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Scientific Research

Evaluation of antioxidant activity and antimicrobial effect of *Nigella sativa* oil on some pathogenic bacteria and its interaction with chloramphenicol antibiotic

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ABSTRACT

Nowadays, with the increase in diseases and consumers' preference for organic foods, the use of natural plant compounds to improve and increase their shelf life is on the rise. In addition, the use of medicinal plants, which can have therapeutic properties, has attracted attention. Black seed oil has attracted a lot of attention in the past due to its unique therapeutic properties. The aim of this study was to investigate the antioxidant activity and evaluate the antimicrobial activity of black seed oil against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus*. The amount of phenolic compounds in black seed oil was measured by Folin-Ciocalteu. The antioxidant activity was determined by DPPH and ABTS radical scavenging methods. The antimicrobial effect of *Nigella sativa* oil was investigated according to well diffusion agar, disk diffusion agar, interaction, minimum inhibitory concentration and minimum bactericidal concentration methods.The *Nigella sativa* oil had 16.25 mg GAE/g phenolic compounds. The antioxidant activity based on DPPH and ABTS radical scavenging were 86.62% and 94.85%, respectively.The *Nigella sativa* oil showed a significant antimicrobial effect on the tested microorganisms. In the combined mode (interaction) of *Nigella sativa* oil with chloramphenicol antibiotic, synergistic mode was observed for all bacterial strains. Given the significant antioxidant activity and antimicrobial effect of *Nigella sativa*, it can be used in food and pharmaceutical industries.

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1- Introduction

Historical medical texts contain extensive information on the use of medicinal plants in treating diseases, especially those related to countries with ancient civilizations such as Iran. Among the valuable information from the Iranians, one can refer to their fields of study in traditional medicine, recognition of medicinal plants, and their therapeutic application even before others [1]. Scientific and technological advancements in recent decades have further underscored the importance and constructive role of medicinal plants, particularly in meeting human pharmaceutical and therapeutic needs [2]. Oil is one form of medication that exists as a liquid or semi-solid. Herbal oils are solutions containing all the beneficial substances found in the plant or the part from which the oil is extracted, such as flavonoids, tannins, minerals, or other compounds that have antibacterial and antioxidant activities and prevent unwanted reactions in food environments. Various antioxidants exist in human blood plasma, but these alone are not capable of combating free radicals produced in the body; therefore, it is necessary to supplement this deficiency with external sources through food. Antioxidants also play a role in reducing the likelihood of stroke and cardiovascular diseases and in preventing cancer [3-9]. Black seed or "Saniz," scientifically known as *Nigella sativa*, is an annual, flowering plant native to Eastern Europe and countries like Bulgaria, Cyprus, Romania, Western Asia, and countries such as Iran, Turkey, and Iraq, but nowadays, it also grows in other regions of Europe and Asia. This plant belongs to the Ranunculaceae family, and its number of petals ranges from five to eight. Its black seeds are located in the middle petals of the flower and have a distinctive scent. The plant grows about 20 to 30 centimeters tall and has a large, three-part, capsule-like fruit containing numerous seeds. These black seeds are used as a spice or an additive in the food industry, including in cooking and confectionery [10, 11].

Yamir et al. (2019) evaluated the polyphenolic and tocopherol content of black seed oil. The results of these researchers showed that black seed is rich in polyphenols and tocopherols, and its oil contains linoleic, oleic, palmitic, and stearic acids [12]. In the studies by Nikavar et al. (2003) [13] and Ramdan and Morsel (2002) [14], the compounds of black seed were examined. During these studies, it was determined that black seeds contain vitamins, minerals, proteins, carbohydrates, phospholipids, carotene, calcium, iron, and potassium. The aim of the present research was to evaluate the antioxidant activity, antimicrobial effect, and interaction of black seed oil in laboratory conditions.

2- Materials and Methods

Black seed oil was obtained from Baran Plant Products Company. All chemicals and solvents used in this research were purchased from Merck Germany with laboratory-grade purity

2-1- Preparation of Black Seed Oil Sample

In this study, the method of Abbasi et al. (2011) was used to measure the phenolic compounds of black seed oil. 1 gram of the oil sample was mixed with 2 milliliters of 90% methanol and vortexed for 4 minutes. The samples were then centrifuged at 3000 g for 5 minutes [15]. The supernatant was used to measure the total phenolic content and to perform the DPPH and ABTS free radical scavenging assays.

