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**Chemical composition and biological activity of *Nepeta cilicica***

## Chemical composition and biological activity of *Nepeta cilicica*

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Article Info	Abstract
<p>Received: 30 March 2017            Accepted: 16 May 2017            Available Online: 20 June 2017            DOI: 10.3329/bjp.v12i2.32010</p> <p>Cite this article:            İřcan G, Göger F, Demirci B, Köse YB. Chemical composition and biological activity of <i>Nepeta cilicica</i>. Bangladesh J Pharmacol. 2017; 12: 204-09.</p>	<p>In the present study, aerial parts of <i>Nepeta cilicica</i> were hydro-distilled for the essential oil. Furthermore, air-dried plant materials were extracted with methanol. According to GC-MS results, 35 compounds were characterized in the oil (totally 92%). Caryophyllene oxide, <math>\beta</math>-caryophyllene and spathulenol (28.2, 8.9 and 4.2%) were found as main components of the oil. Sixteen compounds were detected by LC/MS, most of which were caffeoylquinic acid, luteolin and apigenin derivatives, in methanolic extract of the <i>N. cilicica</i>. Apart from DPPH radical scavenging activity, inhibitory effects on bacterial and yeast growth of essential oil and the methanolic extract were evaluated. Twenty-four different pathogenic bacterial and <i>Candida</i> strains were tested by M7-A7 and CLSI M27-A2 protocols respectively. The crude extract showed better inhibitory effects against bacteria strains than the essential oil. Both oil and the extract demonstrated strong inhibition on <i>C. tropicalis</i> at a concentration of 47.0 <math>\mu</math>g/mL (MIC).</p>

### Introduction

*Nepeta cilicica* is a perennial plant with stem size of 22-100 cm. Some *Nepeta* species are used in the folk medicine for their therapeutic properties such as diaphoretic, diuretic, antiseptic, astringent, antiasthmatic, antitussive, anti-spasmodic, febrifuge, sedative, emmenagogue and stomachic. Flowering aerial parts of several *Nepeta* species are used as medicinal teas for their lemony mint odor and flavor (Duda et al., 2015; Shakeri et al., 2014; Formisano et al., 2013; İscan et al., 2011; Skaltza et al., 2000).

Many compounds have been identified in *Nepeta* species such as monoterpenes, sesquiterpenes, flavonoids, phenolic acid, steroids and essential oils. The chemical composition of the essential oils derived from *Nepeta* can be of two categories. Nepetalactone containing and nepetalactoneless group that are generally rich in 1,8-

cineole (most abundant), pinenes,  $\beta$ -caryophyllene,  $\beta$ -farnesene, caryophyllene oxide,  $\alpha$ -citral and  $\beta$ -citronellol (Asgarpanah et al., 2014; Sharma and Cannoo, 2013; Skaltza et al., 2000). Nepetalactone isomers 4 $\alpha$ ,7 $\alpha$ ,7 $\alpha$ -nepetalactone, 4 $\alpha$  $\beta$ ,7 $\alpha$ ,7 $\alpha$ -nepetalactone, 4 $\alpha$  $\beta$ ,7 $\alpha$ ,7 $\alpha$ -nepetalactone, 4 $\alpha$ ,7 $\alpha$ ,7 $\alpha$ -nepetalactone and 4 $\alpha$ ,7 $\beta$ ,7 $\alpha$ -nepetalactone are considered as biochemical indicator of the nepetalactone-rich group of *Nepeta* essential oils.

Biological efficacy is usually attributed to isoprene derivatives named nepetalactones especially found in the *Nepeta* oils (Kökdil et al., 1999; Bařer et al., 2000; Skaltza et al., 2000; Nestorović et al., 2010). Even though two studies have been reported on the chemical composition of *N. cilicica* essential oil. There has not been any report about the chemical composition of the crude extract and antimicrobial activity of essential oil or the extract. According to previous studies sesquiterpenes ( $\beta$ -caryophyllene, spathulenol and  $\beta$ -caryophyllene oxide) and fatty acids have been determined as main compounds of *N. cilicica* essential oil (Formisano et al.,

2013; Kokdil et al., 1997).

In the present study, hydro-distilled essential oil and crude methanolic extracts of flowering aerial parts of *N. cilicica* were analyzed by chromatographic techniques. GC-MS with GC-FID and LC-MS systems were used for the determination of essential oil and the extract composition respectively. Both oil and extract were subjected to antimicrobial susceptibility assay against 24 pathogenic bacteria and *Candida* strains in comparison with the standard antimicrobial agents and also anti-oxidant assay for the first time.

