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The Antibacterial Property of *Nigella sativa* (Black seed) Oil Against Gram-positive and Gram-negative Bacteria

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ABSTRACT

Antibacetrial resistance and bacterial genetic modification is one of the most challenges that face the world today, and it is heading toward the post-antibiotic era. Traditionally, herbal extracts, medicinal plant oil extracts, and probiotics have been used as an alternative to antibiotics, because most bacteria become resistant agenst antibiotic inspit of drug side effects. This work is designed to estimate the antibacterial efficacy of Nigella sativa (N.sativa) oil extracts against common antibiotic resistant bacteria, including Methicillin-resistant staphylococcus aureus (MRSA) and Pseudomonas aeruginosa (P. aeruginosa). The black seed was purchased from the traditional herbal medicine market. The oil was extracted using Hydro distillation and steam distillation method. The bacteria were obtained from a microbiology company and from clinical samples at the Shar hospital in Sulaimani Provence. The Gram-negative bacteria were; Salmonella enterica (S. enterica), Escherichia coli (E.coli), and Pseudomonas aeruginosa (P. aerouginosa) while the Gram-positive bacteria were; MRSA, Bacillus subtilis (B.subtilis), and Bacillus cereus (B.cereus). The

agar diffusion well technique and minimum inhibitory concentration (MIC) were followed for determining the antibacterial effect of N. sative oil. The black seed oil components were analyzed by the Gas Chromatograph mass spectrum. The results revealed that the oil has direct effect on Gram-positive bacterial growth, including MRSA, which is known as a multidrug resistance bacterium. Bacillus subtilis was more sensitive than other strains, and the significant antibacterial effect of the extracted was observed against E. coil, while it has no significant effect on P. aeruginosa and Salmonella enterica. Determination of Nigella sativa oil minimum inhibitory concentration (MIC) for Gram-positive bacteria is 100 ml for Staphylococcus aureus and Bacillus subtilis, and the MIC was 200 ml and 400 ml for Bacillus cereus and MRSA respectively. This study concludes that oil extract of Nigella sativa is a good natural antimicrobial, it can be used against MRSA and other Gram-positive bacteria.

1. INTRODUCTION

Antibiotics have developed and been used effectively in the 20th century to treat major infectious diseases; however, antibiotic resistance phenomena have emerged against the available antibacterial drugs and even soon after the introduction of the newly discovered drugs [1]. Therefore, it leads researchers to seek a new antibacterial agent with less bacterial resistance and fewer side effects [1]. The black seed is a common traditional herbal medicinal plant that is used in most countries as traditional medicine and food additive. The black seed oil content is a fixed oil, essential oil, proteins, and alkaloids, which consist of several active ingredients with antimicrobial activities and also have anti-inflammatory effects [2]. Black seed is used traditionally for wound healing, which accelerates wound healing especially burn skin due to its antioxidant properties and prevents the peroxidation of membrane lipid into cells due to Thymoquinone action that inhibits cyclooxygenase and 5-lipooxygenase and thereby attenuated inflammation [3, 4]. Furthermore, black seed improves respiratory tract infections and asthma; as researchers revealed that after a daily administration of *Nigella sativa* oil for two weeks, improvement of nasal dryness and obstruction were observed [5,6,7]. The black seed is traditionally applied for treating fever, flu, headache, vermifuge, scorpion stings, and snake bites. It also has been used as a carminative, diuretic, galactagogue [1]. Rheumatoid arthritis is one of the diseases that may be cured by receiving placebo capsules that contain 500 mg of *Nigella sativa* oil. Moreover, an inflammatory biomarker of Rheumatoid arthritis was measured after eight weeks and significant changes in biomarkers were observed, the patients experienced a significant serum interleukin 10 (IL-10) increase, while malondialdehyde [MDA] and nitric oxide [NO] significantly decreased in the serum, which indicates that black seed may improve certain biomarker of inflammation and oxidative stress in Rheumatoid arthritis and improve swelling and tenderness symptoms [8, 9].

Nigella sativa seed is widely used worldwide for medical purposes especially in Islamic medicine, *Nigella sativa* seeds oil or crushed as a powder has therapeutic activities on respiratory, blood circulation, immunity, and endocrine system, in which the thymoquinone has a great role in the oil of black seed that has attributed for pharmacological properties [10].