2-2- Measurement of Phenolic Content

To measure the total phenolic content, the Folin-Ciocalteu reagent was used based on the method of Mehrnia et al. (2021). In this method, 2.5 milliliters of phenol solution were mixed with 1 milliliter of the sample

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extract. After 6 minutes, 2.5 milliliters of 7% sodium carbonate were added, and after one hour, the mixture was transferred to a spectrophotometer and read at a wavelength of 725 nanometers. The total phenol content was reported in mg GAE/g [16].

2-3- Determination of Antioxidant Activity Using DPPH Method

To determine the antioxidant activity of black seed oil, the method of Noshad et al. (2022) was used. 1 milliliter of the sample extract was mixed with 2.9 milliliters of 0.1 mM DPPH in methanol and kept in the dark at room temperature for 30 minutes. The absorbance was then measured at a wavelength of 515 nanometers. Pure methanol was also used as a control sample. The percentage of inhibitory activity was calculated using the following equation [17]:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(Absorbance\ of\ control - Absorbance\ of\ sample)}{(Absorbance\ of\ control)} \times 100$$

2-4- Determination of Antioxidant Activity by ABTS Method

To determine the ABTS free radical scavenging capacity, the method of Susan Gharibond and colleagues (2020) was used [18]. To measure antioxidant activity with ABTS free radicals, the ABTS+ radical was first prepared. An aqueous solution of ABTS at a concentration of 7 mM was prepared, and potassium persulfate was added to reach a final concentration of 2.45 mM in the solution. The resulting solution was left at room temperature in the dark for 16 hours. Then, 300 µL of the extract sample was added to 3 mL of the ABTS radical solution, and after 6 minutes, it was transferred to a spectrophotometer and read at a wavelength of 734 nm. The percentage of radical scavenging activity was calculated using the following equation:

$$\text{ABTS radical scavenging activity (\%)} = \frac{(Absorbance\ of\ control - Absorbance\ of\ sample)}{(Absorbance\ of\ control)} \times 100$$

2-5- Preparation and Standardization of Microbial Strains

For microbiological tests, standard microbial strains from the microbial collection of the Food Microbiology Laboratory, Department of Food Science and Engineering, University of Agricultural Sciences and Natural Resources of Khuzestan, were used. This included 3 Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*) and 3 Gram-positive bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus*). To prepare the microbial suspension for evaluating the antimicrobial activity of black seed oil, the McFarland standard was followed, and ultimately, a microbial suspension containing 1.5×10^8 CFU[1]/mL was prepared [19].

2-6- Agar Disk Diffusion Test

In the disk diffusion test, after preparing the Mueller-Hinton agar medium, the microbial suspension prepared from fresh cultures of pathogenic strains according to the McFarland standard was spread over the surface of the medium. Then, using forceps, 3 disks soaked in black seed oil were placed on the plate, and one disk containing distilled water was placed as a control on each plate. The Petri dishes were incubated at 37 degrees Celsius for 24 hours. After the incubation period, the zone of inhibition or lack of growth around each disk was precisely measured with a ruler in millimeters and recorded [20, 21].

2-7- Agar Well Diffusion Test

۱ Colony forming unit

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In the well diffusion test, after preparing the Mueller-Hinton agar medium, 4 wells with a diameter of 6 mm were created on the surface of the Petri dish using a sterile Pasteur pipette. Using a sampler, 20 µL of the microbial suspension prepared from fresh cultures of pathogenic strains with a McFarland standard concentration was poured into 3 wells, and the fourth well was considered as a control. The Petri dishes were then incubated at 37 degrees Celsius for 24 hours. After the incubation period, the zone of inhibition or lack of growth around each well was precisely measured with a ruler in millimeters and recorded [22, 23].