## Materials and Methods

### Plant material

*N. cilicica* was collected from Anamur-Ermenek Road, İçel, Turkey on June 2014 in full flowering stage. Voucher specimen (ESSE: 14795) was kept in the Faculty of Pharmacy Herbarium, Anadolu University, Turkey.

### Isolation of essential oil

The flowering aerial parts were air-dried at ambient temperature then subjected to a Clevenger-type apparatus for hydro-distillation for 3 hours according to European Pharmacopoeia 8.0, with a yield of 0.02% (on dried weight basis). The oil was dehydrated over sodium sulfate anhydrous and kept at 4°C until analysis.

### Preparation of the extract

Extract of air-dried and grounded plant materials were prepared by the methanol (Merck) in an orbital shaker for 24 hours. After evaporation process at 40°C, dried extract was kept in dark place at 4°C until analysis.

### GC-FID and GC-MS analysis

The GC-FID and GC-MS analysis were carried out with innowax FSC columns (60 m x 0.25 mm, 0.25 mm film thickness) with helium (0.8 mL/min) using Agilent 6890N GC and 5975 GC-MSD systems respectively. FID detector temperature was 300°C. To obtain similar retention times between the GC-FID and GC-MS, synchronous automatic duplicated injection was performed using equal column by setting the same operational parameters. Relative percent of the detected compounds were calculated from the FID chromatograms.

### Identification of the compounds

Characterization and identification of the *N. cilicica* essential oil components were fulfilled by comparison of their relative retention time with those of genuine samples or by comparison of their relative retention indices (RRI) to *n*-alkanes (C<sub>6</sub>-C<sub>16</sub>). Software matching against commercial libraries (Wiley GC-MS and Mass Finder 3) (Koenig et al., 2004; McLafferty and Stauffer, 1989) and in-house "Başer Library of Essential Oil Constituents" created by genuine compounds and

several constituents of the common essential oils, as well as MS data from previous works (ESO, 2000; Joulain and Koenig, 1998) were used for the identification.

### Phenolics analysed by LC-MS/MS method

Applied Biosystems 3200 Q-Trap MS/MS system coupled to a Shimadzu 20A HPLC system was used in the negative ionization mode for the analyses of the phenolic compounds. Octadecyl silica gel analytical column (ODS 150 x 4,6 mm, i.d., 3 µm particle sizes) for the separation operating at 40°C at a flow rate of 1 mL/min. HPLC elution was carried out using a gradient of the solvent mixture: H<sub>2</sub>O:CH<sub>3</sub>OH:CH<sub>2</sub>O<sub>2</sub> (89:10:1, v/v/v) (solvent A) and CH<sub>3</sub>OH:H<sub>2</sub>O:CH<sub>2</sub>O<sub>2</sub> (89:10:1, v/v/v) (solvent B). Solvent B composition was increased from 15 to 40% in 15 min, 40 to 45% in 3 min. It was held at 45% concentration for 12 min. Then increased from 45 to 75% in 5 min and increased from 75 to 85% in 3 min. In one minute, it was increased from 85 to 95% and it reached 100% concentration at 40 min.

### Determination of total phenolic content

Total phenol contents were calculated as gallic acid equivalents (GAE), which means mg gallic acid/g extract. Total phenolics in the oil and extract were determined using with previously described method (Singleton et al., 1999).

### DPPH radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH•) scavenging activity determined according to the previous study of Kumarasamy et al. (2007). Serial dilutions of the stock solution of samples and standards were prepared as half concentrations of previous one. Equal amounts of DPPH• reactive were added to the diluted solutions. After 30 min UV absorbance was recorded at 517 nm.

### Antimicrobial susceptibility tests

Antibacterial and anticandidal properties of the essential oil and methanol extract were evaluated by using partially modified CLSI microdilution broth methods (CLSI-M7-A7 and CLSI-M27-A2). Unlike the reference methods, the essential oil and methanolic extract were diluted between the concentrations of 5.8 to 6000 µg/mL. Ampicillin (Merck), chloramphenicol (Merck), amphotericin B (Sigma-Aldrich) and ketoconazole (Sigma-Aldrich) were used as standard antimicrobial agents. Microorganisms were obtained from ATCC, NRRL and RSK (Refik Saydam National Type Culture Collection, Turkey).

