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Original article

Bioactive extracts of *Carum copticum* L. enhances efficacy of ciprofloxacin against MDR enteric bacteria

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ABSTRACT

The widespread occurrence of extended spectrum β-lactamases (ESβLs) producing enteric bacteria and their co-resistance with flouroquinolones has impaired the current antimicrobial therapy. This has prompted the search for new alternatives through synergistic approaches with herbal extracts. In this study *Carum copticum* (seeds) was extracted first in methanol and then subsequently extracted in different organic solvents. MIC of plant extracts, ciprofloxacin and thymol was determined by broth microdilution method using TTC. Synergism between plant extracts and ciprofloxacin was assayed by the checkerboard method. Chemical constituents of active extracts were analyzed by GC-MS. Methanolic, hexane and ether extract of *Carum copticum* exhibited significant antibacterial activity with MIC values ranged from 0.25 mg/ml to 2.0 mg/ml. Synergy analysis between *Carum copticum* extracts and ciprofloxacin combinations revealed FIC index in the range of 0.093–0.25. About 81% ciprofloxacin resistant ESβL producing enteric bacteria were re-sensitized in the presence of 15.6–250 μg/ml of methanolic extract of *Carum copticum*. Moreover, ciprofloxacin showed 8 to 64 folds reduction in MIC in presence of 250 and 500 μg/ml of hexane extract. Whereas, 4–32 folds reduction in MIC of ciprofloxacin was achieved in the presence of 31.25 and 62.5 μg/ml of ether extract, indicating synergistic enhancement of drug activity. The chemical analysis of hexane and ether extracts by GC-MS revealed the common occurrence of one or more phenolic hydroxyl at different locations on benzene ring. This study demonstrated the potential use of herbal extract of *Carum copticum* in combination therapy against ESβL producing bacteria.

1. Introduction

The emergence and spread of multidrug resistance among bacteria has created an immense clinical global problem and threat to human health. Extended spectrum β-lactamases (ESβLs) is one of the most influential cephalosporin resistance mechanisms among enterobacteriaceae. It is recognized that ESβL producing enteric bacteria harbour transferable plasmids which also confer resistance to other non β-lactam antibiotics, such as fluoroquinolones, aminoglycosides, and chloramphenicol etc. thereby positioning themselves as resistant to almost all available antibiotics (Brolund and Sandegren, 2016).

In the past few years, the growing co-existence of ESβL production and fluoroquinolone resistance has been documented worldwide and considered as serious public health challenge. Recently a global survey on antimicrobial resistance by world health organization has analyzed the data on resistance to third-generation cephalosporins, including resistance conferred by ESβLs, and to fluoroquinolones in *E. coli.* which has been reported higher resistance rates to fluoroquinolones than for the third-generation cephalosporins (WHO, 2014). Another report of SMART study in the Asia-Pacific region have shown greater incidence of fluoroquinolones resistance (ciprofloxacin 82.5% and levofloxacin 79.3%) among ESβL producers than resistance in non-ESβL producing isolates to those agents (31.2% and 28.6%, respectively) (Lu et al., 2012).

Abbreviations: **FIC**, fractional inhibitory concentration; **Ca-CIP**, *Carum copticum*-ciprofloxacin; **Th-CIP**, thymol-ciprofloxacin; **MDR**, multi-drug resistant; **ESβL**, extended spectrum β-lactamase; **PE**, plant extract; **TH**, thymol.

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Similarly, studies from India have also shown high prevalence of ciprofloxacin resistance among ESβL producers in clinical as well as environmental isolates (Tripathi et al., 2012; Maheshwari et al., 2016a, 2016b; Bajaj et al., 2016; Diwan et al., 2012). This is likely due to rise in demand for fluoroquinolones, particularly to treat potentially fatal infections (Wener et al., 2010). The worldwide spread and increasing prevalence of co-resistance to ciprofloxacin among ESβL producing enteric bacteria leave very limited therapeutic options. However, carbapenems seem to be the treatment of choice for infections caused by these resistant bacteria, on the long run, even new antibiotics are rendered ineffective, as microbes continue to acquire resistance due to indiscriminate use of antibiotics in clinical settings for the treatment of severe infectious diseases (Bell et al., 2014). There is also slow progress in the discovery of new antibiotic with novel mode of action. Thus, in the present scenario of antibiotic therapy, there is a continuing quest for the search of either new antimicrobials from other sources or bioactive compounds that potentiate and enhance the efficacy of existing antibiotics and can be used effectively in the treatment of these problematic bacterial infections (Coates et al., 2011; Worthington and Melander, 2013)

Plants are known to produce diverse bioactive substances of chemotherapeutic value. *Carum copticum* is a medicinal plant with promising ethnopharmacological properties. Biological activities of *Carum copticum* such as antimicrobial, antimutagenic, antioxidant, antispasmodic, bronchodilator and hepatoprotective properties have been documented in current literature (Gilani et at., 2005; Boskabady et al., 2014; Kazemi, 2015). In our previous reports, we have demonstrated the antioxidant and antimutagenic properties of this plant (Zahin et al., 2010). In this study, we have investigated synergistic antimicrobial activity of *Carum copticum* extracts with ciprofloxacin against ciprofloxacin resistant ESβL producing enteric bacteria. *In vitro* synergistic interaction is a more effective substitute for existing antibiotic treatment strategies that have become ineffective due to increasing prevalence and co-existence of ciprofloxacin resistance among ESβL producing enteric bacteria.

2. Materials and methods

2.1. Collection of plant material and active ingredient

Plant sample (seeds) was purchased at a local vendor in January 2015 and taxonomic identification of the plant material was confirmed by Professor Shamsul Hayat, Department of Botany, AMU, Aligarh (India). The voucher specimen has been currently archived in the Department of Agricultural Microbiology, Faculty of Agricultural Sciences, AMU, Aligarh, India with acquisition code number [CCM Ag./2015-01]. The active known gradient of the plant, thymol (purity min. 99%) (Refractive index 1.5204 at 25 °C) was purchased from Hi-Media Laboratory Ltd, Mumbai, India.

The organic solvents used in this study were purchased from Thermo fisher scientific India Pvt. Ltd., Mumbai, India.

