

Phytochemical Analysis of *Nigella sativa* L. Utilizing GC-MS Exploring its Antimicrobial Effects against Multidrug-Resistant Bacteria

Fatima A. Saleh¹, Nada El-Darra², Karim Raafat^{3,*}, Iman El Ghazzawi¹

Fatima A. Saleh¹, Nada El-Darra², Karim Raafat^{3,*}, Iman El Ghazzawi¹

¹Department of Medical Laboratory Sciences, Faculty of Health Sciences, Beirut Arab University, LEBANON.

²Department of Nutrition and Dietetics, Faculty of Health Sciences, Beirut Arab University, LEBANON.

³Department of Pharmaceutical Sciences, Faculty of Pharmacy, Beirut Arab University, LEBANON.

Correspondence

Karim Raafat

Pharmaceutical Sciences Department, Faculty of Pharmacy, Beirut Arab University (BAU), 115020 Beirut, LEBANON.

Tel.: +961 1300110;

Fax: +961 1300110 Ext:2599

E-mail: k.raafat@bau.edu.lb,

karim.raafat@yahoo.com

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ABSTRACT

Introduction: The alarming increase in bacterial strains resistant to existing antimicrobial agents has demanded alternative strategies. Medicinal plants are now considered as an alternative treatment because of their secure choice in several diseases. Among them, *Nigella sativa* is a promising traditional herb having rich medical background. The aim of the study is to perform phytochemical analysis of *Nigella sativa* L. Utilizing Gas chromatographic-mass spectrometric (GC/MS) exploring its antioxidant and antibacterial activity against multidrug resistant (MDR) gram-positive and gram-negative bacteria. **Methods:** Total phenolic, tannin, and flavonoid content of *N. sativa* seed extracts and its commercially available oil were determined. Their radical scavenging activity using DPPH was also tested. The antibacterial activity of *N. sativa* seed extracts and its oil against MDR gram-positive and gram-negative bacterial strains was studied using disc diffusion test and the biofilm formation assay. GC-MS studies were also performed. **Results:** Among the different preparations used, *N. sativa* oil showed the highest antioxidant and antibacterial activity against highly resistant gram-positive bacteria with the greatest suppression of biofilm formation, which was attributed to its high bioactive contents. **Conclusion:** This study indicates that *N. sativa* extracts and its oils can be used as natural antibacterial agents to treat infections caused by multidrug resistant bacteria.

Key words: *Nigella sativa*, Antibacterial, Antioxidant, GC-MS analysis, Multidrug resistant bacteria.

INTRODUCTION

Since the discovery of penicillin, the world's first antibiotic, there was the medical belief that this development will lead to the eradication of infectious diseases. However, the overuse and misuse of these antibiotics have led to the emergence of multidrug-resistant bacteria. Recently, antibiotic resistance has reached a dramatic low-point that some infections have developed resistance to even vancomycin, which is considered as the 'antibiotic of last resort'.¹ Therefore, this has initiated an interest towards the development of alternative antibacterial agents and the re-evaluation of the therapeutic uses of herbal extracts and essential oils.²

Herbal extracts and their essential oils are rich in phytochemicals, such as phenolics, flavonoids and tannins,³ which are sources of natural antioxidants and provide protection against predation by microbial pathogens.⁴ Besides, a large number of studies have found a good correlation between the phytochemical content and the antimicrobial efficacy of the herbs investigated, indicating that these secondary metabolites are responsible for the antimicrobial effect.⁵ Among them, *Nigella sativa* (*N. sativa*) belonging to the *Ranunculaceae* family is a promising herb, often referred to as the nature's miracle remedy.⁶

N. sativa, also known as black cumin, grows in many countries around the Mediterranean Sea.⁷ Seeds of *N. sativa* have been used as a condiment in the food industry and as a traditional remedy for a number of diseases including asthma, hypertension, diabetes, eczema, inflammation, rheumatism, jaundice and gastrointestinal problems.⁸ In recent years, both seeds and its oil have been widely investigated and reported to exhibit several pharmacological effects such as analgesic, anti-inflammatory,⁹ anticancer,¹⁰ antiulcer¹¹ and antibacterial activity.¹² Ugur *et al.*¹³ making their treatment difficult. *Nigella sativa* oil is known to be active against Gram-positive cocci, yet its *in vitro* cytotoxicity is rarely investigated, is a proper and powerful candidate for treatment of methicillin-resistant isolates. **OBJECTIVES:** The aim of this study is to evaluate the *in vitro* antibacterial activity and cytotoxicity effect of *N. sativa* oil. **MATERIALS AND METHODS:** The minimal inhibitory concentrations (MICs) showed that *N. sativa* oil has an excellent antibacterial activity against clinical isolates of methicillin-resistant *S. aureus* and methicillin-resistant coagulase-negative *Staphylococci* with very low MIC range of <0.25–1.0 µg/ml.¹³ Other