## Results

### Phytochemical composition

According to GC/MS results, 35 compounds representing 92% of the essential oil were characterized. The oil

was constituted mainly by oxygenated sesquiterpenes (47.5%), sesquiterpene hydrocarbons (25.3%), alkanes (10.2%), oxygenated monoterpenes (3.7%), acyclic-diterpene derivative phytol (2.9%) and fatty acids (2.3%). Caryophyllene oxide (28.2%) and  $\beta$ -caryophyllene (8.9%) were determined as main compounds. Monoterpenes were detected only trace amounts in the essential oil (Table I).

According to the LC-MS/MS analyses, 16 compounds (Figure 1) were determined in the methanol extract of *N. cilicica*. The main compounds of the extract were determined as 5-cafeoylquinic acid, luteolin glucoside, luteolin glucuronide, apigenin glucoside and apigenin glucuronide. Luteolin and apigenin were also determined as aglycons (Table II).

### Biological activity

The extract demonstrated weak DPPH radical scavenging activity at the concentration of  $0.31 \pm 0.05$  mg/mL (Gallic acid was  $0.0025 \pm 0.0002$  mg/mL). Total phenol content was determined as 53.17 mg GAE/g extract.

According to antimicrobial assay results essential oil and methanol extract showed weak to strong antimicrobial effects against 24 different pathogenic *Candida* and Gram positive and negative bacterial strains. The essential oil demonstrated strong activity against food pathogens Gram positive *Bacillus subtilis* and *Listeria monocytogenes* at the concentrations of 94 and 187  $\mu$ g/mL (MIC) respectively (Table III). The strain of ATCC-750 *C. tropicalis* was inhibited by the essential oil having a MIC value of 47  $\mu$ g/mL. The essential oil demonstrated moderate to weak inhibitory effects against all other tested pathogens between the concentrations of 3,000 to 375  $\mu$ g/mL (MIC).

According to broth dilution tests, methanol extract obtained from flowering aerial parts of the *N. cilicica*, demonstrated better antimicrobial effects rather than the essential oil. The methanol extract strongly inhibited the growing of *Proteus vulgaris*, *B. cereus*, *B. subtilis*, *Staphylococcus aureus* and *Listeria monocytogenes* at the concentrations of 94, 187, 94, 47 and 90  $\mu$ g/mL. The essential oil and methanol extract almost showed same effects against *Candida* strains. Similar to essential oil, *C. tropicalis* was inhibited by the methanol extract at the concentration of 47  $\mu$ g/mL (Table IV).

## Discussion

Essential oil composition of the *Nepeta* species has been varying according to the growing region, environmental conditions and soil type, which they are generally divided into two groups nepetalactone containing and nepetalactoneless as reported in

Table I				
Composition of the essential oil of <i>N. cilicica</i>				
No	RRI	Compound	%	Method
1	1032	$\alpha$ -Pinene	Trace	RRI, MS
2	1118	$\beta$ -Pinene	Trace	RRI, MS
3	1132	Sabinene	Trace	RRI, MS
4	1255	$\gamma$ -Terpinene	Trace	RRI, MS
5	1280	<i>p</i> -Cymene	Trace	RRI, MS
6	1466	$\alpha$ -Cubebene	0.6	MS
7	1497	$\alpha$ -Copaene	2.7	MS
8	1535	$\beta$ -Bourbonene	1.5	MS
9	1549	$\beta$ -Cubebene	2.7	MS
10	1611	Terpinen-4-ol	0.3	RRI, MS
11	1612	$\beta$ -Caryophyllene	8.9	RRI, MS
12	1648	Myrtenal	0.1	MS
13	1670	<i>trans</i> -Pinocarveol	0.1	MS
14	1683	<i>trans</i> -Verbenol	1.5	MS
15	1687	$\alpha$ -Humulene	1.3	RRI, MS
16	1726	Germacrene D	3.0	RRI, MS
17	1755	Bicyclgermacrene	1.5	RRI, MS
18	1773	$\alpha$ -Cadinene	0.9	MS
19	1776	$\gamma$ -Cadinene	1.0	MS
20	1804	Myrtenol	0.6	MS
21	1849	Calamenene	1.2	MS
22	1900	<i>epi</i> -Cubebol	2.6	MS
23	2001	Isocaryophyllene oxide	3.8	MS
24	2008	Caryophyllene oxide	28.2	RRI, MS
25	2071	Humulene epoxide-II	3.5	MS
26	2144	Spathulenol	4.2	MS
27	2187	<i>T</i> -Cadinol	1.4	MS
28	2239	Carvacrol	1.1	RRI, MS
29	2289	4-Oxo- $\alpha$ -Ylangene	1.7	MS
30	2392	Caryophylla-2(12),6-dien-5 $\beta$ -ol (=Caryophyllenol II)	2.1	MS
31	2500	Pentacosane	2.5	RRI, MS
32	2622	Phytol	2.9	MS
32	2700	Heptacosane	4.5	RRI, MS
34	2900	Nonacosane	3.2	RRI, MS
35	2931	Hexadecanoic acid	2.3	RRI, MS
		Monoterpene hydrocarbons	Trace	
		Oxygenated monoterpenes	3.7	
		Sesquiterpene hydrocarbons	25.3	
		Oxygenated Sesquiterpenes	47.5	
		Fatty acids	2.3	
		Diterpenes	2.9	
		Alkanes	10.2	
		Total	91.9	