2.2. Preparation of plant extracts

Shade dried seeds of *Carum copticum* (500 gm) were powdered and extracted with 2.5 l of methanol (purity ≥ 99.8%) as described previously (Ahmad et al., 1998). The methanolic extract (yield:10.58%, w/w) was concentrated to dryness under reduced pressure, dissolved in hot distilled water and successively extracted with *n*-hexane (purity ≥ 98.5%), ether (purity 99%), ethyl acetate (purity ≥ 99.5%) and chloroform (purity ≥ 99.8%) as described by Jamil et al., (2012). Each extract was dried under sodium sulphate, filtered and reduced under vacuum to give: hexane extract (yield: 6.54%, w/w), ether extract (yield: 0.62%, w/w), ethyl acetate extract (yield: 1.52%, w/w), and chloroform extract (yield: 0.2%, w/w). The obtained extracts were stored in a refrigerator at +4 °C until use. The dried extracts were reconstituted in 1% DMSO (purity ≥ 99.7%) to prepare stock solutions.

2.3. Bacterial strains

A total of twenty-four enteric bacteria used in this study were previously characterized for their antibiotic susceptibility and ESβL production (Maheshwari et al., 2016a, 2016b). *E. coli* ATCC 25,922 and *K. pneumoniae* ATCC 700,603 were used as reference strains. All the strains were stored at −70 °C in Luria bertani broth (Hi-Media Laboratories Pvt. Ltd., Mumbai, India) containing 40% (w/v) glycerol until use.

2.4. Determination of MIC and MBC

The minimal inhibitory concentration (MIC) of plant extracts and thymol against bacterial strains was determined by broth microdilution susceptibility testing method (Eloff, 1998) using 2,3,5-triphenyltetrazohum chloride [TTC, tetrazolium red, purity min. 99%, SRL Pvt. Ltd. Mumbai, India] dye as a growth indicator. The modification for assessment of plant extracts and essential oil activity in all tests was made by incorporating a final concentration of ≤1.0% (v/v) DMSO into the broth medium to enhance plant extracts and thymol solubility. The concentrations of plant extract were two-fold serial dilutions ranging from 0.0125 to 4 mg/ml and for thymol two-fold serial dilutions ranging from 0.062 to 0.8 mg/ml in a sterile 96-well polystyrene microtitre plates (Axiva Sichem Biotech, Delhi, India). 100 µl of bacterial inoculum (1×10^6 cfu/ml) were added to each well. The covered microtitre plates were incubated for 16 h at 37 °C. To indicate bacterial growth, 40 µl of TTC dissolved in water (2mg/ml w/v) were added to each well and incubated at 37 °C for 30 min. For the confirmation of minimal bactericidal concentration (MBC), 10 µl aliquot from wells was cultured on nutrient agar plates to determine the inhibition of bacterial growth. Each experiment was performed in triplicate and three independent experiments, with *Escherichia coli* ATCC 25,922 and *K. pneumoniae* ATCC 700,603 were used as reference strains.

2.5. Determination of synergistic interaction

2.5.1. Broth microdilution checkerboard method

Synergism between plant extracts and ciprofloxacin (Hi-Media Laboratories Pvt. Ltd., Mumbai, India) was determined by the checkerboard method using sterile 96-well polystyrene microtitre plates (Wagner and Ulrich-Merzenich, 2009). The range of concentrations was determined according to the previously assessed MIC of ciprofloxacin and plant extracts (PE) or thymol (TH) for each of the test isolate. The concentrations of plant extract or thymol and antibiotics were prepared in range $1/4 \times MIC_{PE/TH}$ to $4 \times MIC_{PE/TH}$ and $1/4 \times MIC_{CIP}$ to $4 \times MIC_{CIP}$, respectively. Each well was inoculated with a 100 µl of bacterial inoculum of 1×10^6 cfu/ml. The plates were then incubated at 37 °C for 18 h. An attempt to re-sensitize the bacterial isolates was made by combining antibiotics with plant extract at the above-mentioned concentrations. The synergy method was combined with calculation of fractional inhibitory concentration indexes (FICI) to assess the antimicrobial effects of plant extract/essential oil and antibiotic combinations. The FICI was calculated as $FIC_{PE/EO} + FIC_{AB}$, where $FIC_{PE/EO} = MIC_{PE/EO}$ of the combination/$MIC_{PE/EO}$ alone, and $FIC_{AB} = MIC_{AB}$ of the combination/MIC_{AB} alone. The results were interpreted as a synergistic effect if FICI < 0.5; as an additive or indifferent effect if $0.5 \leq$ FICI ≤ 4 and as an antagonistic effect if FICI > 4 (EUCAST, 2000). All

experiments carried out in triplicate as two independent experiments.

2.5.2. Time kill assay

The effectiveness of the plant extract/essential oil and ciprofloxacin combinations against *E coli* (ECMA2) and *Enterobacter cloacae* (ENM32) was determined by the time-kill curve assay (Verma, 2007) to confirm results obtained by broth microdilution checkerboard method. Changes in bacterial count during incubation period were monitored parallel in four test tubes containing the following: (1) only bacteria (approximately 10^8 CFU ml^{-1}); (2) bacteria and a sub-MIC concentration of antibiotics that showed synergistic effect in combination with PE/TH; (3) bacteria and sub-MIC concentration of plant extract/essential oil that showed synergistic effect in combination with antibiotic; and (4) bacteria, a sub-MIC concentration of antibiotics and plant extract/essential oils. Final volume of each tube was 10 ml and they were incubated at 37 °C for 24 h. The bacterial viable counts were determined after 0, 2, 4, 8, 12 and 24 h of incubation by spreading appropriate dilutions on Muller Hinton agar (Hi Media Laboratories Pvt. Ltd, Mumbai, India). The plates were incubated at 37 °C overnight and bacterial colonies were counted. Procedure was carried out in triplicate as two independent experiments. The results were averaged and expressed as logarithms with corresponding standard errors (me an ± SE). The interaction was considered to be effective and synergistic if the starting bacterial count (cfu/ml) decreased by ≥2 log after 24 h of incubation for the antibiotic-plant extract/thymol combination in comparison to the more active single agent (plant extract/thymol or antibiotic) (Knezevic et al., 2013).

