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Chemical profile by LC-Q-TOF-MS of *Nigella sativa* seed extracts and *in vitro* antimicrobial activity on bacteria which are determined resistance gene and isolated from nosocomial infection

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ABSTRACT: Healthcare-associated infection (HCAI) is a global health challenge and methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum beta-lactamases (ESBL) producing gram-negative bacilli, which were isolated from HCAIs, are the most common antimicrobial-resistant pathogens. The present research aimed to determine the chemical composition and antimicrobial activity of ethyl acetate and methanol extracts of *Nigella sativa* seeds. Antimicrobial activity of plant extracts was observed against 20 clinical yeast (which isolated from swab samples of tongue dorsal surfaces in patients with stomatitis), 18 clinical bacteria isolates (which found from HCAIs), and also eight bacteria, and three yeast standard strains by using a broth microdilution assay. The methanol extract had the highest inhibitory activity against *S. aureus* standard strain at 0.4 mg/mL. The ethyl acetate extract showed the highest antibacterial activity against *Bacillus cereus* standard strain at 0.4 mg/mL. The methanol and ethyl acetate extracts did not show inhibitory activity against Gram-negative bacteria and yeast isolates. The LC-Q-TOF-MS profiles of the extracts were found to contain saponin, alkaloid, and fatty acid. Mass spectra revealed the presence of 14 fatty acids, four saponins, and three alkaloids for methanol extract and 13 fatty acids, three saponins, and two alkaloids for ethyl acetate extract.

KEYWORDS: *Nigella sativa*; antimicrobial activity; clinical isolates; extracts; LC-Q-TOF-MS.

1. INTRODUCTION

Healthcare-associated infections (HCAIs) are acquired in the hospital or healthcare service unit while receiving health care treatment. The term HCAIs known as nosocomial infections in the earlier can also develop in various settings during long-term care, home care, or ambulatory care. HCAIs usually occur within 48-72 hours after hospitalization or three days of discharge or 30 days of operation. Nearly 12–17 microorganisms are responsible for HCAIs, such as *Staphylococcus aureus* and *S. epidermidis* (most frequently reason of prosthetic device infection), *Enterococcus faecalis*/*Enterococcus faecium*, coagulase-negative *Staphylococci*, *Candida albicans*/*Candida glabrata*, gram-negative bacteria including *Pseudomonas* spp., *Citrobacter* spp., *Enterobacter* spp., *Acinetobacter* spp., *Proteus* spp., and *Stenotrophomonas* spp., extended-spectrum beta-lactamases (ESBL)–producing *Escherichia coli*, *Klebsiella pneumoniae*/*K. oxytoca*, and other pathogens. Most of these pathogens may often be resistant to a great many antibacterials. However, HCAIs are frequently caused by multidrug-resistant bacteria species, which differ from bacteria that occurred in community-acquired infections [1-3]. As in the past, bacterial infections are still huge health problems today. Currently, plants have been upheld to be the special sources of the drugs for most of the world's population. Hence, many plant extracts have been examined and tested to develop new antimicrobial agents [4]. *Nigella sativa* L. (black cumin) belongs to the buttercup (Ranunculaceae) family, which is called "Çörek otu" in Turkey, which is used in traditional medicine for the treatment of bacterial diseases. This plant is an annual herb and has light purple

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or white flowers. The fruit of the plant is capsule-shaped, with small black and trigonal seeds. Black cumin is native to the Middle East, South East Asia, and Egypt. It is also cultured in Turkey, Syria, Lebanon, Israel, and Southern Europe. Additionally, the plant seeds are used as a spice, condiment, carminative, and food protective [5-8]. *N. sativa* seeds are used as abortifacient and tea, diuretic, redouble for milk and orexigenic, treatment of respiratory diseases, eczema and skin infections in Turkey [9-11]. The plant has many pharmacological effects such as an antiparasitic, antibacterial, antifungal, antiviral, antiulcer, anticarcinogenic, hepatoprotective, bronchodilator, anti-schistosomiasis, antioxidant, and anti-inflammatory [12-14]. Black cumin seeds include fixed oils, proteins, alkaloids, saponins, and essential oils as main groups. The essential oil of the seed mainly consists of thymoquinone and *p*-cymene. Thymoquinone is one of the most active constituents against the pathogenic gram-positive and gram-negative bacteria [15]. Indazole alkaloids, isoquinoline alkaloids, dolabellane-type diterpene alkaloids, and hederagenin saponins were isolated from *N. sativa*. The polar extracts of seeds of *N. sativa* displayed anticancer, antimicrobial, anti-inflammatory, antiulcer, and spasmolytic effects [16]. Antibiotic-resistant bacteria and fungi cause public health problems. By the development of new antimicrobial agents can be overcome infections caused by antibiotics resistant microorganisms [17]. The present research aimed to compare the chemical composition of ethyl acetate and methanol extracts of *N. sativa* seed by LC-Q-TOF-MS. Also, the antimicrobial activity of extracts was investigated against clinical yeasts isolates (which were isolated from swab samples of tongue dorsal surfaces in patients with stomatitis) and bacteria isolates (which were isolated from HCAIs). There are limited reports against clinical yeasts and bacteria of *N. sativa* seed extracts from Turkey.

