejhany and Najat Magerhy for their help and support.

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