2.6. Gas chromatography-mass spectrometry (GC-MS) analysis of plant extracts

Chemical constituents of hexane and ether extract of *Carum copticum* diluted with methanol (10 µl/ml) were analyzed by Gas chromatography-mass spectrometry analysis (Instrument model GCD 1800A, Hewlett Packard). The sample was injected into a split inlet at 260 °C, with a split ratio 1:10. Helium (purity 99.999%) was used as a carrier, with a constant flow of 1.21 ml/min. The separation was achieved on a 30 m × 0.25 mm × 0.25 m HP-1 column (Thermo scientific), using the following temperature program: start at 70 °C and hold for 2 min, 5 °C/min to 250 °C and hold for 2 min, 10 °C/min to 280 °C and hold for 17.0 min (total run time 50 min). Elute was delivered to the mass spectrometer with Ion source temperature 230 °C and interface temperature 270 °C. Data was acquired in Scan mode (m/z range 40–650). The compounds were identified by mass spectra comparison with libraries (Wiley Registry of Mass Spectral Data 7th ed. (McLafferty, 2005), and NIST/EPA/NIH Mass Spectral Library 05 (NIST/EPA/NIH, 2005). Relative amounts of components, expressed in percentages, were calculated by normalization measurement according to peak area in total chromatogram.

3. Results

A total of twenty-four ESβL producing MDR enteric bacteria including *E. coli, Enterobacter cloacae* and *Klebsiella pneumoniae* were used in this study as depicted in Table 1. In order to investigate antibacterial efficacy of *Carum copticum* and thymol, MIC and MBC values were determined against twenty-four ESβL producing enteric bacteria. Methanolic extract of *Carum copticum* showed considerable antibacterial activity against ESβL producing bacterial strains with MIC values ranged from 0.25 mg/ml to 2.0 mg/ml (Table 2). Moreover, thymol was effective against ESβL producing enteric bacteria with MIC in the range from 0.05 mg/ml to 0.2 mg/ml (Table 2). Further, antibacterial activity of fractionated methanolic extracts of *Carum copticum* in hexane, ether, ethyl acetate, and chloroform were tested against selected ESβL producing bacterial strains. As presented in Table 3, antibacterial potency (MIC values) of the extracts was in order of hexane extract > ether extract > ethyl acetate extract followed by chloroform extract against ESβL producing strains. Hexane extract showed promising antibacterial activity against these strains with MIC in the range from 0.25 mg/ml to 0.5 mg/ml while MIC values of ether extract was found to be in the range of 0.25 mg/ml to 1.0 mg/ml. Ethyl acetate extract exhibited moderate activity with MIC range between 1.0 mg/ml to 2.0 mg/ml. Whereas, chloroform extract showed little or no activity against these isolates with MIC > 4 mg/ml.

Table 1
Antibiotic resistance profile of ESβL producing enteric bacteria used in this study against twenty four tested antibiotics.

Strain designation	Bacterial identification	Antibiotic resistance profile	
		β-lactams	Non β-lactams
ENM36	*Enterobacter cloacae*	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	CIP,NX,TE,DO,CO,CLM, RIF,NA
ENM32	*E. cloacae*	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	CIP,NX,TE,AZM,E,CO, CLM,RIF, NA
ECMA2	*E. coli* O97	AMX,CAZ,CTX,CX, CXM,CTR,CPD,AT	CIP,NX,NIT,RIF,NA
ECM49	*E. coli* O145	AMX,CAZ,CTX,CX, CXM,CTR,CPD,AT	CIP,NX,TE,DO,E,CO,RIF, NA
ECM4	*E. coli* rough	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	CIP,NX,TE,DO,E,CO,RIF, NA
ECMW9	*E. coli* rough	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	HLG,AZM,E,CO,NIT, CLM,RIF,NA
ECMW6	*E. coli* O2	AMX,CTX,CX,CXM, CTR,CPD,CPM,AT	CIP,NX,TE,DO, CO,RIF, NA
ECMW30	*E. coli* rough	AMX,CTX,CX,CXM, CTR,CPD,CPM,AT	CIP,NX,TE,DO,E,CO, NIT,CLM,RIF,NA
ECM8	*E. coli* rough	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	CIP,TE,DO,CO,CLM, NIT,RIF,NA
ECMW31	*E. coli* O147	AMX,CTX,CX,CXM, CTR,CPD,AT	CIP,NX,TE,DO, RIF,NA
ECM18	*E. coli* O2	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	CIP,NX,TE,DO,E,CO, CLM,RIF,NA
ECM16	*E. coli* O2	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	CIP,NX,TE,DO,E,CO,RIF, NA
ECMW21	*E. coli* rough	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	NIT,CO, CLM,RIF,NA
ECMW41	*E. coli* O86	AMX,CAZ,CTX,CX, CXM,CTR,CPD,AT	CIP,NX,TE,DO, AZM, CO,RIF,NA
ECMW5	*E. coli* O97	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	CIP,NX,E, CO,RIF,NA
ECMA20	*E. coli* UT	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	CIP,NX,TE,DO,E,CO, CLM,RIF,NA
KPMA19	*K. pneumoniae*	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	E,RIF,NA
KPM27	*K. pneumoniae*	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	TE,DO,E, CO,CLM,RIF, NA
KPMA9	*K. pneumoniae*	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	TE,DO,E,RIF,NA
KPMS1	*K. pneumoniae*	AMX,CAZ,CTX,CX, CXM,CTR,CPD,AT	TE,DO,E,CLM, RIF,NA
KPMA14	*K. pneumoniae*	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	TE,DO,E, AZM,RIF,NA
KPMA17	*K. pneumoniae*	AMX,CAZ,CTX,CX, CXM,CTR,CPD,AT	TE,DO,E, RIF,NA
KPMEA17	*K. pneumoniae*	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	HLG,CIP,NX,TE,DO,E, CO,NIT,CLM,RIF,NA
KPM3	*K. pneumoniae*	AMX,CAZ,CTX,CX, CXM,CTR,CPD,CPM,AT	CIP,NX,TE,DO,E,CO, NIT,CLM, RIF,NA

AMX-Amoxycillin;CX-Cefoxitin;CXM-Cefuroxime;CTR-Ceftriaxone;CTX-Cefotaxime; CAZCeftazidime;CPD Cefpodaxime;CPM-Cefepime;AZ-Aztreonam;MPM-Meropenem; IMP-Imipenem;TC-Tetracycline;DO-Doxycycline;CIP-Ciprofloxacin;NX-Norfloxacin; E-Erythromycin;AZM-Azithromycin;NIT-Nitrofurantoin;CLM-Chloramphenicol;RIF-Rifampicin;NA-Nalidixicacid;COT-trimethoprim/sulphamethaxazole;HLG Gentamicin.

Table 2
Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of methanolic extract of *Carum copticum* and thymol against ESβL producing bacterial strains.