Nigella sativa consists of several bioactive compounds such as flavonoids, alkaloids, tannins, and phenolic, which have hypoglycemic, anti-inflammatory, anticancer, neuroprotective, antihistamine, and bronchodilator effects. and the investigation showed that *Pseudomonas aeruginosa* and *Enterobacter aerogens* resist black seed oil while the most sensitive strain was

S. epidermidis [11]. Depending on many studies, they indicate that black seed contains thymohydroquinone and thymoquinone that have inhibitory activity against bacteria especially Gram-positive bacteria [1]. Many studies indicate that herbal medicine is an alternative for treatment exactly against bacterial infection in Kurdistan [12,13]. Therefore, In the current work, the antibacterial activity of black seed has been studied against seven strains of bacteria, including Gram-positive bacteria strains and Gram-negative bacteria with the determination of MICs.

2. METHODS AND MATERIALS

2.1 Preparation of Black Seed Oil

The black seed *(N.sativa*) was purchased from the traditional herbal medicine market in Sulaimani. The hot extraction (Hydro distillation) of the oil was performed according to [14]; Briefly, 100 gm of dried black seed was crushed then used for extraction. Then the Clevenger - type apparatus was used for hydro-distillation of 100 gm seeds for 3 hours, and 500 ml distal water was added. The relative percentage was adapted to calculate oil content as a (v/w). The collected oil was poured into a dark bottle and preserved at 4 °C. Finally, the filtrating and concentrating of oil extracts was performed evaporating the solvents under reduced pressure. Dimethylsulphoxide (DMSO) was used for the preparation of stock solution (50 mg/ml) and then aliquots.

2.2 Bacterial Strains and Cultures

Seven clinical strains of both Gram-positive and Gram-negative bacteria were purchased from the American Type Culture Collection company (ATCC), which are *E. coli* ATCC® 8739™, *P.aeruginosa* ATCC® 9027™, *S.entrica* NCTC 6017, *B. subtilis* ATCC® 6633™, *S.aureus* ATCC® 6538P™, while the MRSA, and *B. cereus* was isolated from clinical samples (blood and urine) from patients admitted to Shar-hospital in Sualaimani province. The isolated bacteria (MRSA and *B. cereus*) were identified based upon biochemical tests according to standard microbiological techniques with their colony characteristics, bacterial culture, and biochemical [15,16], in which a single colony from each strain was picked, transferred, and suspended into a 5 ml of Muller-Hinton broth (MHB) tube, and then incubated for six hours at 37°C to get 0.5 McFarland standard turbidity. Before the use, the cultures were diluted 1in 10 with Müller-Hinton broth.

2.3 Antibacterial Activity

The agar disc-diffusion assay was used (antibacterial susceptibility tests) to determine the Black seed oil effect as described by Bauer [16]; The diluted bacterial culture (100 µl) was inoculated on sterile common bacterial medium (Müller-Hinton agar plates) by spread technique method. The wells at 4 mm diameter were cut in Müller-Hinton agar. Each well was filled with 20 µL of *Nigella sativa* oil, the then incubated at 37°C for 1 day. The most common antibiotic used as a positive control was Gentamicin (10µg) and tween (20 µl) was regarded as Negative control, then incubated overnight. The diameter zones of inhibition of the oil were measured in (mm) manually by a common ruler.

2.4 Minimum Inhibitory Concentration (MIC)

MIC of *N. Sativa* oil was achieved by using different dilutions of *N. sativa* oil against Methicillin-Resistance *Staphylococcus aureu*s, *Bacillus subtilis*, *B. cereus*, and *staphylococcus aureu*s. Serial dilution was prepared as 50, 100, 200, 400, 600, 800µL concentrations of the oil using (Tween 20 BIOCHEM company) as a solvent. The agar disk diffusion technique was used to evaluate the antibacterial activity of *N.sativa* oil on the bacteria, and pure tween 20 is considered as a control, then incubated at 37°C for 1 day [16].