2. RESULTS

Antimicrobial activity of ethyl acetate and methanol extracts of *N. sativa* seeds was investigated against 20 clinical yeasts isolates, 18 clinical bacteria isolates, eight bacteria, and three standard yeast strains by using a broth microdilution assay. The resistance genes of the bacterial isolates included in this study are shown in Table 1. The results of antibiotic susceptibility tests with standard antibiotic discs against gram-positive and gram-negative bacteria are given in Table 2 and Table 3. The mean inhibition zone diameters of amphotericin B against yeast isolates were 19.9 ± 2.1 (13-30) mm. The methanol and ethyl acetate extracts did not show inhibitory activity against gram-negative bacteria and yeast isolates. The methanol extract had the highest inhibitory activity against *S. aureus* standard strain at 0.4 mg/mL and *B. cereus* standard strain at 1.6 mg/mL. The ethyl acetate extract had the highest inhibitory activity against *B. cereus* standard strain at 0.4 mg/mL and *S. aureus* standard strain at 3.2 mg/mL. Also, the methanol and ethyl acetate extracts showed inhibitory activity against *S. aureus* (3), *E. faecium* (3) clinical isolates, and *S. epidermidis* standard strain at 6.4 mg/mL (Table 4). CLSI recommends dimethyl sulfoxide (DMSO) as a solvent for water-insoluble extracts. In the present study, DMSO was used as a solvent to dissolve extracts. Also, the methanol, ethyl acetate, and DMSO solutions were investigated against bacteria and yeast isolates. These solvents did not indicate inhibitory activity against bacteria and yeast isolates.

Table 1. Bacteria isolated from HCAIs and their resistant genes.

Bacteria name	Number	Resistant gene
E. coli	1	NDM-1 and OXA-48
K. pneumoniae	1	NDM-1
	1	NDM-1 and OXA-48
	1	OXA-48
E. cloacae	1	OXA-48
	1	NDM-1
P. aeruginosa	3	PER-1 and OXA-10
A. baumannii	3	OXA-58 and OXA-51
S. aureus	3	mecA
E. faecium	3	vanA
Total	**18**	

Table 2. Resistance patterns of Gram-negative bacteria for antibiotics.

Bacteria Name (Number)	Resistance Gene	IMP	MEM	ETP	AK	CN	CIP	CTX	TZP	CAZ	SXT
E. coli (1)	NDM-1, OXA-48	R	-	R	R	R	R	R	R	R	-
K. pneumoniae (1)	NDM-1	R	-	R	R	R	R	R	R	R	-
K. pneumoniae (1)	NDM-1, OXA-48	R	-	R	R	R	R	R	R	R	-
K. pneumoniae (1)	OXA-48	R	-	R	R	R	R	R	R	R	-
E. cloacae (1)	OXA-48	R	-	R	R	R	S	R	R	R	-
E. cloacae (1)	NDM-1	R	-	R	R	R	S	R	R	R	-
P. aeruginosa (1)	PER-1, OXA-10	R	R	-	S	R	S	-	S	R	-
P. aeruginosa (1)	PER-1, OXA-10	S	S	-	S	S	R	-	S	S	-
P. aeruginosa (1)	PER-1, OXA-10	S	R	-	S	S	S	-	S	S	-
A. baumannii (1)	OXA-58, OXA-51	R	R	-	R	R	R	R	R	R	R
A. baumannii (2)	OXA-58, OXA-51	S	S	-	R	R	S	R	R	R	R

IMP; imipenem, MEM; meropenem, ETP; ertapenem, AK; amikacin, CN; gentamicin, CIP; ciprofloxacin, CTX; cefotaxime, TZP; piperacillin/tazobactam, CAZ; ceftazidime, SXT; trimethoprim/sulfamethoxazole.