Bacterial isolates	Methanolic extract of *Carum copticum*		Thymol	
	MIC[a]	MBC[b]	MIC	MBC
ENM32	1	1	0.1	0.1
ENM36	1	2	0.1	0.2
ECMA20	2	2	0.2	0.4
ECMW30	1	2	0.1	0.2
ECMW6	1	2	0.2	0.4
ECM W31	2	2	0.2	0.2
ECM18	1	2	0.2	0.4
ECM16	0.5	1	0.1	0.2
ECMA2	1	2	0.2	0.2
ECMW41	0.5	1	0.2	0.2
ECM 4	1	2	0.2	0.4
ECM 8	1	1	0.2	0.2
ECMW9	1	2	0.1	0.2
ECM49	1	2	0.1	0.2
ECMW21	1	1	0.2	0.2
ECMW5	0.25	0.5	0.05	0.1
KP MA19	0.5	1	0.1	0.2
KPM27	0.25	0.5	0.05	0.1
KPM3	0.5	1	0.2	0.2
KPMA9	0.5	1	0.2	0.4
KPM S1	1	1	0.2	0.2
KPMA14	0.5	0.5	0.1	0.2
KPMA17	0.5	1	0.1	0.2
KPMEA17	1	1	0.2	0.2
ATCC25922	0.125	0.125	0.025	0.025
ATCC700603	1	2	0.2	0.2

[a] MIC minimum inhibitory concentration, values given as mg/ml.
[b] MBC minimum bactericidal concentration, values given as mg/ml.

Primarily, synergistic interactions of methanolic extract of *Carum copticum* with ciprofloxacin against sixteen ciprofloxacin-resistant ESβL producing enteric bacteria were evaluated *in vitro* according to the calculated FIC indexes (FICI) from checkerboard analyses. The results in Table 4 showed that the synergistic activity was detected against all the tested enteric bacteria. However, varying level of synergy was observed with FICI ranged from 0.093 to 0.25. Among enteric bacteria, the synergistic interaction of methanolic extract of *Carum copticum* with ciprofloxacin was found to be most effective against *E. coli* isolates (FICI ranged from 0.093 to 0.250) in which MIC of ciprofloxacin was reduced in the range from 1/8 × MIC to 1/64 × MIC in the presence of 15.6 to 250 μg/ml of methanolic extract of *Carum copticum*. For *Enterobacter cloacae* isolates (ENM32 and ENM36) MIC of ciprofloxacin in the combination was reduced to 1/8 × MIC and 1/32 × MIC respectively in the presence of 125 μg/ml of methanolic extract of *Carum copticum*. Similarly, in case of *Klebsiella pneumoniae* isolates, MIC for ciprofloxacin was reduced to 1/16 × MIC in the presence of 62.5 and 125 μg/ml of methanolic extract of *Carum copticum* (Table 4). Whereas, 16 to 128 folds reduction in MIC of ciprofloxacin was achieved in presence of 12.5 to 50 μg/ml MIC of thymol (Table 5).

As per CLSI guidelines for antibiotic susceptibility (Clinical and Laboratory Standards Institute, 2014), thirteen ciprofloxacin resistant ESβL producing enteric bacteria turned out to be ciprofloxacin sensitive in the presence of 15.6 to 250 μg/ml of methanolic extract of *Carum copticum* indicating synergistic enhancement of drug activity.

The effect of combination of methanolic extract of *Carum copticum* and ciprofloxacin sub-inhibitory concentrations against ciprofloxacin-resistant ESβL producing enteric bacteria is shown by time kill curve (Fig. 1). For Ca-CIP combination against ECMA2 (*E coli*:O97), the reduction in bacterial cell count was ~ 3 log as compared to initial count of bacterial cell for the first 4 h. Moreover, no growth was found at 12 h of incubation. This is 7 log greater reduction rates as compared to the administration of ciprofloxacin alone at the same sub-MIC concentration (Fig. 1**A**). Whereas in case of Th-CIP combination, there was sharp decrease in the number of viable cell with 5 log reduction as compared to initial count of bacterial cell for the first 4 h. However, in the next 4 h, the count started to increase from 3.2 to 6 log and for the twelfth hour of incubation, the cell count was reduced again and final bacterial count was under 3 log after twenty four hour of incubation (Fig. 1**B**). Against *Enterobacter cloacae* strain ENM32, For Ca-CIP combination, the reduction in viable cell count was greater than 2 log as compared to its initial count for the first 2 h of incubation. However, in the next 2 h, the count started to increase from 5.75 to 6.0 log. After 4 h incubation, the viable cell count reduced again and at 8 h and 12 h incubation, the reduction in viable cell was greater than 4 and 5 log respectively as compared to bacterial initial count. Final viable cell count was observed to be under 2 log after 24 h incubation (Fig. 1**C**). Similarly, For Th-CIP combination, initial reduction in viable cell count was 1.5 log as compared to its initial count for the first 2 h of incubation. However, in the next 2 h, the viable cell count was slightly increased from 6.7 to 7.17 log. In the next 4 h incubation, there was sharp decrease in viable cell count from 7.17 to 4.62 log reduction. After that, gradual decrease in viable cell was observed and final bacterial cell count was under 4 log (Fig. 1**D**).

Further, different extracts of *Carum copticum* were evaluated for its synergy with ciprofloxacin against selected ciprofloxacin-resistant ESβL producing enteric bacteria including *E. coli* and *Enterobacter cloacae*. Among them, hexane extract, ether extract showed synergistic interaction with ciprofloxacin against these strains. Reduction in MICs of ciprofloxacin with hexane extract,

Table 3
Antibacterial activity of different extracts of *Carum copticum* against selected ESβL producing bacterial strains.

Bacterial strains	Methanol extract		Hexane extract		Ether extract		Ethyl acetate extract		Chloroform extract	
	MIC[a]	MBC[b]	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
ENM32	1	1	0.25	0.25	0.5	1	1	2	>4	>4
ENM36	1	2	0.25	0.5	1	1	2	4	>4	>4
ECMA2	1	2	0.5	1	0.25	0.5	1	1	>4	>4
ECMW9	1	2	0.5	1	0.25	1	1	2	>4	>4
ECM4	1	2	0.5	0.5	0.25	0.5	1	1	>4	>4
ECM49	1	2	0.25	0.5	0.5	1	2	4	>4	>4
KPMA19	0.5	1	0.25	0.5	0.5	1	1	2	>4	>4
ATCC25922	0.5	1	0.125	0.25	0.25	0.25	2	2	>4	>4
ATCC700603	1	2	0.5	1	0.5	1	2	4	>4	>4

[a] MIC minimum inhibitory concentration, values given as mg/ml,
[b] MBC minimum bactericidal concentration, values given as mg/ml.