RRI relative retention indices; Trace (<0.1%), %calculated from FID data; Identification method based on the relative retention indices (RRI) of authentic compounds on the HP innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the wiley and mass finder libraries and comparison with literature data

Table II			
Phenolic compounds of the <i>N. cilicica</i> methanol extract			
Rt	[M-H] -m/z	Fragments	Identified as
5.7	353	191, 173	1-caffeoylquinic acid
7.5	315	153	Protocatechuic acid hexoside
9.2	353	191, 179, 173	5-Caffeoylquinic acid
11.5	337	191, 173	5-Coumaroylquinic acid
13.0	448	287, 151, 135	Eriodictiol glucoside
13.2	447	285, 151, 133	Luteolin glucoside
13.6	461	357, 285, 151, 133	Luteolin glucuronide
15.1	431	269, 240, 151, 117	Apigenin glucoside
15.7	445	269, 175, 113	Apigenin glucuronide
16.3	359	197, 161	Rosmarinic acid
18.3	593	323, 269, 151, 117	Apigenin derivative
18.9	487	269, 217, 113	Apigenin derivative
20.0	277	259, 233, 203	Unknown
21.0	285	133	Luteolin
23.4	277	177, 151	Naringenin
23.9	269	151, 117	Apigenin

literature data. Apart from mono- and sesquiterpenes in essential oils, diterpenes, triterpenes, iridoids and their glucosides, were reported as major constituents of *Nepeta* species (Asgarpanah et al., 2014; Formisano et al., 2013; Sharma and Cannoo 2013; Başer, 2000; Skaltza et al., 2000).

Table III					
Antibacterial activity of <i>N. cilicica</i> essential oil and methanol extract (MIC, $\mu\text{g/mL}$ )					
Bacteria	Sources	Nc <sup>1</sup>	Nc <sup>2</sup>	St-1	St-2
<i>Pseudomonas aeruginosa</i>	ATCC 27853	3000	1500	16	4
<i>Enterobacter aerogenes</i>	NRRL 3567	3000	3000	8	1
<i>Proteus vulgaris</i>	NRRL B-123	750	94	1	4
<i>Bacillus cereus</i>	NRRL B-3711	375	187	2	4
<i>Bacillus subtilis</i>	NRRL B-4378	94	94	1	1
<i>Serratia-amarcenscens</i>	NRRL B-2544	3000	3000	16	4
<i>E. coli</i>	ATCC 8739	3000	3000	2	1
<i>Salmonella typhimurium</i>	ATCC 14028	3000	3000	1	1
<i>Staphylococcus aureus</i>	ATCC43300	750	47	1	8
<i>E. coli O157:H7</i>	RSSK 234	3000	3000	1	1
<i>Listeria monocytogenes</i>	ATCC 19111	187	90	1	2
<i>S. epidermidis</i>	ATCC 14990	750	187	1	1

Nc<sup>1</sup>: Essential oil of *N. cilicica*; Nc<sup>2</sup>: Methanol extract of *N. cilicica*; St-1: Ampicillin; St-2: Chloramphenicol