Table 3. Resistance patterns of gram-positive bacteria for antibiotics.

Bacteria Name (Number)	Resistance Gene	CIP	LEV	LZD	TEL	E	DA	TEC	CN	P	SXT	AMP	VA
S. aureus (3)	Mec-A	S	S	S	S	S	S	S	S	R	S	-	-
E. faecium (3)	Van-A	-	-	S	-	R	-	R	-	-	-	R	R

CIP; ciprofloxacin. LEV; levofloxacin, LZD; linezolid, TEL; telithromycin, E; erythromycin, DA; clindamycin, TEC; teicoplanin, CN; gentamicin, P; penicillin, SXT; sulfamethoxazole/trimethoprim, AMP; ampicillin, VA; vancomycin.

Table 4. MIC values of methanol and ethyl acetate extracts of *N. sativa* against both clinical isolates and standard strains.

Bacteria Name (Number)	*N. sativa* (µg/mL)	
	Methanol extract	Ethyl acetate extract
S. aureus (3)	6.4	6.4
P. aeruginosa (3)	>6.4	>6.4
A. baumannii (3)	>6.4	>6.4
E. cloacae (2)	>6.4	>6.4
E. coli	>6.4	>6.4
E. faecium (3)	6.4	6.4
K. pneumonia (3)	>6.4	>6.4
E. coli ATCC 25922	>6.4	>6.4
S. epidermidis ATCC 12228	6.4	6.4
K. pneumonia ATCC 700603	>6.4	>6.4
P. vulgaris ATCC 8427	>6.4	>6.4
B. subtilis ATCC 6051	>6.4	>6.4
B. cereus ATCC 14579	1.6	0.4
S. aureus ATCC 29213	0.4	3.2
S. typhimurium ATCC 14028	>6.4	>6.4

In the current study, Thermo Hypersil Gold column was used for chromatographic separation. Peaks were identified tentatively via MassHunter METLIN Metabolite PCDL (version 7) and published literature data [18-20]. The spectra of most intense peaks were searched through the database based on generated molecular formula using accurate mass data with product ions and isotopic pattern. Mass error of 5 out of 25 formula had mass error between 5-10 ppm and rest were all less than 5 ppm. The LC-Q-TOF-MS profiles of the extracts were found to contain saponins, alkaloids and fatty acids. A total of 18 and 21 peaks were detected from ethyl acetate and methanol extracts, respectively (given in Table 5, Figure 1 and 2). Mass spectra revealed the presence of 14 fatty acids, four saponins and three alkaloids for methanol extract and 13 fatty acids, three saponins and two alkaloids for ethyl acetate extract. Norargemonine, tauroside H2 and dihydroxyoctadecanoic acid compounds were not detected in the ethyl acetate extract. Mass spectral interpretation allowed the identification of alkaloid peaks marked by their high-resolution masses and higher response in positive ionization mode (Figure 2).

Table 5. Secondary metabolites of ethyl acetate (EA) and methanol (MeOH) extracts of *Nigella sativa* seeds by LC-Q-TOF-MS.