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studies reported that *N. sativa* seed oil is rich in phenolic compounds with strong antioxidant properties.¹⁴ The phytochemical composition of *N. sativa* oil was further characterized by many studies. Farag *et al.* showed that *N. sativa* oil contains more than 52 Metabolites including 8 saponins, 10 flavonoids, 6 phenolics, 10 alkaloids, and 18 fatty acids that might be utilized for their health benefits.¹⁵

The present study was conducted to determine total phenolic, tannin, and flavonoid content of *N. sativa* seed extracts and its commercially available oil and to carry gas chromatographic–mass spectrometric studies to describe the essential oil and fixed oil (fatty acid) composition of the different *N. sativa* preparations. Their antiradical activity using DPPH method and the antibacterial activity against multidrug resistant strains were also performed.

Therefore, the aim of the current study was to perform phytochemical analysis of *Nigella sativa* L. utilizing GC-MS exploring its antioxidant and antibacterial activity against multidrug resistant (MDR) gram-positive and gram-negative bacteria.

MATERIALS AND METHODS

Nigella sativa preparation

N. sativa seeds (imported from India) were purchased from a local herb store in Beirut, Lebanon. Seeds were identified and authenticated with reference sample and were given voucher specimen number (NS-16-14) and stored in the Faculty herbarium. The seeds were grounded to a powder shape using an electric grinder for 5 min, and then the grounded samples (50 g) were soaked overnight in 200 ml of distilled water or methanol under shaking for the preparation of aqueous and methanolic extracts, respectively. The extracts were then centrifuged, filtered twice then filter sterilized using nitrocellulose membrane filter. Sterile-filtered oil and capsules oily content of *N. sativa* (Pharco Pharmaceuticals, Egypt) were included in this study.

Bacterial strains, culture media and growth conditions

Eleven multiple antibiotic resistant bacterial strains were included in this study. They are clinical isolates provided by the Faculty of medicine, Alexandria University, Egypt. The isolates tested include the following gram-positive strains: 2 Methicillin-resistant *Staphylococcus aureus* (MRSA), 2 Methicillin-resistant *Staphylococcus epidermidis* (MRSE), high-level aminoglycoside-resistant (HLAR) Enterococci (*Enterococcus faecalis* and *Enterococcus faecium* which was also vancomycin-resistant (VRE)). Furthermore, the following gram-negative strains including 1 strain of *Pseudomonas aeruginosa*, 2 strains of *Klebsiella pneumoniae* and 2 strains *Acinetobacter baumannii* were also studied. The strains of *Klebsiella pneumoniae* and *Acinetobacter baumannii* were previously proved to be extended-spectrum β -lactamases (ESBL) as well as Carbapenemase producers. All strains were previously validated at the Microbiology laboratory, Faculty of Medicine, Alexandria University, Egypt. MRSA and MRSE by doing cefoxitin sensitivity test, HLA enterococci by using gentamicin discs and Vancomycin resistant enterococci by vancomycin sensitivity test. All gram-negative strains were identified by different biochemical reactions. The ESBL strains were identified by double synergy test while Carbapenemase production by modified Hodge tests.

Total Phenolic Content determination

The total phenolic content was determined according to FolinCiocalteu (FC) method.¹⁶ 0.2 ml of standard (gallic acid) or diluted sample, 1.0 ml of FC reagent, and 0.8 ml of Na₂CO₃ solution (7.5%) were mixed and allowed to stand for 2 h at room temperature. Light absorption was measured at 750 nm by a spectrophotometer UV-VIS against a blank similarly prepared, but containing distilled water instead of extract. The total phenolic content (Ye) was expressed in mg/ml.

Determination of tannin concentration

Total tannin content (g/l) was determined according to Ribéreau-Gayon and collaborators.¹⁷ Total tannin assay is based on the heating process of tannins in acidic medium leading to the formation of cyanidins. Two tubes were prepared, each containing 1 ml of the sample, 0.5 ml of water and 1.5 ml of 12 N HCl. The first tube was mixed and heated in a water bath at 100°C for 30 min. The second was kept at room temperature. Following the rapid cooling, 0.25 ml of ethanol was added to the mixture and the resulting absorbance was recorded at 520 nm.