Table 4
Synergistic interaction of *Carum copticum* with ciprofloxacin against ciprofloxacin-resistant ESβL producing enteric bacteria.

Bacterial isolates	Methanolic extract of *Carum copticum*			Ciprofloxacin			FICI
	MIC_A[a]	MIC_C[b]	FIC	MIC_A	MIC_C	FIC	
ENM32	1000	125	0.125	64	2	0.031	0.156
ENM36	1000	125	0.125	8	1	0.125	0.250
ECMA2	1000	125	0.125	32	2	0.062	0.187
ECMA20	2000	250	0.125	128	2	0.015	0.140
ECM4	1000	62.5	0.062	16	0.5	0.031	0.093
ECM16	500	62.5	0.125	64	2	0.031	0.156
ECMW30	1000	250	0.25	128	8	0.062	0.312
ECM18	1000	125	0.125	128	2	0.015	0.140
ECMW41	500	31.25	0.062	128	4	0.031	0.093
ECMW5	250	15.6	0.062	4	0.125	0.031	0.093
ECM49	1000	125	0.125	64	1	0.015	0.140
ECMW6	1000	125	0.125	128	2	0.015	0.140
ECM8	1000	125	0.125	128	2	0.015	0.140
ECMW31	2000	250	0.125	64	8	0.125	0.250
KPM3	500	62.5	0.125	8	0.5	0.062	0.187
KPMEA17	1000	125	0.125	32	2	0.062	0.187

[a] MIC_A Minimum inhibitory concentration (μg/mL) of agent alone.
[b] MIC_c Minimum inhibitory concentration (μg/mL) of agent in combination.

Table 5
Synergistic interaction of most active extracts of *Carum copticum* with ciprofloxacin against selected ciprofloxacin-resistant ESβL producing enteric bacteria.

Bacterial isolates	Hexane extract		Ciprofloxacin		FICI	Ether extract		Ciprofloxacin		FICI	Thymol		Ciprofloxacin		FICI
	MIC_A[a]	MIC_C[b]	MIC_A	MIC_C		MIC_A	MIC_C	MIC_A	MIC_C		MIC_A	MIC_C	MIC_A	MIC_C	
ENM32	250	62.5	64	2	0.281	500	62.5	64	2	0.156	100	25	64	2	0.281
ENM36	250	62.5	8	1	0.375	1000	31.25	8	2	0.281	100	25	8	0.5	0.312
ECMA2	500	125	32	0.5	0.265	250	31.25	32	2	0.187	200	50	32	0.25	0.257
ECM49	250	62.5	64	2	0.281	500	62.5	64	2	0.156	100	12.5	64	4	0.187
ECM4	500	62.5	16	0.5	0.156	250	31.25	16	1	0.187	200	25	16	0.25	0.140

[a] MIC_A Minimum inhibitory concentration (μg/mL) of agent alone.
[b] MIC_C Minimum inhibitory concentration (μg/mL) of agent in combination.

ether extract is summarized in Table 5. Ciprofloxacin showed 8 to 64 folds reduction in MIC in presence of 250 and 500 μg/ml of hexane extract. Whereas, in case of ether extract and ciprofloxacin combination, 4 to 32 folds reduction in MIC of ciprofloxacin was achieved in the presence of 31.25 and 62.5 μg/ml of ether extract.

In order to identify major components of the most active extracts of *Carum copticum*, hexane and ether extract were subjected to Gas chromatography–mass spectrometry analysis (Supplementary Figs S1 and S2). The results obtained by GC-MS analysis are listed in Table 6. Major ingredients of hexane extract as revealed by GC-MS analysis is thymol (84.46%). While in case of ether extract, the major components as identified by GC-MS analysis were 3-Methoxy-2, 4, 6-trimethylphenol (48.85%), Benzene-1,4-diol (10.11%), 4-tert-butyl-Pyrocatechol (6.84%), 2,3, 5,6-Tetramethylhydroquinone (3.36%). The structural analysis of the major components as identified by GC-MS analysis revealed the common occurrence of one or more phenolic hydroxyl at different locations on benzene ring (Fig. 2) indicated the role of free hydroxyl group with delocalized electrons in the synergistic antimicrobial activity of *Carum copticum.*

4. Discussion

The emergence of ciprofloxacin resistance among ESβL producing enteric bacteria has become a serious concern to public health and infection control strategies as they restrict the use of fluoroquinolones for the treatment of fatal infectious diseases caused by these problematic bacteria. The present global scenario of multiple antibiotic resistances has led to the investigation of new combinations and possible alternative treatment strategies. Medicinal plant extracts and phytocompounds have been considered as potential source of such antimicrobial agents. In this study, *Carum copticum* has been analysed to explore its efficacy against MDR ESβL producing enteric bacteria with special reference to its synergy with ciprofloxacin. In order to identify the contribution of active known component of this plant in the synergistic antimicrobial potential of *Carum copticum*, thymol has also been tested for its efficacy against these isolates. Our study clearly indicated overall high potency (in terms of mean MIC values) by methanolic extract of *Carum copticum* and thymol irrespective of the drug resistance pattern of the test bacteria. The antimicrobial activity of *Carum copticum* and thymol has also been reported in other studies (Boskabady et al., 2014; Jafarpour et al., 2013; Xu et al., 2008). As methanolic extract of *Carum copticum* exhibited promising antibacterial activity, bio-guided fractionation of this extract using organic solvent system was done and it was shown that hexane extract and ether extract exhibited considerably significant antibacterial activity with no remarkable differences.

A GC-MS analysis of hexane and ether extract reflected the presence of a number of low molecular weight phenolic compounds. Structural analysis of these phenolic components revealed the presence of one or more hydroxyl groups at different locations on the phenolic ring. The presence of free hydroxyl groups and a system of delocalized electrons is essential for antimicrobial activity of different components of plant extracts as it plays an important role to depolarize membrane potential (Ultee et al., 2002).