Retention time	Compound	EA	MeOH	Class	Molecular formula	Molecular ion (m/z) (±)	Error (ppm)
14.66	Norargemonine	-	+	Alkaloid	$C_{20}H_{23}NO_4$	+342.1693	2.2
20.62	Sieboldianoside A	+	+	Saponin	$C_{64}H_{104}O_{30}$	1351.6590	4.0
20.92	Tauroside H2	-	+	Saponin	$C_{59}H_{95}O_{26}$	1219.6117	0.3
22.34	Pentadecanoic acid hexoside	+	+	Fatty acid	$C_{21}H_{40}O_7$	403.2718	4.5
22.69	Trihydroxy octadecenoic acid	+	+	Fatty acid	$C_{18}H_{34}O_5$	329.2346	3.9
25.50	Sapindoside B	+	+	Saponin	$C_{46}H_{74}O_{16}$	881.4907	0.5
26.00	Tauroside E	+	+	Saponin	$C_{41}H_{66}O_{12}$	749.4494	1.6
26.85	Dihydroxy-octadecenoic acid	+	+	Fatty acid	$C_{18}H_{34}O_4$	313.2399	4.5
28.46	Dihydroxy-octadecanoic acid	-	+	Fatty acid	$C_{18}H_{36}O_4$	315.2553	4.1
29.46	Hydroxy-octadecadienoic acid	+	+	Fatty acid	$C_{18}H_{32}O_3$	295.2304	8.1
30.12	Hydroxy-octadecatrienoic acid	+	+	Fatty acid	$C_{18}H_{30}O_3$	293.2141	6.9
30.37	Hydroxy-octadecenoic acid	+	+	Fatty acid	$C_{18}H_{34}O_3$	297.2460	8.2
30.91	Nigellamine A2	+	+	Alkaloid	$C_{39}H_{42}N_2O_7$	+651.3039	4.1
32.27	Nigellamine A1	+	+	Alkaloid	$C_{40}H_{43}NO_7$	+650.3091	3.6
33.38	Octadecatrienoic acid	+	+	Fatty acid	$C_{18}H_{30}O_2$	277.2183	3.4
33.98	Hexadecenoic acid	+	+	Fatty acid	$C_{16}H_{30}O_2$	253.2199	10.0
35.59	Palmitic acid	+	+	Fatty acid	$C_{16}H_{32}O_2$	255.2330	0.5
35.79	Octadecenoic acid	+	+	Fatty acid	$C_{18}H_{34}O_2$	281.2489	1.2
35.89	Eicosadieneoic acid	+	+	Fatty acid	$C_{20}H_{36}O_2$	307.2648	1.5
37.04	Stearic acid	+	+	Fatty acid	$C_{18}H_{36}O_2$	283.2650	0.5
37.14	Eicosaenoic acid	+	+	Fatty acid	$C_{20}H_{38}O_2$	309.2802	0.9

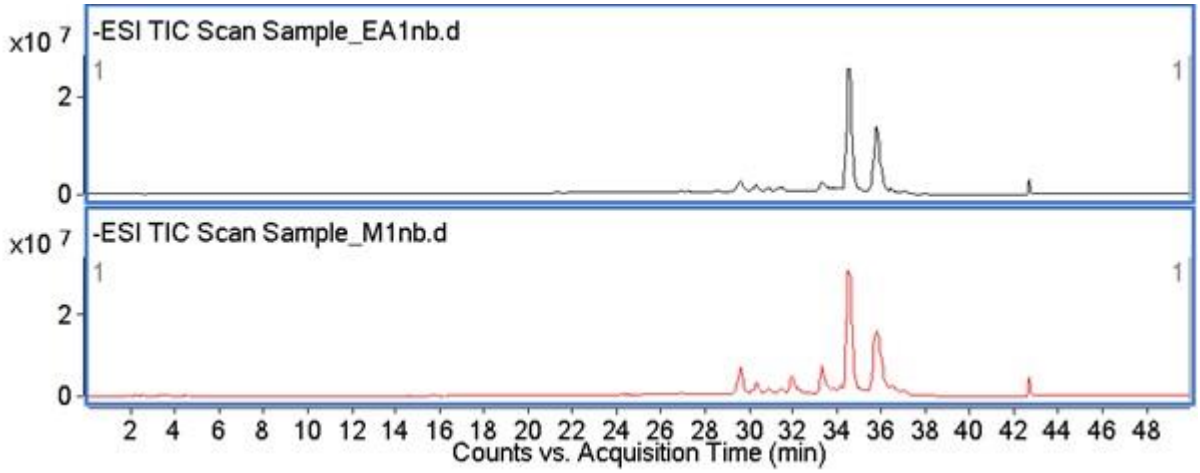

Figure 1. Total Ion Chromatogram (TIC) of ethyl acetate (EA1nb) and methanol extracts (M1nb) (Negative ESI-MS).