Free radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical was used in the present study for the screening of the radical scavenging activity of the extracts.¹⁸ The DPPH radical scavenging activity was measured using the spectrophotometer UV-VIS (Libra S32, Biochrom, France). The samples were tested at a concentration of 20 mg/ml then mixed with 1000 μ l of 0.1 mMDPPH-ethanol solution and 450 μ l of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50 μ l) was used for blank measurements in this experiment. After 30 min of incubation at room temperature, the reduction of the DPPH free radical was measured by reading the absorbance at 517 nm. Butylhydroxytoluene (BHT) (a synthetic antioxidant) was used as a positive control. The inhibition ratio (percent) was calculated according to the following equation:

$$\% \text{ inhibition} = \left[\frac{(\text{absorbance of control} - \text{absorbance of test sample})}{\text{absorbance of control}} \right] \times 100$$

Determination of total flavonoids (TF)

The total flavonoids (TF) assay was done as previously described by Zhuang and coworkers¹⁹ after bringing minor modifications. A volume of 1 ml of diluted extract or standard solution of catechin was placed in a 10 ml volumetric flask already containing 4 ml of H₂O. Five minutes later, 0.3 ml of NaNO₂ (5%) and 1.5 ml of AlCl₃ (2%) were added. The mixture was shaken for 5 min then 2 ml of 1 M solution of NaOH were added and the mixture was well shaken again. The absorbance was measured at 510 nm against the blank. The results were calculated according to the calibration curve for catechin (R² = 0.99). The content of TF was expressed as mg of catechin equivalent (CE) per 1 g of dry matter content. All samples were analyzed in duplicate.

Disc diffusion test

For the preparation of bacterial suspension of each strain to be tested, 5 colonies were picked from fresh blood agar culture and inoculated onto 3 ml saline then left for 5 minutes. Each strain suspension was swabbed onto plates of Muller Hinton agar and 5 mm sterile filter paper discs were placed on top. Aqueous extract, Methanolic extracts (undiluted, diluted 1:1), Oil (crude, diluted 1:1 in ethylene glycol (EG) and diluted 1:1 in dimethyl sulfoxide (DMSO) and capsule content were tested for their inhibition effect on the strains. This was performed by pipetting 5 μ l of all preparations on 5 mm sterile discs. Negative controls were included. After overnight incubation, the inhibition zones were recorded.²⁰

Biofilm formation

An overnight broth culture of each bacterial strain (2 MRSA, 1 MRSE) tested were diluted 1:5 in tryptic soya broth. For each bacterial strain, five sterile beakers containing a 2.5 cm coverslip were used, where 300 μ l bacterial suspension was added. An equal volume of methanolic extract, crude oil, oil diluted in EG and capsule content, was added to each beaker, leaving one untreated to serve as a positive control. All beakers were incubated overnight at 37°C. Then, each beaker was washed 3x with distilled water, fixed with 95% of ethanol for 30 min and stained with

0.1% crystal violet for an hour. After a final wash, the coverslips were air-dried and microscopically visualized for biofilm formation.²¹

GC-MS Analysis

The GC-MS investigation of the *Nigella sativa* oil, capsule, methanolic and aqueous extracts have been made in an Agilent instrument (Agilent, Japan) under computer control at 70 eV. About 1 μ l of each sample was injected into the GC-MS and the scanning has been done for 25 min. The oven temperature has been maintained at 100°C. Helium gas has been utilized as carrier and eluent. The helium flow rate has been set at 1 ml/min. HP-5MS capillary-column (30 m \times 0.25 mm; 0.25 μ m film-thickness) has been utilized as the GC-column for components separation. The identity and quantifying of the oil components have been assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and the reference standards calibration curves.²²

Statistical analysis

The result are expressed as mean \pm SD. Analysis of variance (ANOVA) was used to test for differences in the groups and multiple range test of significance was used.