As *Carum copticum* was proven efficient in antimicrobial analysis, we further expanded this study to explore its synergistic effect with ciprofloxacin against ciprofloxacin resistant strains. To the best of our knowledge, the present study is the first report on *Carum copticum* seed extracts inducing synergic enhancement of the efficacy of a ciprofloxacin drug against ESβL producing enteric bacterial isolates. We found a marked reduction in MIC of

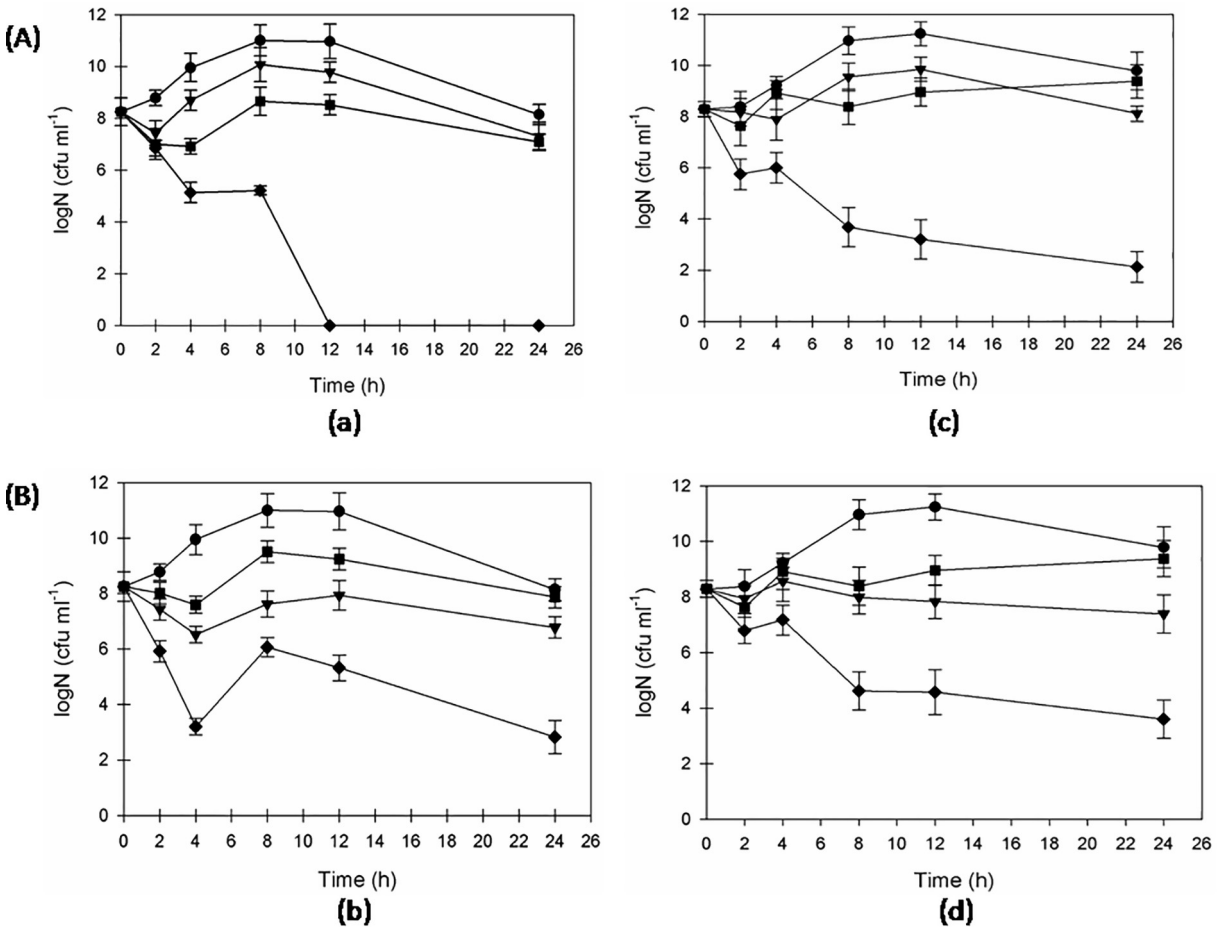

Fig. 1. (**A**) Time kill curve showing synergistic interaction of *Carum copticum* and ciprofloxacin against (a) *E coli*:O97 (ECMA2) (c) *Enterobacter cloacae* ENM32 **(B)** Time kill curve showing synergistic interaction of Thymol and ciprofloxacin against (b) *E coli*:O97 (ECMA2) (d) *Enterobacter cloacae* ENM32; ●() Bacteria without treatment; (■) Ciprofloxacin; (▲) *Carum copticum* or Thymol; (◆) Ca-CIP or Th-CIP combination.

Table 6
Major components of most active extracts of *Carum copticum* as identified by Gas chromatography–mass spectrometry.

Peak no.	Retention time	Area%	Components	Chemical class
Hexane extract				
11	14.63	84.46	Thymol	Monoterpenes
24	33.40	7.11	cis-Vaccenic acid	Fatty acid
23	33.24	2.20	cis-linoleic acid methyl ester	Fatty acid
19	29.95	1.53	Pentadecanoic acid	Fatty acid
Ether extract				
63	43.675	48.85	3-Methoxy-2,4,6-trimethylphenol	phenol
32	14.31	10.11	Benzene-1,4-diol (Hydroquinone)	phenol
48	21.36	6.84	4-tert-butyl-Pyrocatechol	Phenol
64	44.49	3.36	2,3,5,6-Tetramethylhydroquinone	Phenol
46	19.86	2.38	(2E)-5-Hydroxy-3,4,4-trimethyl-2-hexenoic acid	Carboxylic Acid
61	37.91	2.23	5-Isopropyl-2-methylphenyl acetate (Carvacrol acetate)	Monoterpenes
33	14.52	2.12	Thymol	Monoterpenes
6	7.65	1.85	3-hydroxy-4,4-dimethyldihydro-2(3h)-furanone	Furanes
35	15.11	1.70	4a-methyldecahydro-1-naphthalenyl acetate	Ester
26	12.54	1.60	3-(2 Hydroxyphenyl) acrylic acid	Coumaric acid
45	19.14	1.39	cis-.beta.-Terpineol	Monoterpenes
60	33.32	1.35	cis-Vaccenic acid	Fatty acid

ciprofloxacin in presence of *Carum copticum* and its extracts. Even, in most of the cases, MIC was reduced to below the breakpoints for this antibiotic. This implies that the combination increases bacterial sensitivity to ciprofloxacin i.e. reduced the effective antibiotic concentration and reflect a significant synergistic interaction between *Carum copticum* bioactive extracts and ciprofloxacin. Many studies have confirmed the synergistic action between fluoroquinolones and different plant extracts (Dey et al., 2012; Rosato et al., 2007). Moreover, ether extract of *Carum copticum* showed most promising synergistic antimicrobial activity than thymol and hexane extract respectively. The presence of different kind of phenolic compounds in methanolic and ether extract of *Carum copticum* can promote their synergistic effect and results in a greater synergy with ciprofloxacin than hexane extract.