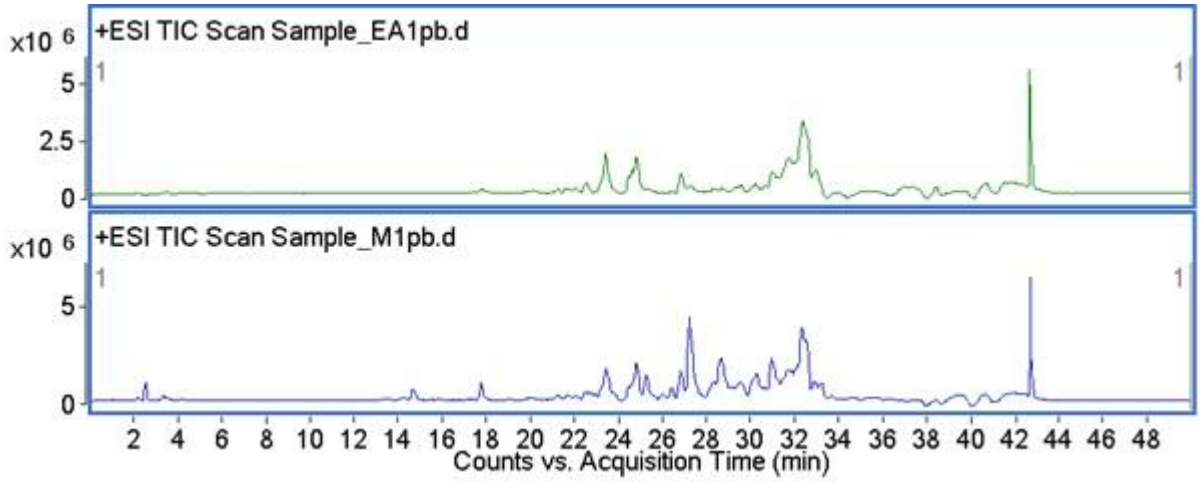

Figure 2. Total Ion Chromatogram (TIC) of ethyl acetate (EA1pb) and methanol extracts (M1pb) (Positive ESI-MS).

3. DISCUSSION

The chemical composition of the seed extracts of *N. sativa* possessed remarkable diversities in the main compounds. These diversities could be related to different geographical origins and morphological characteristics. Previous studies indicated that *N. sativa* seeds have genetic differences in different countries. These genetic differences revealed that the plant has distinct varieties, species, and subspecies. Also, differences in chemical composition and antimicrobial activities of *N. sativa* may be related to species, subspecies, and varieties of the plant [21]. Singh et al. reported that the chemical compositions of the essential oil and acetone extract of *N. sativa* seed from India were determined by gas chromatography-mass spectrometry (GC-MS) [22]. Also, the antimicrobial activity of the acetone extract and essential oil was studied. The acetone extract had linoleic acid (53.6%), thymoquinone (11.8%), and palmitic acid (10.0%) as the main compounds. The acetone extract was effective on gram-positive (*Bacillus subtilis* and *S. aureus*) and gram-negative (*Pseudomonas aeruginosa*) bacteria and molds (*Aspergillus ochraceus*, *A. niger*, *A. flavus*, *A. terreus*, *Fusarium graminearum*, *F. monoliforme*, *Penicillium citrium*, and *P. viridicatum*). The main compounds of the extract may be responsible for antimicrobial activity. In the current study, the ethyl acetate and methanol extracts were not effective against gram-negative bacteria and clinical yeast isolates. Linoleic acid and thymoquinone were not determined in the extracts of the present study. Palmitic acid was found in the extracts in the present study. The present research displayed dissimilar chemical profiles from the previous study. These controversial activity results can be related to constituents of extracts and microorganisms types.

In another research, the antimicrobial activity of the essential oil and oleoresins (*n*-hexane, ethyl acetate, and ethanol) of *N. sativa* seed from India was studied. The chemical compositions of the essential oil and

oleoresin extracts were analyzed by GC-MS. The main compounds of the oleoresin extracts were linoleic acid (27.0-43.9%) and glyceryl linoleate (21.9-27.7%). Fatty acid derivatives were a dominant group of oleoresin (*n*-hexane, ethyl acetate, and ethanol) extracts. Oleoresin extracts displayed activity against yeast isolates (*A. niger*, *A. flavus*, *F. monoliforme*, and *P. viridicatum*) and gram-positive bacteria (*B. subtilis* and *S. aureus*) except *B. cereus*. Additionally, oleoresin extracts were not effective against gram-negative bacteria (*E. coli* and *P. aeruginosa*) [23]. In the present study, ethyl acetate and methanol extracts mainly consisted of fatty acid derivatives with hydroxyl groups. The dominant group of the previous study showed a similar chemical profile with the present study. But the main compounds of the previous study were not found in the extracts of the present study. Due to the difference of the main components, the antimicrobial activity of both studies had dissimilar results.

