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Effects of *Nigella sativa L.'s* Essential Oils on Multi Drug Resistant *Escherichia coli* Isolates

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Abstract

Escherichia coli is one of the most important human pathogens and is a major cause of nosocomial infections. In this study, it was aimed to investigate the antimicrobial efficacy of *Nigella sativa L.* against multi drug-resistant *E. coli* isolates. This study was carried out on multi drug resistant *E. coli* isolates. In this study, seeds of *Nigella sativa* L. obtained from spice market were used as material .Multidrug resistant *E. coli* strains were identified by conventional methods. The antibacterial activity of *Nigella sativa* L.'s essential oils was performed by disk diffusion test. In addition, when needed, automated systems were used for rapid identification of microorganisms and susceptibility testing identification. The ESBL production of these strains was confirmed by double disc synergy test.In ESBL producing *E.coli* strains, the MIC range of ampicillin was 4-32 μg/ml and the MIC range of cefuroxime was found to be 2-16 μg/ml. MIC values of ampicillin were range from 0.25 to 4 μg/ml and MIC of levofloxacin range were 1 to 8 μg/ml and MIC of cefuroxime range were found to be 0.25-4 μg/ml. Following treatment with essential oils of *Nigella sativa,* resistance rates of GSBL producing *E.coli* strains against all these three antibiotics (ampicillin, levofloxacin and cefuroxime) have been found to decrease statistically significantly ($p<0.05$). *Nigella sativa L.'s* essential oils showed complete zone of inhibition of the standart *E.coli* strains ($p<0.05$). Besides this, in experiments with multi drug resistant *E.coli* isolates, it was found that the inhibition zones of *Nigella*-treated GSBL producing *E.coli* strains differed from non-treated strains. There was a statistically significant increasing in inhibition zones in drug *E.coli* isolates ($p<0.01$).The essential oils of *Nigella sativa* L. may contain promising antimicrobial components in the treatment of multi drug-resistant *Ecoli* isolates. This is very important for the discovery of new antimicrobial agents. More extensive clinical trials should be undertaken in this regard.

Keywords: *Escherichia coli*, *Nigella sativa* L., drug, resistance, essential oil.

Резюме

Escherichia coli е един от най-важните човешки патогени и е основна причина за нозокомиални инфекции. Целта на настоящето проучване е да се изследва ефикасността на антимикробното действие на *Nigella sativa* L. срещу множество лекарствено резистентни изолати от *E. coli.* Експериментите са проведени с резистентни към много лекарства изолати на *E. coli*, използвани са семена от *Nigella sativa* L. от пазара на подправки. Резистентните *E. coli* щамове са идентифицирани с конвенционални методи. Антибактериалната активност на етеричните масла на *Nigella sativa* L. се извършва чрез дисково дифузионен тест. Освен това, когато е необходимо, са използвани автоматизирани системи за бързо идентифициране на микроорганизми и тест за чувствителност. Продукцията на ESBL от тези щамове е потвърдена чрез тест за двойно дисково синергично действие. Диапазонът на MIC

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при ESBL произвеждащите *E. coli,* е между 4 и 32 мкг/мл а този на цефуроксим е от 2 16 16 мкг/мл. Стойностите на MIC на ампицилин са в диапазона от 0.25 до 4 мкг/мл, на левофлоксацин е 1 до 8 мкг/мл, а на цефуроксим - 0.25-4 мкг/мл. След въздействието с етерични масла от *N. sativa*, степента на резистентност на щамове *E. coli*, произвеждащи GSBL срещу изпитаните три антибиотика (ампицилин, левофлоксацин и цефуроксим) е намаляла статистически значително (p<0,05). Етеричните масла на *N. sativa* L. показват зона на пълно инхибиране на стандартните *E. coli* щамове (p <0.05). Освен това, при експерименти с мултирезистентни *E. coli* изолати, е установено, че зоните на инхибиране при третирани с *Nigella* GSBL произвеждащи *E. coli* щамове се различават от нетретираните. Наблюдава се статистически значимо увеличение в зоните на инхибиране на изолатите на *E. coli* (p <0,01). Етеричните масла на *N. sativa* L. вероятно съдържат перспективни антимикробни компоненти за лечение на мултирезистентни *E. coli* изолати. Получените резултати са важен принос при откриването на нови антимикробни агенти.

Introduction

Nosocomial infections are quite important and a widespread as the public health problem in our country like all over the world. One of the most important agents of hospital infections is *Escherichia coli*, which is the most important cause of hospital and community acquired urinary system infections. *E. coli* infections cause significant morbidity and mortality (Bereket *et al*., 2012; Totsika *et al*., 2012).

In recent years, increasing resistance profiles have been reported in *E. coli* strains as with all hospital infections agents. In particular, there are serious difficulties in the treatment of infections caused by *E. coli* strains producing broad spectrum β-lactamase (ESBL). Due to the increased antibiotic resistance, new drug investigations are continuing intensively in our country as well as all over the world. For this purpose, new drug research studies are being carried out intensively on plant-based active components due to low toxicity (Bereket *et al*., 2012; Kang *et al*., 2012; Totsika *et al*., 2012; Prestinaci *et a*l., 2015; Padmini *et al*., 2017). Due to the wide range of pharmacological activities of *Nigella sativa* L., significant and intensive studies are being carried out in this area. *N. sativa* is an endemic species that grows naturally in our country and especially in our region and is cultivated (Venkatachallam *et al*., 2010).

In this study, we aimed to investigate the antimicrobial activity of essential oils of *N. sativa* L. on multiple drug resistant *E. coli* strains.