2-8- Minimum Inhibitory Concentration (MIC)

To determine the minimum inhibitory concentration, the original solution of black seed oil was prepared with a dilution of 512 mg/mL, and different concentrations were prepared by diluting the original solutions with Mueller-Hinton broth (Merck, Germany). 20 µL of the suspension prepared according to the McFarland standard was injected into the wells of a 96-well plate, then 200 µL of different concentrations of the solution prepared from black seed oil was added to the well. The plate was incubated at 37 degrees Celsius for 24 hours. After that, 20 µL of a 5% solution of triphenyl tetrazolium chloride was added to each well and incubated again for 30 minutes. After this time, the 96-well plate was checked for color change in each well, and the first concentration at which no color change was observed was reported as the minimum inhibitory concentration [24, 25].

2-9- Minimum Bactericidal Concentration (MBC)

To determine the minimum bactericidal concentration, 100 µL from the wells where no color change was observed was transferred to solid medium (Mueller-Hinton agar) and maintained at 37 degrees Celsius for 24 hours. The first concentration at which no growth was observed was considered as the minimum bactericidal concentration [26, 27].

2-10- Interaction of Black Seed Oil with Chloramphenicol Antibiotic

The interaction of black seed oil with chloramphenicol antibiotic discs was investigated using the sub-inhibitory concentration method, which was equivalent to a 1:2 MIC dilution. The prepared dilution was added to Mueller-Hinton agar medium. The culture medium containing black seed oil was poured into a Petri dish, and with the help of a sampler, 100 µL of microbial suspension prepared from fresh cultures of pathogenic strains with a McFarland standard concentration was added to the culture medium, and the chloramphenicol antibiotic disc was placed on the medium. Petri dishes containing Mueller-Hinton agar medium were used to examine the effect of the chloramphenicol antibiotic disc. The Petri dishes were incubated at 37 degrees Celsius for 24 hours. After this time, the diameter of the inhibition zones was measured and recorded in millimeters. Subsequently, the diameter of the inhibition zones created in the culture medium containing black seed oil was compared with the condition of the culture medium without black seed oil, and the type of interaction was reported [28, 29].

2-11- Statistical Analysis

The tests were repeated three times, and the data were analyzed using one-way analysis of variance with the help of Minitab software (version 17). The difference between the mean results was examined using the Tukey test at a 95% confidence level ($p < 0.05$).

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3- Results and Discussion

The number of phenolic compounds per gram of black seed oil extract was 16.25 ± 0.57 mg GAE/g. The results of the tests examining antioxidant activity in terms of DPPH and ABTS radical scavenging were 86.62 ± 0.59% and 94.85 ± 0.83%, respectively (Figure 1).

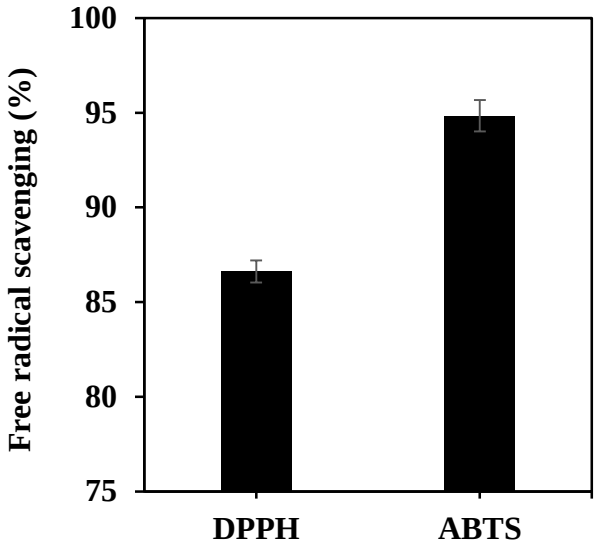

Figure 1. The antioxidant activity of *Nigella sativa* oil based on DPPH and ABTS radical scavenging methods.