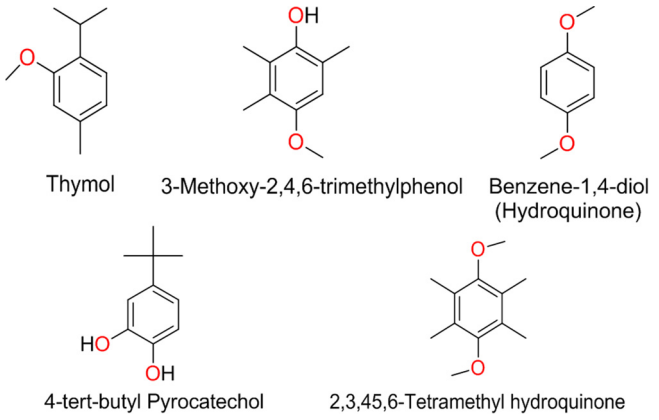

Fig. 2. Chemical structures of the major components of hexane and ether extract of *Carum copticum* identified by GC-MS analysis.

Whereas, synergistic interaction of hexane extract was shown to exhibit due to the presence of thymol. Most of the researches have shown that whole plant extracts or combinations of compounds are more effective antimicrobials than isolated constituents (Lee and Lee, 2010; Garvey et al., 2011; Ncube et al., 2012; Wang et al., 2014), which are accordance with the results of the present study.

In order to examine the antibacterial effect of combinations during time, two ciprofloxacin resistant isolate *E coli* (ECMA2) and *E. cloacae* (ENM32) were selected for time kill curve analysis. The obtained results of antibacterial effect of combinations revealed that this combination of agents resulted very efficient synergy against ciprofloxacin resistant *E coli* isolate, completely reducing cell count after 12 h of incubation. In the scenario when the same concentration of ciprofloxacin was administered alone, no significant reduction in viable count of *E. coil* was observed. Whereas, in case of *E. cloacae* strain ENM32, when *Carum copticum* has been combined with ciprofloxacin, a sharp reduction in bacterial count after 2 h incubation followed by the temporary regrowth after 4 h incubation and final significant bacterial count reduction have been observed after 8 h onwards and no significant reduction of viable bacterial counts have been found on the same sub-MIC concentration of ciprofloxacin. These cases support this work regarding synergism of *Carum copticum* and ciprofloxacin. Similar synergistic pattern has been exhibited by thymol-ciprofloxacin combination for both of the bacterial isolates. However, methanolic extract of *Carum copticum* has shown more efficient synergism with ciprofloxacin in terms of reduction of bacterial viable count.

The mechanisms of synergistic interactions of antimicrobial agents are not fully explored in current literature. However, chemical complexity of herbal extracts and multi-target nature of herbal extract-antibiotic combinations could enhance its therapeutic potential (Hemaiswarya et al., 2008; Bone and Mills, 2013). Ciprofloxacin inhibits DNA replication targeting DNA gyrase (Zhao et al., 1997) and *Carum copticum* is responsible for the loss in membrane integrity, increase membrane permeability results the leakage of protons and potassium which finally leads to the loss of membrane potential of bacteria and affecting cell envelopes as first barrier for antibiotics (Xu et al., 2008). Thus, *Carum copticum* due to its active phytoconstituents and thymol probably provides the open path for ciprofloxacin; facilitate penetration and activity of this antibiotic.

5. Conclusion

The results of synergistic action of *Carum copticum* with ciprofloxacin demonstrate the potential use of *Carum copticum* to enhance ciprofloxacin action especially against ciprofloxacin resistant enteric bacteria. This synergy reduced ciprofloxacin minimum efficient dose and thus can minimize antibiotic side effects or prevent the emergence of ciprofloxacin resistance among ESβL producing enteric bacteria. Further, extraction and purification of active compounds from bioactive extracts is needed to explore the exact synergistic mechanisms and its possible therapeutic potential in combination therapy.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.sjbs.2017.12.008.

References

Ahmad, I., Mehmood, Z., Mohammad, F., 1998. Screening of some Indian medicinal plants for their antimicrobial properties. J. Ethnopharmacol. 62, 183–193.
Bajaj, P., Kanaujia, P.K., Singh, N.S., Sharma, S., Kumar, S., Virdi, J.S., 2016. Quinolone co-resistance in ESBL or AmpC-producing *Escherichia coli* from an Indian urban aquatic environment and their public health implications. Environ. Sci. Pollut. Res. 23 (2), 1954–1959.
Bell, B.G., Schellevis, F., Stobberingh, E., Goossens, H., Pringle, M., 2014. A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. BMC Infect. Dis. 14 (1), 13.
Bone, K., Mills, S., 2013. Principles and practice of phytotherapy: Modern Herbal Medicine. Churchill Livingston Elsevier, London.
Boskabady, M.H., Alitaneh, S., Alavinezhad, A., 2014. *Carum copticum* L.: a herbal medicine with various pharmacological effects. Bio. Med. Res. Int. 2014, 1–11.
Brolund, A., Sandegren, L., 2016. Characterization of ESβL disseminating plasmids. J. Infect. Dis. 48 (1), 18–25.
Clinical and Laboratory Standards Institute, 2014. Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement. Document M100-S24. Wayne, PA: CLSI.
Coates, A.R., Halls, G., Hu, Y., 2011. Novel classes of antibiotics or more of the same? Brit. J. Pharmacol. 163 (1), 184–194.
Dey, D., Debnath, S., Hazra, S., Ghosh, S., Ray, R., Hazra, B., 2012. Pomegranate pericarp extract enhances the antibacterial activity of ciprofloxacin against extended-spectrum b-lactamase (ESBL) and metallo-b-lactamase (MBL) producing Gram-negative bacilli. Food Chem. Toxicol. 50, 4302–4309.
Diwan, V., Chandran, S.P., Tamhankar, A.J., 2012. Identification of extended-spectrum -lactamase and quinolone resistance genes in *Escherichia coli* isolated from hospital wastewater from central India. J. Antimicrob. Chemother. 67, 857–859.
Eloff, J.N., 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med. 64, 711–713.
Eucast definitive document E, 2000. Def 1.2. Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID).
Garvey, M.I., Rahman, M.M., Gibbons, S., Piddock, L.J., 2011. Medicinal plant extracts with efflux inhibitory activity against Gram-negative bacteria. Int. J. Antimicrob. Agents 37, 145–151.
Gilani, H., Jabeen, Q., Ghayur, M., Janbaz, K., Akhtar, M., 2005. Studies on the antihypertensive, antispasmodic, bronchodilator and hepatoprotective activities of the *Carum copticum* seed extract. J. Ethnopharmacol.. 98, 127–135.
Hemaiswarya, S., Kruthiventi, A.K., Doble, M., 2008. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine 15, 639–652.
Jafarpour, M., Golparvar, A.R., Lotfi, A., 2013. Antibacterial activity of essential oils from *Thymus vulgaris*, *Trachyspermum ammi* and *Mentha aquatica* against *Erwinia carotovora in vitro*. J. Herbal Drugs (Int. J. Med. Herbs). 4 (3), 115–118.
Jamil, M., Haq, I., Mirza, B., Qayyum, M., 2012. Isolation of antibacterial compounds from *Quercus dilatata* L. through bioassay guided fractionation. Ann. Clin. Microbiol. Antimicrob. 11, 1–11.
Kazemi, M., 2015. Chemical composition and antimicrobial, antioxidant activities and anti-inflammatory potential of *Achillea millefolium* L., *Anethum graveolens* L., and *Carum copticum* L. essential oils. J. Herb Med. 5 (4), 217–222.