In accordance with our results, sesquiterpenes were the

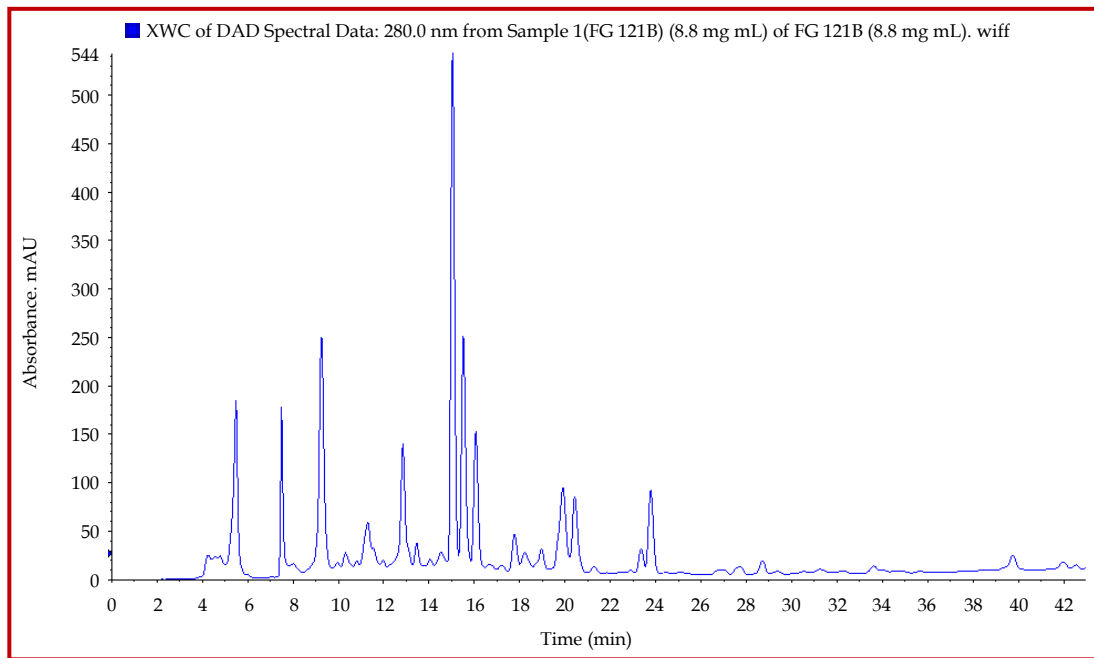


Figure 1: HPLC chromatogram of *Nepeta cilicica* methanol extract at 280 nm

Table IV					
Anticandidal activity of <i>N. cilicica</i> essential oil and methanol extract (MIC, µg/mL)					
<i>Candida</i> strain	Sources	Nc <sup>1</sup>	Nc <sup>2</sup>	St-1	St-2
<i>C. albicans</i>	ATCC 10231	375	750	0.5	0.1
<i>C. utilis</i>	NRRL Y-900	375	187	0.3	0.1
<i>C. krusei</i>	NRRL Y-7179	750	750	0.5	0.3
<i>C. zeylanoides</i>	NRRL Y-1774	750	750	0.5	0.1
<i>C. parapsilosis</i>	NRRL Y-12696	375	187	0.5	0.1
<i>C. glabrata</i>	ATCC 2001	750	1500	1	0.1
<i>C. glabrata</i>	ATCC 66032	375	1500	1	0.1
<i>C. tropicalis</i>	ATCC 1369	750	750	0.5	0.1
<i>C. albicans</i>	ATCC® 24433	750	750	0.5	0.1
<i>C. tropicalis</i>	ATCC 750	47	47	1	0.1
<i>C. parapsilosis</i>	ATCC 22019	750	750	0.5	0.1
<i>C. krusei</i>	ATCC 6258	750	750	1	0.3

Nc<sup>1</sup>: Essential oil of *N. cilicica*; Nc<sup>2</sup>: Methanol extract of *N. cilicica*; St-1: Amphotericin-B; St-2: Ketoconazole

major compounds (73%) of *N. cilicica* oil. According to a few previous works on *N. cilicica* essential oil, sesquiterpenes such as spathulenol (15.1%), hexadecanoic acid (14%) and  $\gamma$ -cadinene (5.5%) were found as main compounds (Formisano et al., 2013). In the other study  $\beta$ -caryophyllene oxide (41%),  $\beta$ -caryophyllene (15.7%) and  $\alpha$ -copaene (2.7%) were reported as main components of the *N. cilicica* essential oil similar to our results (Köküldil et al., 1999).