Some medicinal plants are rich in phenolic compounds and have strong antioxidant activity. Various studies indicate differences in the number of phenolic compounds and the antioxidant properties of similar medicinal plants. Numerous factors can be cited to justify this issue. Climatic conditions, including water, air, soil, and altitude, are among the factors affecting plant growth and the change in their chemical compositions [30, 31]. Various methods of measuring phenolic compounds are another factor justifying the difference in the results of different studies [31]. Studies show that the high content of phenolic compounds is the main reason for the high antioxidant activity of some medicinal plants. This is because, based on existing evidence, there is a positive correlation between the number of phenolic compounds and the antioxidant power of plants. On the other hand, it seems that phenolic compounds, which are widely found in plants and have high antioxidant power, are more extractable through their plant oils. The key role of phenolic compounds as free radical scavengers has been reported in several articles. It is worth mentioning that phenolic compounds effectively act as hydrogen donors and function as an effective antioxidant [32-34]. However, studies conducted by Tavaha and colleagues (2010) [35] and Chen and colleagues (2010) [36] have shown that there is no direct relationship between flavonoid content and antioxidant activity.

There are numerous studies and various methods for determining antioxidant capacity. These methods, influenced by different conditions such as access to laboratory materials and the solubility ratio of antioxidants between aqueous and organic phases, temperature, oxidation conditions, oxidizing substance, and the amount of oxidation, yield different results; therefore, using a single method to measure antioxidant capacity is not appropriate [37]. The basis of the DPPH method is based on the reduction of free radicals by antioxidants in the absence of other free radicals in the environment, which results in color formation in the environment that can be measured with a spectrophotometer. The DPPH radical has little resemblance to the peroxyl radical, but due to the ease and speed of this test, this method is usually used to examine the amount of antioxidant in plants or oilseeds. The reaction of the DPPH free radical is reversible, which means that the antioxidant capacity of many antioxidants is usually read less than their specified limit. Another indicator of antioxidant activity is the peptides resulting from enzymatic hydrolysis, the inhibition of the

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ABTS cationic radical, and the evaluation of free radical scavenging capacity. Since antioxidants inhibit radicals resulting from the oxidation of linoleic acid, they prevent the interaction between these radicals and beta-carotene, thereby reducing the color loss of beta-carotene as a result of this reaction. Therefore, there is a direct relationship between the antioxidant power of the substances participating in this test and the extent to which they prevent the reduction of beta-carotene color [30, 31].

The results of the antimicrobial activity of black seed oil by the agar disk diffusion method are presented in Figure 2. The results showed that with an increase in the concentration of black seed oil from 64 to 512 mg/mL, the diameter of the inhibition zone increased, such that at a concentration of 512 mg/mL, the diameter of the inhibition zone was in the following order: *Listeria monocytogenes* > *Bacillus cereus* > *Pseudomonas aeruginosa* > *Staphylococcus aureus* > *Salmonella typhi* > *Escherichia coli*. The statistical comparison results showed that there is a significant difference at the 5% statistical level between each concentration level of black seed oil for all the tested bacteria.

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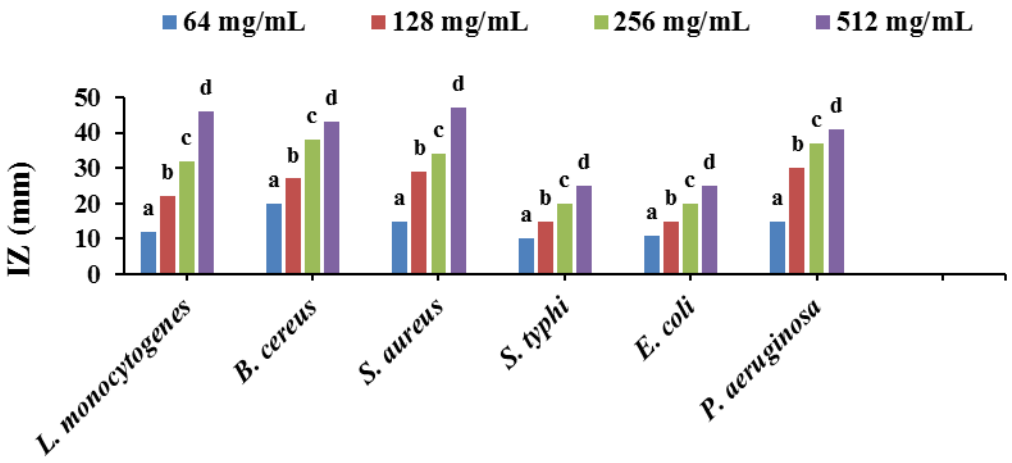

2. Figure

Antibacterial activity of *Nigella sativa* oil based on disc diffusion agar method. Means with different superscripts at each concentration are significantly different ($p<0.05$).