RESULTS AND DISCUSSION

Polyphenol, tannins and flavonoids content of different *N. sativa* preparations

Figure 1 shows that the highest concentration of total phenolic compounds and flavonoids were found in the capsules and oil followed by the methanolic and aqueous extracts. The amount of polyphenols in oil and capsules (248 mg/ml) was higher than the one found in seeds extracts (21 mg/ml for the aqueous extract and 39 mg/ml for the methanolic extract). Similar results were obtained for the flavonoids with a concentration of 19.8 mg/ml for the oil and capsules followed by the methanolic (2.98 mg/ml) and the aqueous extract (0.11 mg/ml). However, for the tannins, the highest amount (8.3 mg/ml) was noted for the capsules, followed by the oil (3.4 mg/ml), and methanolic extract (1.5 mg/ml) while the aqueous extract contains only 0.49 mg/ml. These results are in accordance with a study by Cheikh-Rouhou *et al.*, 2007²³ which showed that *N. sativa* oil is a potential source of natural phenolic compounds.

Antiradical activity of *N. sativa* Extracts against DPPH radical

The assessment of the antiradical activity of the aqueous and methanolic extracts, oil and capsules was also performed (Figure 2). DPPH is a stable radical, widely used to evaluate the free radical scavenging activity in many plant extracts.²⁴ The assessment of antiradical activity showed that all *N. sativa* preparations were able to scavenge this radical. Oil and capsules displayed a higher activity, 16% and 17% inhibition of the radical, respectively. Whereas, methanolic and aqueous extracts exhibited a lower antiradical activity (12% and 7%). The high antiradical activity of the oil and capsules could be mainly attributed to their high phenolic content that act as a free radical scavenger.²⁵ found that antiradical activity of *N. sativa* extracts increased proportionally to the polyphenol content with a linear relationship between radical inhibition and total polyphenols. Similar studies by^{26,27,28} showed concentration-dependent radical scavenging activity of polyphenol extracts.

Antibacterial activity of *Nigella sativa* preparations

The aqueous extract, methanolic extracts (undiluted, diluted 1:1), oil extracts (crude oil, oil in ethylene glycol 1:1, oil in DMSO 1:1) and capsule content of different *N. sativa* samples were evaluated for their antibacterial activities against different resistant gram-positive and gram-negative bacterial strains using the disc diffusion method by measuring the inhibition zone (Table 1). As shown in Table 1 the aqueous extract did not exhibit any antibacterial activity. However, the other preparations

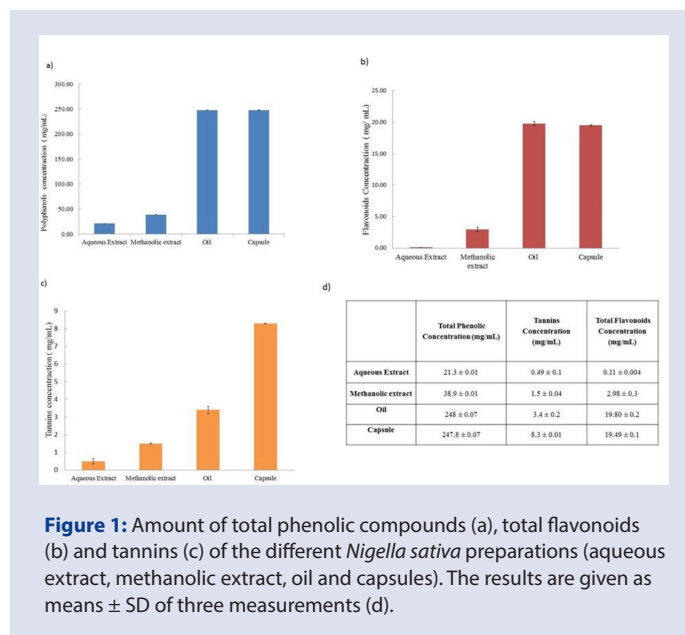


Figure 1: Amount of total phenolic compounds (a), total flavonoids (b) and tannins (c) of the different *Nigella sativa* preparations (aqueous extract, methanolic extract, oil and capsules). The results are given as means \pm SD of three measurements (d).