Ugur et al. reported that the essential oil of *N. sativa* seed from Turkey showed significant activity against clinical isolates of MRSA and methicillin-resistant coagulase-negative staphylococci (MRCoNS) at a very low minimum inhibitory concentration (MIC) value [21]. Thymoquinone, thymohydroquinone, dithymoquinone, and thymol were the most active compound in the essential oil, and these compounds were responsible for antimicrobial activity against MRSA. Fatty acid derivatives may also have minor antimicrobial activities. In our study, the ethyl acetate and methanol extracts had activity against MRSA at 6.4 mg/mL. Our findings were in agreement with the literature. But the current study extracts had higher MIC values against MRSA when compared with the literature. The difference in MIC values is due to the difference in the active compound in essential oil and extracts. Thymoquinone, thymohydroquinone, dithymoquinone, thymol, and *p*-cymene were not detected in the extracts of the current study.

Although there are other studies investigating antibacterial and antifungal activity of *N. sativa* against clinical strains [4, 6, 8, 14, 15, 21], this is the first study evaluating the effectiveness against clinical bacterial strains that have resistant genes such as NDM-1 and OXA-48 in Enterobacteriaceae; PER-1, OXA-10, OXA-58, and OXA-51 in nonfermenting gram-negative bacilli; mecA and vanA in gram-positive cocci. Bacterial resistance against antibiotics is a great problem worldwide today. The results of the present study indicate that the extract of *N. sativa* seeds have antibacterial activity against most common bacterial strains involving even multiple antibiotic resistant isolates.

4. CONCLUSION

The antibacterial and antifungal activity of *N. sativa* was evaluated against clinical bacterial strains with resistant genes, standard bacterial strains as well as clinical and standard yeast strains in this study. Besides this, the chemical composition of ethyl acetate and methanol extracts of *N. sativa* seed was compared by the LC-Q-TOF-MS technique. The methanol and ethyl acetate extracts of black cumin seeds showed antibacterial activity against gram-positive bacteria. These extracts may be used as an alternative and cheap medicine. Additionally, these extracts can open a new way for investigation in food and product development. Further studies on extracts of *N. sativa* are required to improve the extraction procedure and to isolate active compounds of extracts that are responsible for its antibacterial activities. Both extracts did not show activity against gram-negative bacteria and yeasts. This result indicates a higher concentration of the extracts is required to inhibit the growth of gram-negative bacteria and yeasts.

5. MATERIALS AND METHODS

5.1. Plant material

N. sativa seeds were purchased from the local herbal shop in Bakirkoy, Istanbul, Turkey. The plant materials were identified by Dr. Ahmet Dogan. Herbarium specimen of the plant was deposited in the Marmara University Herbarium (Herbarium number: MARE22475). The seeds (80 g for each solvent) were extracted (three times each solvent) with ethyl acetate (160 mL, 99.5% purity, Merck) and methanol (160 mL, 99.8% purity, Merck) by using the maceration method (three days each extract), respectively. Each extract was filtered by using filter paper and concentrated at 45^{o}C under reduced pressure using a rotary evaporator (Buchi, Labortechnik, Flawil, Switzerland). 3.5 g crude ethyl acetate extract and 5.0 g crude methanol extract were obtained from *N. sativa* seeds.

5.2. Bacteria and yeast isolates

Gram-positive cocci and bacilli, gram-negative bacilli, and yeast strains isolated from clinical specimens were contained in the present research. These bacteria and yeasts isolates were; three *S. aureus*, three *P.*

aeruginosa, three *A. baumannii*, two *E. cloacae*, one *E. coli*, three *E. faecium* (vancomycin-resistant *Enterococcus*; VRE) three *K. pneumoniae*, five *C. albicans*, five *C. glabrata*, five *C. tropicalis*, five *C. parapsilosis*, and two *C. kefyr*. All clinical isolates were isolated and identified in the Bacteriology and Mycology Laboratories at the Department of Medical Microbiology of Istanbul Faculty and Medicine, years between 2007 and 2017. Then, the resistance genes of all bacteria isolated from HCAIs were determined by using polymerase chain reaction (PCR) method (unpublished work). Also, the standard microorganisms used in this study were as follows; *E. coli* ATCC 25922, *S. epidermidis* ATCC 12228, *K. pneumonia* ATCC 700603, *P. vulgaris* ATCC 8427, *Bacillus subtilis* ATCC 6051, *Bacillus cereus* ATCC 14579, *S. aureus* ATCC 29213, *Salmonella typhimurium* ATCC 14028, *C. albicans* ATCC 90028, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019.