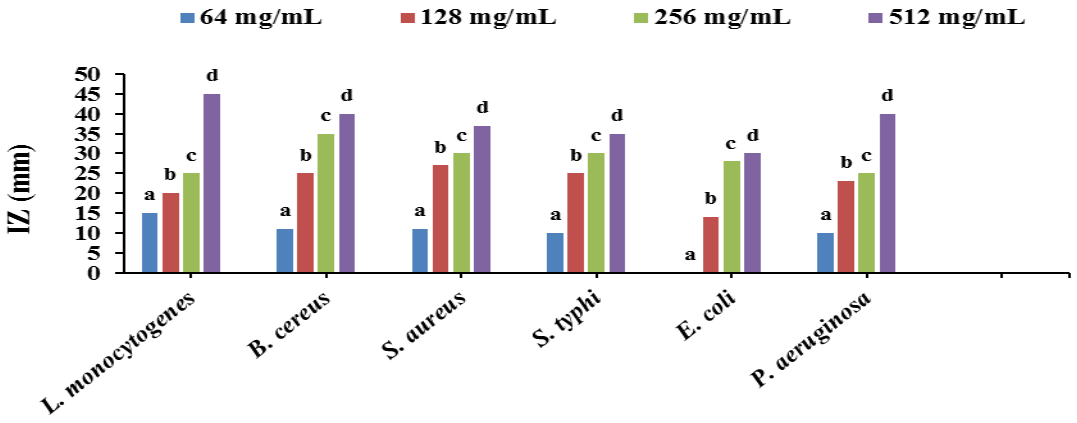

Figure 3. Antibacterial activity of *Nigella sativa* oil based on well diffusion agar method. Means with different superscripts at each concentration are significantly different ($p<0.05$).

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In Figure 3, the results of the antimicrobial activity of black seed oil by the agar well diffusion method are shown. The obtained results indicated that with an increase in the concentration of black seed oil from 64 to 512 mg/mL, the diameter of the inhibition zone increased, such that at a concentration of 512 mg/mL, the diameter of the inhibition zone was in the following order: *Staphylococcus aureus* > *Listeria monocytogenes* > *Bacillus cereus* > *Pseudomonas aeruginosa* > *Salmonella typhi* ≥ *Escherichia coli*. The statistical comparison showed that there is a significant difference at the 5% statistical level for all the tested bacteria.

The results of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of black seed oil on the studied pathogenic microorganisms are shown in Table 1. The results showed that the MIC of black seed oil for *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, and *Pseudomonas aeruginosa* was 2, 2, 1, 2, 32, and 1 mg/mL, respectively. The results also showed that the MBC of black seed oil for *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, and *Pseudomonas aeruginosa* was 256, 512, 256, 512, greater than 512, and 256 mg/mL, respectively.

Table 1. Antibacterial activity of *Nigella sativa* oil based on MIC and MBC methods

Microorganisms	**MIC (mg/mL)**	**MBC (mg/mL)**
L. monocytogenes	2	256
B. cereus	2	512
S. aureus	1	256
S. typhi	2	512
E. coli	32	> 512
P. aeruginosa	1	256

The results of the inhibition zone diameter of chloramphenicol antibiotic discs and their interaction with black seed oil on pathogenic bacteria are presented in Table 2. The results showed that the addition of black seed oil to the culture medium resulted in a synergistic effect for all the tested bacteria.