Knezevic, P., Curcin, S., Aleksic, V., Petrusic, M., Vlaski, L., 2013. Phage antibiotic synergism: a possible approach to combating *Pseudomonas aeruginosa*. Res. Microbiol. 164, 55–60.

Lee, O.H., Lee, B.Y., 2010. Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract. Bioresour. Technol. 101, 3751–3754.

Lu, P.L., Liu, Y.C., Toh, H.S., Lee, Y.L., Liu, Y.M., Ho, C.M., Huang, C.C., Liu, C.E., Ko, W.C., Wang, J.H., 2012. Tang HJ. Epidemiology and antimicrobial susceptibility profiles of Gram-negative bacteria causing urinary tract infections in the Asia-Pacific region: 2009–2010 results from the Study for Monitoring Antimicrobial Resistance Trends (SMART). Int. J. Antimicrob. Agents 40S1, S37–S43.

Maheshwari, M., Ahmad, I., Althubiani, A.S., 2016a. Multidrug resistance and transferability of bla CTX-M among extended-spectrum β-lactamase-producing enteric bacteria in biofilm. J. Glob. Antimicrob. Resist. 6, 142–149.

Maheshwari, M., Yaser, N.H., Naz, S., Fatima, M., Ahmad, I., 2016b. Emergence of ciprofloxacin-resistant extended-spectrum β-lactamase-producing enteric bacteria in hospital wastewater and clinical sources. J. Glob. Antimicrob . Resist. 5, 22–25.

McLafferty, F.W., 2005. Wiley RegistryTMof Mass Spectral Data. JohnWiley & Sons Ltd, New York, NY, USA.

Ncube, B., Finnie, J.F., Van Staden, J., 2012. In vitro antimicrobial synergism within plant extracts combinations from three South African medicinal bulbs. J. Ethnopharmacol. 139, 81–89.

NIST/EPA/NIH, 2005. Mass Spectral Library with Search Program: (Data Version: NIST 05, Software Version 2.0d). National Institute of Standards and Technology, New York, NY, USA.

Rosato, A., Vitali, C., De Laurentis, N., Armenise, D., Milillo, M.A., 2007. Antibacterial effect of some essential oils administered alone or in combination with norfloxacin. Phytomedicine 14, 727–732.

Tripathi, A., Dutta, S.K., Majumdar, M., Dhara, L., Banerjee, D., Roy, K., 2012. High Prevalence and Significant Association of ESBL and QNR Genes in Pathogenic *Klebsiella pneumoniae* Isolates of Patients from Kolkata. India. Indian J. Microbiol. 52 (4), 557–564.

Ultee, A., Bennink, M.H.J., Moezelaar, R., 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Appl. Environ. Microb. 68, 1561–1568.

Verma, P., 2007. Methods for determining bactericidal activity and antimicrobial interactions: synergy testing, time-kill curves, and population analysis. In: Schwalbe, R., Steele-Moore, L., Goodwin, A.C. (Eds.), Antimicrobial Susceptibility Testing Protocols. CRC Press, New York, pp. 275–299.

Wagner, H., Ulrich-Merzenich, G., 2009. Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine. 16, 97–110.

Wang, S.Y., Sun, Z.L., Liu, T., Gibbons, S., Zhang, W.J., Qing, M., 2014. Flavonoids from *Sophora moorcroftiana* and their synergistic antibacterial effects on MRSA. Phytother Res. 28 (7), 1071–1076.

Wener, K.M., Schechner, V., Gold, H.S., Wright, S.B., Carmeli, Y., 2010. Treatment with fluoroquinolones or with β-Lactam-β-Lactamase inhibitor combinations is a risk factor for isolation of Extended-Spectrum β-Lactamase-Producing *Klebsiella* Species in Hospitalized Patients. Antimicrob. Agents Chemother. 54 (5), 2010–2016.

World Health Organization (WHO), 2014. Antimicrobial Resistance: Global report on surveillance. Geneva.

Worthington, R.J., Melander, C., 2013. Combination approaches to combat multidrug-resistant bacteria. Trends Biotechnol. 31 (3), 177–184.

Xu, J., Zhou, F., Ji, B.P., Pei, R.S., Xu, N., 2008. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. Lett. Appl. Microbiol. 47, 174–179.

Zahin, M., Ahmad, I., Aqil, F., 2010. Antioxidant and antimutagenic activity of *Carum copticum* fruit extracts. Toxicol. In vitro 24 (4), 1243–1249.

Zhao, X., Xu, C., Domagala, J., Drlica, K., 1997. DNA topoisomerse targets of the fluoroquinolones: a strategy for avoiding bacterial resistance. Proc. Natl. Acad. Sci. USA 94, 13991–13996.