2.5 Gas- Chromatography-Mass Spectrometry of N. sativa

The bioactive of the oil was analyzed by GC-MS (QP 2010 Plus SHIMADZU). The temperatures were set at 220°C and 250°C for injector and detector respectively. The dilution of oil was performed in methanol, and then a 1µl oil diluted oil sample was injected. For Analysis, the company instruction was followed, the comparison with those of authentic

reference compounds was applied for the identification of the different compounds for identification of the chemical compounds found in the black seed.

3. RESULT

3.1 Antibiotic Property of N. sativa

The Data of three replicate tests revealed that *Nigella sativa* oil extract has a significant antibacterial effect mostly toward Gram-positive bacteria. Including, MRSA, which is regarded as a multidrug resistance bacterium was susceptible to the oil extract. The result (mean of three replicates) and standard division of zone of inhibition against *Bacillus subtilis* were (26 ± 1.9) mm, *Staphylococcus aureus* was (22 ± 0.5) mm, *Bacillus cereus* was (20 ± 0.7) mm and methicillin-resistant *Staphylococcus aureus* was (14 ± 1.2) mm (Figure 1).

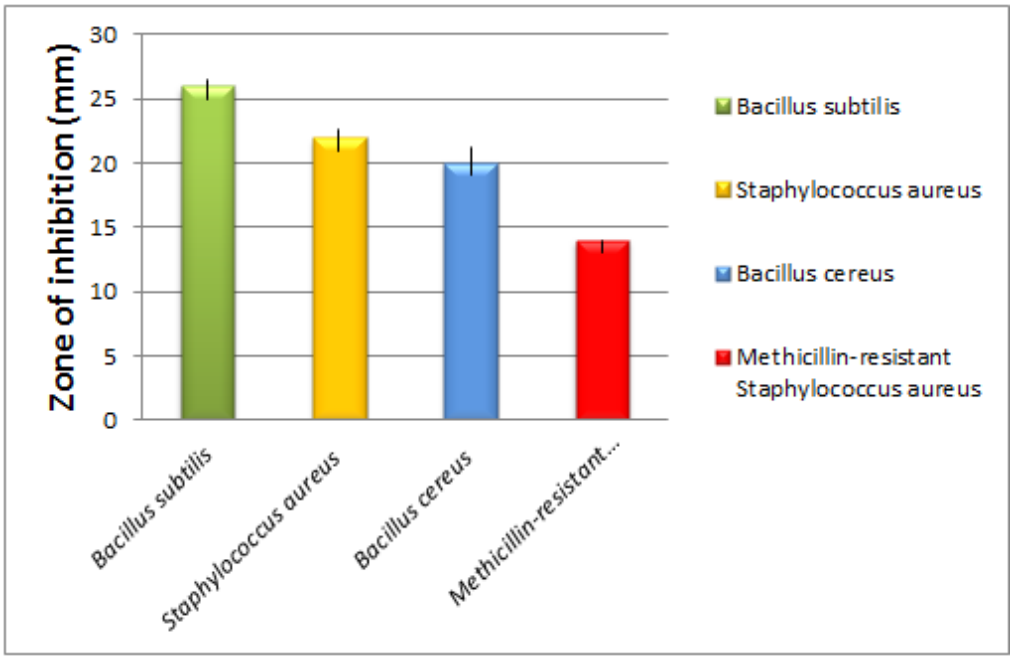

Figure 1: The inhibition zone of *N. sativa* oil against G^{+ve} bacteria using three replicate for each sample

The inhibition zone of the oil against (Gram-negative bacteria) *Escherichia coli* ATCC® 8739™ was (11.5 ± 1) mm while there was no zone of inhibition against *Pseudomonas aeruginosa* ATCC® 9027™, *Salmonella entrica* NCTC6017 (Figure 2and appendix 1).