5.3. The minimum inhibitory concentration (MIC) Assay

The microorganisms were stored at -20°C till analysis day. The bacteria and yeast isolates were cultured on Tryptone Soya Agar (bacteria) and Sabouraud Dextrose Agar (yeasts) and incubated overnight at 37°C. For the MIC method, each microorganism was suspended in sterile saline and diluted at 1.5 x 10^8 colony-forming unit (CFU) per mL according to McFarland 0.5 turbidity standard. The extracts of *N. sativa* were solved by using DMSO to prepare a stock extract solution of 256 µg/mL. Two-fold dilutions of the extracts were prepared in 0.1 mL volumes of sterile Mueller Hinton Broth to give a range of concentrations from 6.4 to 0.03125 mg/mL of the extracts in 96 well plate. The same process was performed for methanol, ethyl acetate, and DMSO. The whole plates incubated at 37°C for 24 h, and then were checked for growth. The MIC values of each extract was determined as the lowest concentration that showed no growth [24]. Antifungal susceptibility testing was also performed by using RPMI 1640 medium, which contains 106 CFU yeast per mL. After plates were incubated at 35°C for 48 h, the MIC value was defined as the lowest concentration (µg/mL), which inhibited yeast growth [32]. The range of extracts concentration was 0.03125-6.4 mg/mL in 96 well plate. The same process was performed for methanol, ethyl acetate, and dimethyl sulfoxide (DMSO).

5.4. Antimicrobial susceptibility testing for referent antimicrobial drugs

Standard antibiotics (imipenem (30 µg/disc), meropenem (10 µg/disc), ertapenem (10 µg/disc), amikacin (30 µg/disc), gentamicin (5 µg/disc), ciprofloxacin (5 µg/disc), cefotaxime (30 µg/disc), piperacillin/tazobactam (100/10 µg/disc), ceftazidime (30 µg/disc), trimethoprim/sulfamethoxazole (1.25/23.75 µg/disc), levofloxacin (5 µg/disc), linezolid (30 µg/disc), telithromycin (15 µg/disc), erythromycin (15 µg/disc clindamycin (10 µg/disc), teicoplanin (30 µg/disc), penicillin (10 µg/disc), ampicillin (10 µg/disc), vancomycin (30 µg/disc) were tested on Mueller Hinton agar plates by using Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute criteria [25]. The inhibition zone was measured as sensitive, intermediate sensitive, or resistant per the standard criteria. Antifungal susceptibility tests of *Candida* spp. were studied for amphotericin B (10 µg/100 µL) by the disc diffusion method. After incubation at 37°C for 24-48 hours, inhibition zone diameters were measured [26].

5.5. LC-Q-TOF-MS analysis of the extracts

Agilent 1260 Series HPLC system with a Binary Pump, High Performance HiP Autosampler, Column Thermostat and a UV dedector coupled with an Agilent G6550A iFunnel Quadrupole Time-of-Flight LC-MS system with a Dual Spray Agilent Jet Stream electrospray ionisation source (Agilent Technologies, Incorporated, CA, USA). The instrument was operated in 2 GHz Extended Dynamic Range mode with negative and positive electrospray ionisation. All ions mode with collision energies 0 eV, 10 eV, and 30 eV were used during the run, the acquisition rate was 1 spectra/second. The mass range was adjusted to 40-1700 amu for mass scan. Optimized ion source parameters were as follows: drying gas temperature: 200°C; drying gas flow: 14 L/min; nebulizer pressure: 40 psi; capillary voltage: 1500; sheat gas temperature: 350°C; sheat gas flow: 11 L/min; nozzle voltage: 1000 V. The reference ions were 980.0147, 1033.9881 for negative run, 922.0098 for positive run.

Analytical column used for separation was Thermo Hypersil Gold C18 (4.6 mm x 150 mm x 3 µm). The data was processed by Agilent MassHunter Software B 06.00. The gradient flow using mobile phase A: 5mM ammonium acetate in water and mobile phase B: acetonitrile. Flow rate was determined as 0.6 mL/min, analysis time was 50 min, the injection volume was 5 µL and the column temperature was 35°C. The gradient started with 5%B, hold for 3 min; increased to 10%B within 8 min and then to 100% B in 32 min and kept there for 6 min.

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Conflict of interest statement: The authors declared no conflict of interest.

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