Table 2. The mean inhibition zone diameter (mm) of chloramphenicol and its interaction with *Nigella sativa* oil on *bacterial pathogenesis*

Microorganisms	Chloramphenicol	Chloramphenicol + *Nigella sativa* oil	Interaction
L. monocytogenes	20.10 ± 0.58	77.30 ± 0.40	Synergistic
B. cereus	25.20 ± 0.43	74.40 ± 0.37	Synergistic

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S. aureus	30.50 ± 0.52	78.50 ± 0.50	Synergistic
S. typhi	23.30 ± 0.67	70.90 ± 0.43	Synergistic
E. coli	26.10 ± 0.36	73.10 ± 0.33	Synergistic
P. aeruginosa	24.10 ± 0.41	72.20 ± 0.29	Synergistic

Much time has passed since the antimicrobial activity of oils, essences, and plant extracts was proven; however, in recent years, the increased interest in developing organic and natural processes has led to the resumption of scientific studies related to these substances [38-40]. In this study, the inhibitory effect of black seed oil on the growth of several pathogenic bacteria under laboratory conditions was established; therefore, it can be used as a plant compound to inhibit the growth of pathogenic bacteria. The difference in the inhibitory effect of black seed oil on the growth of different bacteria can be attributed to the different mechanisms of microorganisms against growth-limiting agents. The experiments conducted in this research showed that black seed oil has a desirable antibacterial effect. These results indicate that the concentration difference is very important for the strains under study.

4- Conclusion

Plants have been used as medicine since ancient times, but recently, the use of plants and materials produced from them as preservatives and natural medicine has gained more attention. The results of the current research showed that black seed oil contains phenolic compounds, and the antioxidant activity of black seed oil was also proven. The results of the antimicrobial activity of black seed oil showed that this oil can prevent the growth of both Gram-positive and Gram-negative bacteria under laboratory conditions. Given the significant antioxidant and antibacterial properties of black seed oil, it can be used in the food and pharmaceutical industries.

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ارزیابی فعالیت آنتی‌اکسیدانی و اثر ضدمیکروبی روغن سیاه‌دانه بر برخی از باکتری‌های بیماری‌زا و برهمکنش آن با آنتی-بیوتیک کلرامفنیکل

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چکیده

امروزه با افزایش بیماری‌ها و تمایل مصرف‌کنندگان به استفاده از غذاهای ارگانیک، استفاده از ترکیبات طبیعی گیاهی برای بهبود و افزایش عمر ماندگاری آن‌ها رو به افزایش است. علاوه بر این، استفاده از گیاهان دارویی که دارای خاصیت درمانی نیز هستند، مورد توجه قرار گرفته است. روغن سیاه‌دانه به دلیل خواص درمانی، از گذشته توجه زیادی را به خود معطوف داشته است. هدف از این پژوهش، بررسی فعالیت آنتی‌اکسیدانی و ارزیابی فعالیت ضدمیکروبی روغن سیاه‌دانه بر باکتری‌های *اشرشیا کلی، سودوموناس ائروژینوزا، سالمونلا تیفی، استافیلوکوکوس اورئوس، لیستریا مونوسیتوژنز و باسیلوس سرئوس* بود. میزان ترکیبات فنولی روغن سیاه‌دانه با معرف معرف فولین – سیوکالتو اندازه‌گیری شد. فعالیت آنتی‌اکسیدانی روغن سیاه‌دانه توسط روش‌های مهار رادیکال **DPPH** و **ABTS** تعیین گردید. اثر ضدمیکروبی روغن سیاه‌دانه، مطابق روش‌های ضدمیکروبی چاهک آگار، دیسک دیفیوژن آگار، برهمکنش، حداقل غلظت مهارکنندگی و حداقل غلظت کشندگی بررسی شد. روغن سیاه‌دانه دارای مقدار ترکیبات فنولی **mg GAE/g** ۱۶/۲۵ بود. فعالیت آنتی‌اکسیدانی بر حسب مهار رادیکال **DPPH** و **ABTS** به ترتیب ۸۶/۶۲ و ۹۴/۸۵ درصد بود. روغن سیاه‌دانه، اثر ضدمیکروبی قابل توجهی بر میکروارگانیسم‌های مورد بررسی از خود نشان داد. نتایج برهمکنش روغن سیاه دانه با آنتی‌بیوتیک کلرامفنیکل نشان داد که برای تمامی باکتری‌های بیماری‌زا اثر سینرژیستی مشاهده شد. با توجه به فعالیت آنتی‌اکسیدانی و ضدمیکروبی قابل‌توجه روغن سیاه‌دانه، می‌توان از این روغن در صنایع غذایی و دارویی استفاده کرد.

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