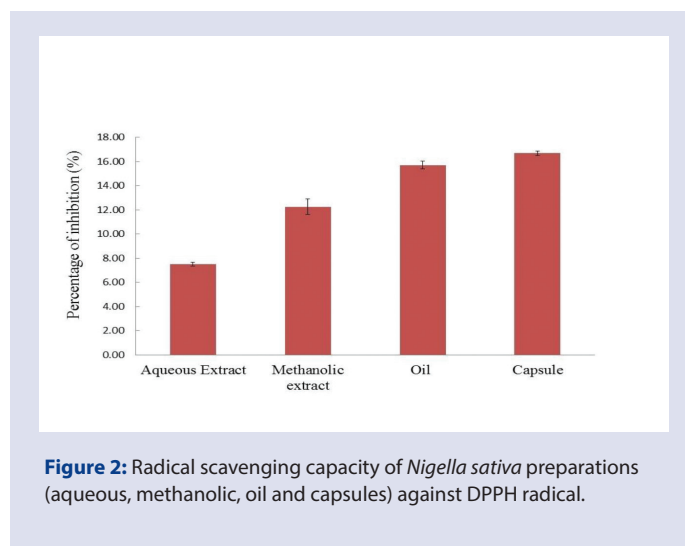


Figure 2: Radical scavenging capacity of *Nigella sativa* preparations (aqueous, methanolic, oil and capsules) against DPPH radical.

showed antibacterial activities with different efficacy. These results are in agreement with a study by,²⁹ which reported lower antibacterial activity for the aqueous extract of high phenolic content plant like rosemary compared to its methanolic extract. The inhibitory effect for the different extracts is higher for the gram-positive strains than for the gram-negative strains. Our findings are in concordance with the findings of³⁰ which showed that phenolic compounds have enhanced activity against gram-positive strains compared to gram-negative, due to the presence of an outer membrane in the cell wall of the latter, thus acting as permeability barrier and reducing the uptake of polyphenols.³¹ The capsules showed the highest inhibitory activity against the different tested gram-positive strains. The extracts in pure methanol showed inhibitory activity against the different tested gram-positive and gram-negative strains. Other extracts (diluted methanol, oils (crude and diluted)) were found to be active against all the tested species of gram-positive bacteria whereas the gram-negative bacteria remained unaffected. Our results are in accordance with the study by,³² that showed that methanolic extracts were more effective against both gram-positive and gram-negative bacteria tested, which could be explained by a better extraction of

Table 1: Inhibition zone of the different *Nigella sativa* preparations against different bacterial strains (gram + and gram -)

Bacteria/POMs	Inhibition zome (mm)						
	Acqueous	Methanolic undiluted	Methanolic diluted (1:1)	Crude oil	Oil in Ethylene Glycol (1:1)	Oil in DMSO (1:1)	Capsules
Methicillin Resistant Staphylococcus aureus (MRSA 1294) (gram *)	0	11	7	26	14	0	40
Methicillin Resistant Staphylococcus aureus (MRSA 1295) (gram +)	0	11	9	35	22	0	55
(Methicillin-resistant Staphylococcus epidermidis MRSE 1297) (gram +)	0	13	7	Complete inhibition	Complete inhibition	Complete inhibition	Complete inhibition
(Methicillin-resistant Staphylococcus epidermidis MRSE 1296) (gram +)	0	10	9	Complete inhibition	Complete inhibition	Complete inhibition	Complete inhibition
Enterococci HLA vancomycin resistant VRE 45 (gram +)	0	15	12	Complete inhibition	Complete inhibition	0	34
Enterococci HLA 21 (gram +)	0	11	0	0	0	0	35
Pseudomonas aeruginosa (gram -)	0	10	0	0	0	0	0
Klebsiella 126 (gram -)	0	9	0	0	0	0	0
Klebsiella 129 (gram -)	0	11	0	0	0	0	0
Acinetobacter 13 (gram -)	0	0	0	0	0	0	0
Acinetobacter 12 (gram -)	0	0	0 </tr				

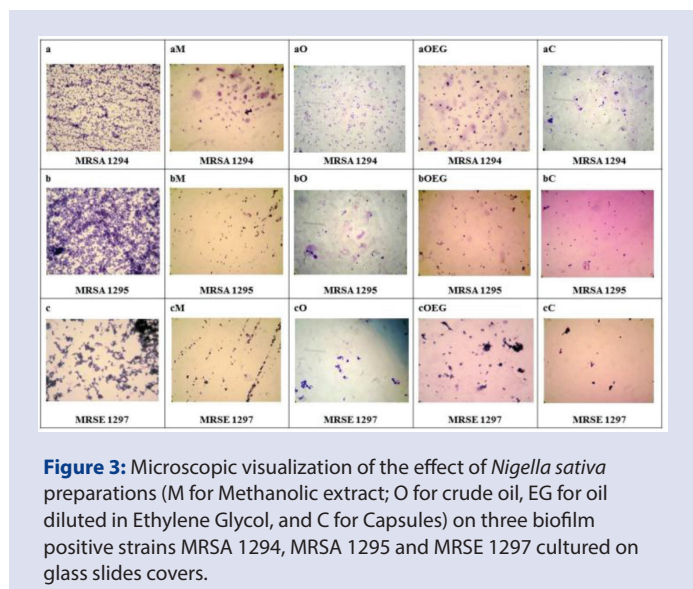


Figure 3: Microscopic visualization of the effect of *Nigella sativa* preparations (M for Methanolic extract; O for crude oil, EG for oil diluted in Ethylene Glycol, and C for Capsules) on three biofilm positive strains MRSA 1294, MRSA 1295 and MRSE 1297 cultured on glass slides covers.