To the best of our knowledge, this is the first study about phenolic content of *N. cilicica*. According to LC-MS/MS analyses, 16 compounds were identified. The hydroxybenzoic acid derivatives (compounds 1-4) were identified using the identification keys of Clifford (Clifford et al. 2003). Caffeoylquinic acids are the ester of caffeic acid and (-)-quinic acid. Caffeoylquinic derivatives (chlorogenic acids) present characteristic molecular ion peak at  $m/z$  353 [M - H]<sup>-</sup>. Compounds 1 and 3 were identified as 1-O-caffeoylquinic acid, 5-O-caffeoylquinic acid respectively. Both of the compounds showed a base peak at  $m/z$  191 ([quinic acid-H]<sup>-</sup>) discrimination were done according to the retention times where 1-O-caffeoylquinic acid elute earlier than 5-O-caffeoylquinic acid. MS fragmentation of compound 4 showed a molecular ion peak at  $m/z$  337 and a base peak ion at  $m/z$  191, being characterized as 5-p-coumaroylquinic acid. Compound 2 showed characteristic MS fragmentation of gentisic acid hexoside and protocatechuic acid hexoside with its pseudo molecular

ion peak at  $m/z$  315. Further fragmentation of  $m/z$  315 resulted in an ion at  $m/z$  153 because of the loss of a glucose moiety. Protocatechuic and gentisic acid differing only in their UV spectra characteristic (gentisic acid presents UV max at 330 nm while protocatechuic acid shows UV max at 260nm). Low UV intensity at 330 nm was let us to identify the compound 2 protocatechuic acid hexoside. Compound 14 showed pseudo molecular ion peak at  $m/z$  285 [M-H]<sup>-</sup> and identified as luteolin. Confirmation of compound 14 with authentic luteolin standard. Compound 6 and 7 presented an aglycon ion as luteolin. Compound 6 showed a molecular ion peak at  $m/z$  447 [M-H]<sup>-</sup> and its MS/MS spectrum presented deprotonated luteolin aglycon ion at  $m/z$  285 due to loss of a glucose unit (162 amu). Compound 7 presented a pseudo molecular ion peak at  $m/z$  461 which fragmented a product ion at  $m/z$  285 by the loss of a glucuronic acid moiety (-176 amu). So, the compound 6 and 7 were identified as luteolin glucoside and luteolin glucuronide respectively. In a similar way, compound 16 identified as apigenin and compound 8 and compound 9 were identified as apigenin glucoside and apigenin glucuronide respectively. Compound 11 and compound 12 was not fully identified but determined as apigenin derivative. Compound 10 was determined as rosmarinic acid with its molecular ion peak at  $m/z$  359 [M-H]<sup>-</sup> and further fragmentation was observed at  $m/z$  197 due to the elimination of a caffeoyl moiety. The confirmation of compound 10 was done using the standard rosmarinic acid. Apigenin glucoside, apigenin glucuronide, luteolin glucoside and 5-caffeoylquinic acid were determined as the major compounds of the extract.

Total phenol content was determined as 53.17 mg GAE/g extract. In different study *Nepeta* total phenol content was determined as 2.1- 23.1 mg GAE/g extract (Kraujalis, et al. 2011). We calculated the highest total phenol content similar to previously calculated in *N. catarica*, *N. bulgaricum*, *N. transcaucasia* (Kraujalis et al. 2011) and *N. praetervis* (Fareed et al. 2013).

The essential oil and the crude methanolic extract of *N. cilicica* were evaluated for their antibacterial and anticandidal properties for the first time here. The methanol extract found more active than essential oil against all tested bacteria species. Except *C. tropicalis* both essential oil and the methanol extract did not show remarkable effects pathogen *Candida* panel. According to all antibacterial results, both essential oil and the crude methanolic extract were slightly more active against Gram positive bacteria.

The study mainly focused on the bioactivity of the essential oil and total methanolic extract of *N. cilicica* used as food as well as medicine. Sesquiterpenes were found as major compounds of the essential oil where the caffeoylquinic acid, luteolin and apigenin derivatives were found in the extract. The essential oil showed

strong inhibitory effects especially against food pathogen bacteria *B. subtilis* and *Listeria monocytogenes*. The total extract has stronger activity against all tested bacteria species. With the remarkable antimicrobial properties and phenolic content, *N. cilicica* total extract may be considered as a potential food preservation additive after further analysis.

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