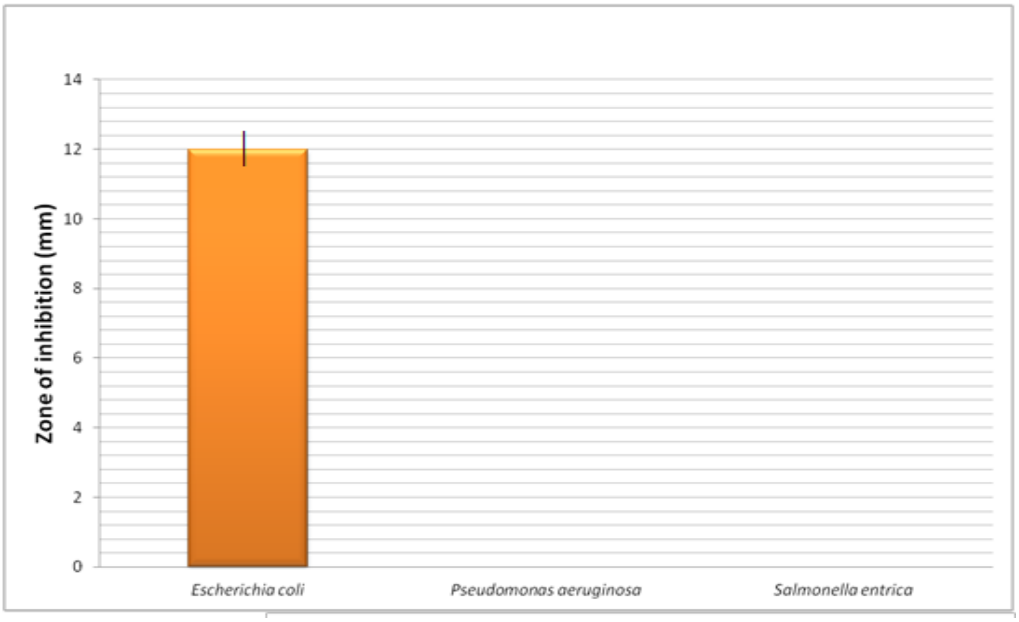

Figure 2: The antimicrobial activity of the *N. sative* oil toward G^{-ve} bacteria ((*Escherichia coli, Pseudomonas aeruginosa*, and *Salmonella entrica)*

The high effects of *N. Sativa* oil toward MRSA were observed in comparison with the common antibiotic disks which are used in this study. The results revealed the inhibition zone of *N. sativa* oil was (14 ± 1.2) mm. While the inhibition zone of tetracycline was (16 ± 0.8) mm, the MRSA was resistant to Erythromycin, Ampicillin, and Gentamicin and there was no zone of inhibition (Figure 3).

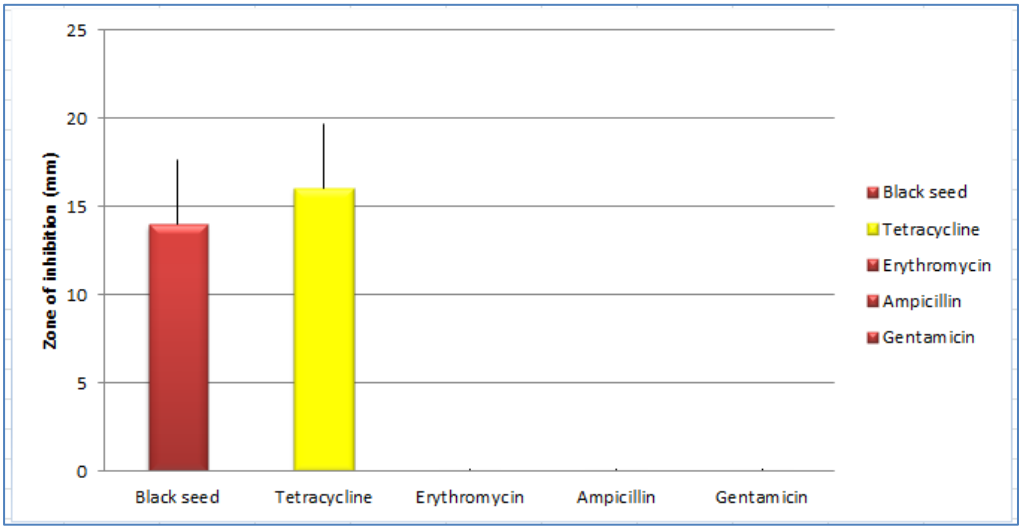

Figure 3: Comparison of *N. sativa* oil effect with some antibiotics (Tetracycline, Erythromycin, Ampicillin, Gentamicin). against MRSA.