phenolic and flavonoid components at alcoholic concentration of 500 mg/ml. The extracts diluted in methanol showed as well an antibacterial activity but lower than in pure methanol. The crude oil and the diluted ones showed an antibacterial activity. However, the crude oil demonstrated better results than the diluted one. Capsules showed the best effect with the highest inhibition zone suggesting that the antibacterial efficacy of *N. sativa* preparations is associated with their high bioactive contents. Those results are in good agreement with previous studies by,³³ α -thujene (5.6% reporting that the essential oils like thymoquinone are responsible for the strong antimicrobial activity of *N. sativa* oil³³ α -thujene (5.6%. However, there is increasing amount of evidence that the activity of *N. sativa* oils may not rely exclusively on the main active components present, but also on the interactions between those components and other constituents in the oil.

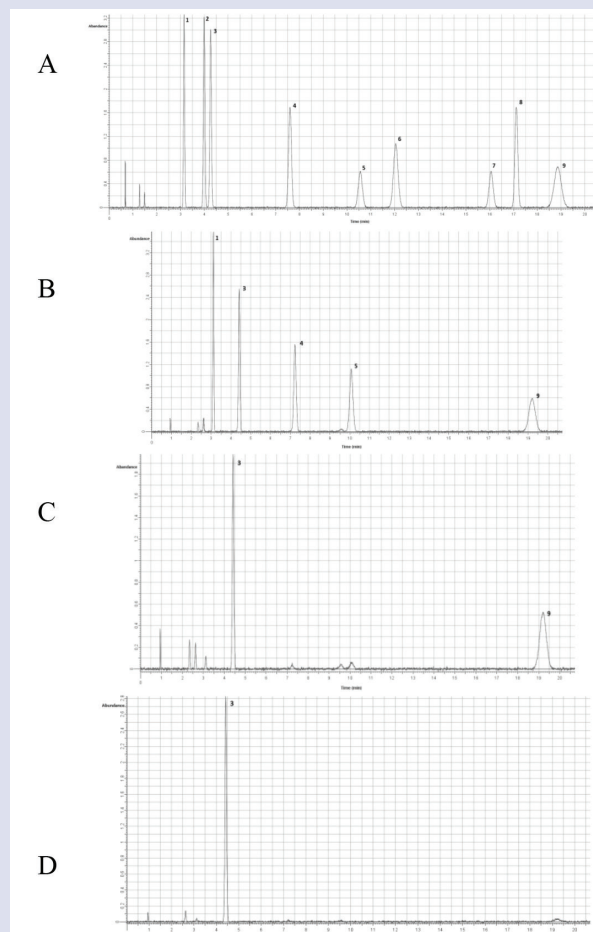


Figure 4: GC-MS analysis of *Nigella sativa* preparations: (A) Oil (B) Capsule (C) Methanolic extract (D) Aqueous extract.

Table 2: Chemical compositions of *N. sativa* preparations:oil, capsule, methanolic and aqueous extracts major peaks

N	Compound	RT	Oil	Capsule (%)	Methanolic extract (%)	Aqueous extract (K)
1	Beta-Pinene	3.1	9.1	10.0	–	–
2	O-Cymene	4.0	8.7	–	–	–
3	Thymoquinone	4.3	7.8	8.6	37	–
4	Palmitic acid	7.6	5.0	5.6	–	–
5	Oleic acid	10.2	7.2	8.1	–	–
6	Linoleic acid	12.1	9.5	–	–	–
7	9,12-Octadecadienoic acid (Z-Z)-, methyl ester	16.1	7.1	–	–	–
8	1-Heptatriacotanol	17.2	6.9	–	–	–
9	Thymol	18.9	9.4	10.3	6.4	–

Effect of *Nigella sativa* extracts on biofilm formation on glass microscope slide covers

N. sativa preparations were tested for their potential to prevent biofilm formation of three gram-positive resistant bacterial strains (MRSA 1294, MRSA 1295 and MRSE 1297). Prevention of biofilm formation by methanolic extracts, crude oil, oil diluted in EG and capsule content was shown by microscopic visualization. *N. sativa* preparations reduced the biofilm formation of all bacterial strains tested compared to the positive controls (non-treated bacteria, Figure 3a, b, c). A moderate biofilm formation reduction was noted for the methanolic extracts and oil diluted in EG. Whereas, the highest suppression of biofilm formation was observed for crude oil and capsule content. Our results are in accordance with the study of ²¹ who showed that thymoquinone, an active principle of *N. sativa* is able to prevent bacterial biofilm formation. To confirm this, we performed GC-MS analysis of the different preparations.

GC-MS Analysis

N. sativa oil GC-chromatogram has shown the presence of 9 major peaks (Figure 4A), and the components corresponding to the peaks have been determined: Beta-Pinene (9.1%), O-Cymene (8.7%), Thymoquinone (7.8%), Palmitic acid (5.0%), Oleic acid (7.2%), Linoleic acid (9.5%), 9,12-Octadecadienoic acid (Z-Z)-, methyl ester (7.1%), 1-Heptatriacotanol (6.9%), and Thymol (9.4%) (Table 2). Moreover, *N. sativa* capsule GC-chromatogram has shown the presence of 5 major peaks (Figure 4B), and the components corresponding to the peaks have been determined: Beta-Pinene (10.0%), Thymoquinone (8.6%), Palmitic acid (5.6%), Oleic acid (8.1%), and Thymol (10.3%) (Table 2). Furthermore, *N. sativa* methanolic extract GC-chromatogram has shown the presence of 2 major peaks (Figure 4C), and the components corresponding to the peaks have been determined: Thymoquinone (3.7%) and Thymol (6.4%) (Table 2). Nevertheless, *N. sativa* aqueous extract GC-chromatogram has shown the presence of one major peak, Thymoquinone (6.9%) (Table 2). As shown in Table II, Thymoquinone is the common bioactive component of all *N. sativa* preparations, which is in agreement with the literature³⁴ and Beta-Pinene is the major constituent of *N. sativa* oil. Our results are quite different from the work of ³⁵ who reported that *p*-cymene (37.3%) and thymoquinone (13.7%) were the major components of *N. sativa* oil.³⁶ also reported that *N. sativa* oil contains *p*-cymene as the major component. Our results show that the oil and the capsule contain considerable amounts of bioactive compounds (Beta-Pinene, Thymoquinone, thymol and Oleic acid). However, these compounds were absent in *N. sativa* methanolic and aqueous extracts, and hence the antiradical and antimicrobial activities of the oil and capsule could be attributed to the complex interactions between the individual components.

CONCLUSION

In this work, we proposed the use of *N. sativa* preparations as a potential source for antiradical and antibacterial activities. All *N. sativa* preparations have shown the ability to act as antioxidants by free radical inhibition, with the oil and capsules content showing the higher activity. *N. sativa* was also assessed for its antibacterial activity against highly resistant gram-positive and gram-negative bacteria. Generally, all preparations were strongly active against bacterial growth and biofilm formation for gram-positive bacteria. *N. sativa* capsule and oil were found to present the highest antibacterial activity, mainly attributed to their rich bioactive components. Therefore, *N. sativa* preparations can be suggested as a useful alternative for some infectious diseases, such as a decolonizing agent for gram-positive bacteria if it is incorporated in a local ointment as well as natural preservatives in food industry.

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CONFLICTS OF INTEREST

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

ABBREVIATION USED

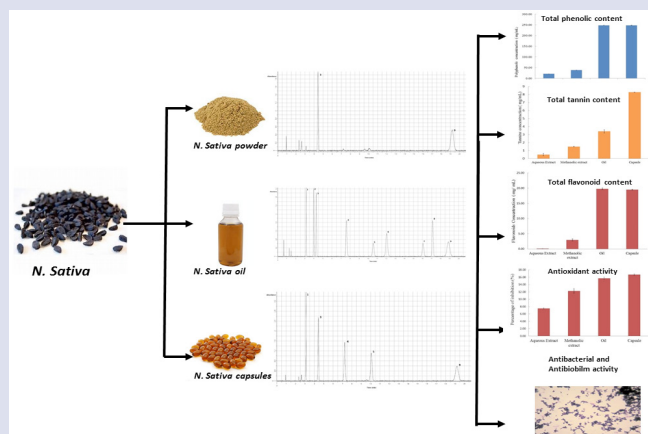
MDR: Multidrug resistant; **GC-MS:** Gas chromatographic-mass spectrometric; **MICs:** The minimal inhibitory concentrations; **ESBL:** extended-spectrum β -lactamases; **Ye:** Total phenolic content; **DPPH:** 1,1-diphenyl-2-picrylhydrazyl); **BHT:** Butylhydroxytoluene; **CE:** Catechin equivalent; **EG:** Ethylene glycol.