***3.2 Minimum Inhibitory Concentrations** (MICs)*

The MICs were determined for each bacteria using three replicate for each bacteria and the mean of them was calculated firstly the serial dilution was prepared (100 µl, 800 µl, 600 µl,

400 µl, 200 µl, 100 µl, and 50 µl). The result of MIC shows that the activity of oil extract of black seed on MRSA is at the concentration of 400 µl with (7 ± 1) mm of inhibition zone and 800 ml concentration was (10 ± 0.9) mm. While the concentration from 200 µl to 50 µl of oil has no inhibition zone. Furthermore, the MICs for *B. subtilis* bacteria, the lowest concentration of the oil is 200 µl with an inhibition zone of (9 ± 0.8) mm and there was an increase of inhibition zone with increasing the concentration of the oil; For instance, 400 µl and 800 µl concentrations, the inhibition zones were (18 ± 1.2) mm and (22 ± 1.9) mm respectively. However, with 100 µl and 50 µl concentrated there was no observable inhibition zone. In the case of *S. aureus*, 100 µl concentration of the extracted oil is considered as the lowest concentration at which the zone of inhibition was (9 ± 0.8) mm and there was an increase of inhibition zone with increasing the concentration of the oil; for instance, 200 µl and 400 µl and 800 µl concentrations have the inhibition zones of(15 ± 1.2) mm, (18 ± 0.4) mm and (20 ± 1.6) mm respectively and there was no inhibition zone with 50 ml concentration. Finally, 200 µl of the extracted oil was considered as the lowest concentration at the inhibition zone of (11 ± 1) mm was observed with *B. cereus* and for 400 µl and 800 µl concentrations, the inhibition zones were (14 ± 0.5) mm and (17 ± 1.1) mm respectively, while there was no observed inhibition zone with 100 µl and 50 µl concentration, the MICs of the *N. sativa* oil were shown in [figure 4].

3.3 Chemical constituent's analysis of N. sativa oil extract

GC–MS analysis data detected several constituents found in *N. sativa* oils. Most of the components in the extracted oil in methanol were shown in [Figure 5]. The determination of oil extract complex was performed by comparison of the retention time mass spectral data of compounds with the references compounds according to the NIST Library.

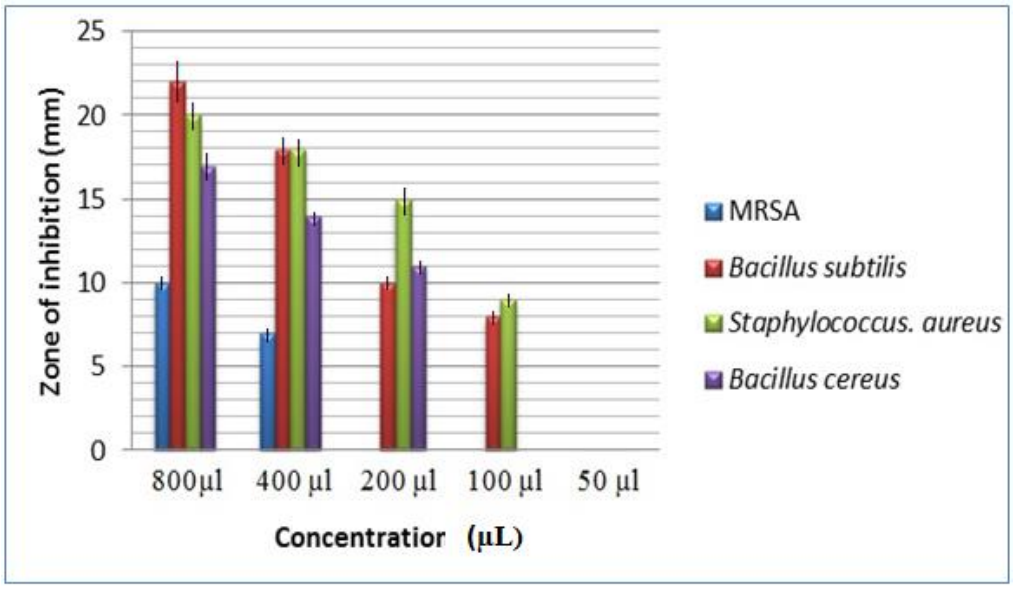

Figure 4: Determination of MIC for *N. sativa* oil against some Gram-positive bacteria.