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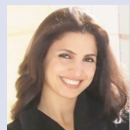
GRAPHICAL ABSTRACT



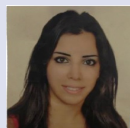
SUMMARY

- In this study, total phenolic content, tannin, and flavonoid content of *N. sativa* seed extracts and its commercially available oil were determined. Their radical scavenging activity using DPPH method was also tested. The antibacterial activity of *N. sativa* seed extracts and its oil against different multidrug resistant gram-positive and gram-negative bacterial strains was studied using disc diffusion test and the biofilm formation assay. Gas chromatographic-mass spectrometric studies were also performed to describe the essential oil and fixed oil (fatty acid) composition of the different *N. sativa* preparations.

ABOUT AUTHORS



Dr. Fatima A. Saleh: I obtained my BSc in Pharmacy with honors from Beirut Arab University, Lebanon in 2004. I earned my M.Sc. in Pharmacology and Biotechnology from Sheffield Hallam University, UK in 2005, then worked as a researcher at the Biomedical Tissue Research Group, University of York, UK. I was subsequently awarded a BBSRC CASE scholarship to pursue my PhD in biological sciences at University of York, UK. After completing my PhD in 2011, I worked as a research associate at Hull York Medical School (HYMS), UK. I have a number of publications in peer-reviewed journals. In 2013, I returned to Lebanon to be appointed as an assistant professor at Medical Laboratory Technology department, Faculty of Health sciences, at Beirut Arab University.



Dr. Nada El Darra: I obtained my BSc in Life and Earth Sciences from Saint-Joseph University, Lebanon in 2007. I earned my M.Sc. in Food chemistry with honors from Saint-Joseph University, Lebanon in 2009. Then worked as a Quality manager at Conserves Modernes Chtaura_Lebanon. In 2013, I obtained a certificate entitled "ISO 22000:2005" Food safety management System Lead Auditor from RABQSA. I was subsequently awarded a scholarship to pursue my PhD under a joint program between Saint-Joseph University and University of Technology of Compiègne_France. I obtained in 2013 a PhD in Food chemistry from Saint-Joseph University and a PhD in Industrial Process Engineering and Sustainable Development from University of Technology of Compiègne_France. After completing my PhD, I worked in 2014 as a Quality manager at Abido Spices_Neemeh_Lebanon. I have a number of publications in peer-reviewed journals. In 2014, I was appointed as an assistant professor at Nutrition & Dietetics department, Faculty of Health sciences, at Beirut Arab University.



Dr. Karim M. Raafat: Is an Associate Professor of Phytochemistry and Pharmacognosy at Faculty of Pharmacy, Beirut Arab University. He has completed his PhD from German University in Cairo, New Cairo, Egypt, under the channel system and joint supervision scheme between The German University in Cairo (GUC) and Johann Wolfgang Goethe-University Frankfurt, Germany and postdoctoral studies from Beirut Arab University (BAU) and German University in Cairo. He is a visiting Scientist of Johann Wolfgang Goethe-University, Frankfurt, Germany. He is the Head of Phytochemistry Research Team and Junior Research Team, BAU, Lebanon. He has published more than fifty books, patent, and peer reviewed journal articles and presentations in scientific conferences. He has been honored with several national and international awards in the scientific field and public service.



Prof. Iman El Ghazzawy: I obtained my Bachelor of Medicine (MBBCH) from Alexandria University in 1976. Then my MSc from the same university in 1980. In 1986, I earned my PhD, Faculty of Medicine- Alexandria University. I have been teaching from 1992 till end of 2015 in Alexandria University, Egypt. I have been also a visiting professor for eight times at the Faculty of Medicine, Beirut Arab University from 1998 till 2015 as well as Gryounis university at Benghazi, Libya for two successive academic years 2005-2006 for postgraduate medical students. In 2015-2016, I was assigned as the Head of Medical Laboratory Technology Department, Faculty of Health Science at Beirut Arab University.

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