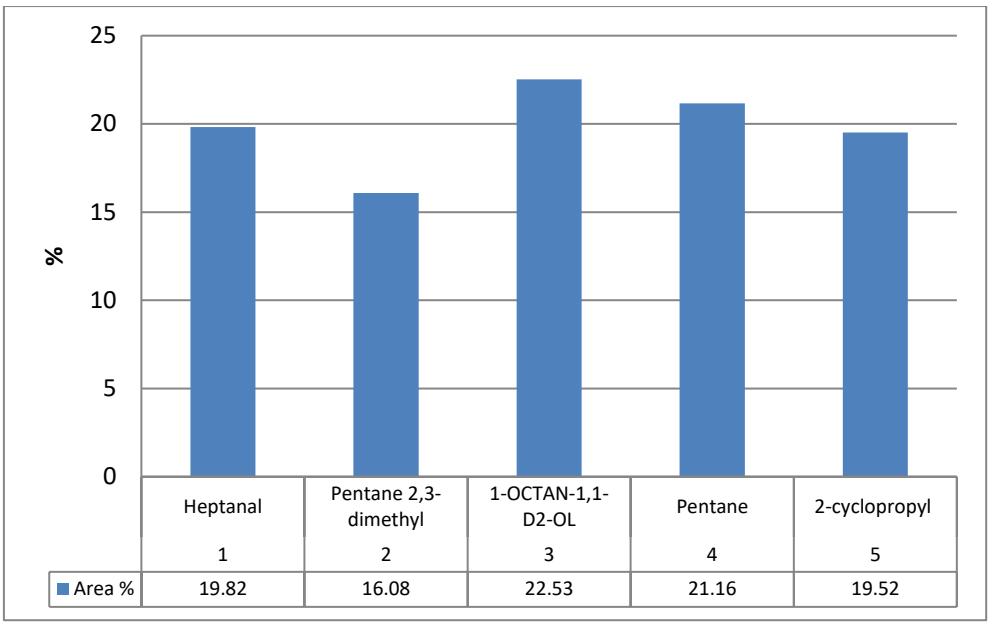

Figure 5: *Nigella sativa* oil extract analyzed by GC-MS shows the common compounds.

4. DISCUSSION

Black seed (*N.sativa*) is a herbal medicinal plant, which has a flat, trigonous, and angular appearance, it contains some bioactive substances which have medical importance [17]. The black seed has many nutritional and pharmaceutical roles and especially in curing several clinical conditions; respiratory problems, diarrhea, hyperglycinemia, and many cancer cells [18, 19].

Many studies indicated that black seed has bactericidal and pharmacological effects, they stated that many bacterial infections and especially gram-positive bacteria can be killed or inhibit the growth of bacteria by black seed oil also it has an anti-inflammatory effect [17-19] while our study evaluates the effect of *N.sativa* oil on the most common resistant bacteria to the antibiotics, and it is known as MARSA.

Turkmani *et al*., state that among many herbal medicinal plants the *N. sativa* is emerging as a miracle herb this related to a rich historical and religious background because oils have an important role in human health, curative and protective actions like possessing antitumor activity, In addition, it is used to the backing stomach, intestinal health as well as kidney and liver work [20].

The current investigation emphasized a number of the previous researches that have achieved for determining the antimicrobial action of the *N. sativa* oil extract. The results revealed oil extract of *N. sativa* has effective antibacterial action against Gram-positive bacteria; however, such effect did not show with Gram-negative bacteria except *E. coli* that is effectively inhibited by *N. sativa* oil, and these results are in agreement with other researches [19] they indicated that the black seed oils can inhibit the growth of *S. aureus* and this effect may be due to the two important active compounds that present in *N. sativa* oils, which are thymoquinone and melanin. in the case of *E. coli*, our results are not agreed because there was a strong zone of inhibition by black seed oil against *E. coli*. This difference in results may be due to using a different strain of the bacteria as the bacterial strain of *E. coli* was not mentioned in his study [19].

Regarding the effect of black seed oil on MRSA growth, our finding was supported by Othman *et al.*[21] they indicated a remarkable antibacterial effect of *N. sativa* oil against pathogenic Gram-positive, Therefore, the potential antibacterial action of black seed oil against MRSA requires serious attention because MRSA is considered as an opportunistic

bacterium among cutaneous microflora while it is responsible for a variety of infections such as wound infections and septicemia, which has likely a threat to human health. Moreover, black seed oil revealed an inhibitory effect against *S. aureus*, and MIC was 200 ml concentration. This result is similar to Emeka *et al.*[22] that demonstrated a dramatic inhibition of bacterial growth by active components found in *N. sativa* oil.

The results obtained from this study showed the predominant spectrum of black seed oil antibacterial action against *B. subtilis* and *B. cereus*, These results are supported by Javed *et al* [23] and Zuridah and his colleagues [24] they stated that the phenols and thymoquinone which are soluble in methanol and it reveals a great antibacterial effect against *B. subtilis*, Also a study by Shova, showed a similar result against *B. cereus* even at a very low concentration [25]. But Safhi *et al.* [26] disagreed with our results they showed negligible activity of *N. sativa* against *B. subtilis*.

Despite growth inhibition of *E. coli* by black seed oil; however, such effect was not observed against *P. aeruginosa* and *S. entrica*. This result is agreed with the finding by Ferdous *et al.*[27], which stated that *E. coli* is greatly susceptible to *N. sativa* oil; whereas, *P. aeruginosa* showed resistance for such oil. Many factors may affect the oil and antibiotic's action on the bacteria such as mode of action of the antibacterial, the bacterial structure, virulence factors, presence of the toxin, and presence of spore.

The effect of black seed oil was scientifically demonstrated in the extensive research done by Emeka *et al*. [22] Which showed that the significant influence of oil on the multidrug-resistant *Staphylococcus aureus*. MRSA mostly causes skin untreatable infections and abscesses. In the United States, about 40% of nosocomial infection typically involves MRSA [28]. MRSA has instantly become a major health concern globally because it resists various *anti-staphylococcal* agents. Urgently newer antimicrobials infectious agents are genuinely needed to combat this potential problem.

In our study, the GC-MS analysis of the *N. Sativa* composed of 5 essential compounds all of them is an extraordinary mixture of organic compounds alkaloid and shows high biologically active such as (Heptanal, Benton 2,3-dimethyl, 1-OCTAN-1,1-D2-OL and Pentane, 2-cyclopropyl). Black seed qualitative analysis compounds are undoubtedly shown that black seed inhibits bacterial growth revert to the chemical composition, The present study indicates that one of the main characteristics of black seed is a very good natural antibiotic for gram-positive bacteria that was similar to a study by Krishnapura [29]. The identification structure of the compound using a mass spectrometer to determine the compounds contained in the essential oil samples (*Nigella Sativa* L.) analyzed can be seen from the relative abundance of mass fractions of molecules of the molecular ion. Therefore, the purpose behind GC- MS analysis is to identify the chemical components in a proper way depending on the mass transfer of substance into the solvent also electron ionization plays a series role in the routine analysis of small molecules [30]. The basic components of *N.Sativa* oil consist of the varied mixture (thymoquinone, p-cymene, and 4-terpineol) as the highest amount of a chemical compound [31] These results differ with our results because we investigate (Heptanal, Pentane 2,3-dimethyl, 1-OCTAN-1,1-D2-OL and Pentane, 2-cyclopropyl) this due to environmental factors like location and genetic makeup.

5. CONCLUSION

The current study concludes that *N. sativa* oil is a good antimicrobial agent especially against Gram-positive bacteria and more interestingly the common resistant bacteria (MRSA) is significantly inhibited by this oil. Therefore, *N. sativa* oil may be a good medication for bacterial skin infections and it will be medically used alone or mixed with the skin lesion or cream after approvement with the global drug agency.

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Appendix 1: The oil extract effect through antibiotic sensitivity test against MARSA, *S.cereas, B.subtilis and S. aureas*