Materials and Methods

Isolation of the essential oils

Oven-dried at 35°C to constant weight, 100 g seeds of *N. sativa* L. were extracted by hydrodistillation with 1 L of distilled water for 3 h using Neo-Clevenger apparatus. The oils were dried over anhydrous sodium sulphate and then stored in dark color (amber) glass bottles and kept at 4°C ready for GC-MS analysis.

GC-MS analysis of essential oil

Analysis of the essential oils was carried out by using Thermo Scientific Focus Gas Chromatograph equipped with MS, auto sampler and TG-WAX MS-A (5% Phenyl Polysilphenylene-siloxane, 0.25 mm x 30 m i.d, film thickness 0.25). The carrier gas was helium (99.9%) at a flow rate of 1 mL/min; ionization energy was 70 eV. Mass range m/z 50-650 amu. Data acquisition was scan mode. MS transfer line temperature was 250° C, MS Ionization source 319 temperature was 220° C, the injection port temperature was 220° C. The samples were injected with 250 split ratio. The injection volume was 1 µl. Oven temperature was programmed in the range of 50 °C to 220 ° C at 3° C /min. The structure of each compound was identified by comparison with their mass spectrum (Wiley). The data were handled using Xcalibur software program.

Isolation and ıdentification

Multidrug resistant *E. coli* strains were identified by conventional methods. The antibacterial activity of *N. sativa* L.'s essential oils was performed by disk diffusion test. Also, when needed, automated systems (Vitek-32, BioMerieux, France) were used for rapid identification of microorganisms and susceptibility testing identification. The ESBL production of these strains was confirmed by double disc synergy test.

Bacteria

In this study, *E. coli strains* were provided from the bacteriological laboratory of Mustafa Kemal University of Department of Microbiology, Turkey. *E. coli* strains were incubated in Mueller Hinton Agar (MHA, Oxoid) and incubated at 37°C for overnight. Then, McFarland standards were used as a final concentration of approximately 10^5 CFU per ml. Later, the Mueller Hinton Agar plates

were inoculated with the essential oils of *N. sativa L* and incubated at least 48 hours at 37℃.

Determination of antibacterial activity

Conventional methods (colony morphology, gram staining properties, according to biochemical tests) were used for the identification of bacterial strains. When Bacteria couldn't be identified by conventional methods, automatic system (Vitec 2.0, bioMerieux, France) was used.

In this study, standard bacterial strain (*E. coli* ATCC 25922) was also used as a standard strain. The antimicrobial activity of *N. sativa* L. against multi-drug resistant *E. coli* strains was evaluated with disc difusion and broth microdilution methods. Minimal inhibition concentration values of *N. sativa* L.' essential oils were measured according to the CLSI (Clinical and Laboratory Standards Institute) guidelines (CLSI 2011).

Mueller-Hinton Broth was used for testing bacterial strains. The inoculum density was selected as $1x10^5$ cfu/ml. The essential oils of *N. sativa* L. were dissolved in DMSO. Then, the concentrations of *N. sativa* L.'s essential oils ranged from 256 to 0.25 µg/ml. The plates were incubated at 37ºC and evaluated after 48 hours. The MIC values of *N. sativa* L. against multi-drug resistant *E. coli* strains were determined as the lowest concentrations of the substance that had no visible turbidity.

E. coli strains showing a clear zone of inhibition >10 mm were considered to be sensitive. All experiments were carried out in triplicates for each combination of EOs (essential oils) of *N. sativa* L. The antibacterial activity of EOs was evaluated by measuring the diameter of the inhibition zone.

Drug resistant *E. coli* strains isolated from clinical specimens were used in the study. Modified disk synergy test was used for the detection of broad spectrum beta lactamase (ESBL) presence of strains.

Disc diffusion test

For this purpose, $1x10^7$ cell per ml was spread on the Mueller-Hinton agar. Then, discs containing different concentrations of *N. sativa* L. were placed on agar medium. In the study, sterile DMSO was chosen as the negative control. The plates were incubated for 24 hours at 37 °C, then the activity was assessed by measuring the inhibition zone diameters in mm.

Determination of MIC values

MIC values were determined as the lowest concentration which is able to inhibit the bacterial growth. The MIC concentrations of *N. sativa* L. were determined according to the microdilution broth method. Dilutions of the essential oils of *N. sativa L.* were prepared in Mueller-Hinton broth to obtain a final concentration of 16, 8, 4, 2, 1, 0.5, 0.25 µl, respectively. And then bacteria adjusted to 0.5 McFarland standards were added to all the tubes containing different concentrations of essential oils. All the bacterial tubes were incubated at 37°C for 24 hours. At the end of incubation, bacterial specimens from each tube were inoculated onto Mueller-Hinton agar plates. The plate without growth was determined as MIC value.

Results and Discussion

Nigella is an annual herbaceous (Gharby *et al* 2015), which belongs to the botanical family of *Ranunculaceae* that commonly grows in Eastern Europe, the Middle East, and Western Asia. It is a small shrub with tapering green leaves and rosaceous white and purplish flowers (Shalih *et al*., 2015). It is reported that there are twelve species of *Nigella* in Turkey. *N. sativa* is one of the most important cultivar produced. It is generally known that *Nigella* seeds are economically valuable. *N. sativa* seeds contain 20% protein, 30% fixed fat and 35% carbohydrate (Baydar, 2016). It has been reported that *N. sativa* seeds contain 0.4-2.5% essential oils. In previous studies, it has been shown that *N. sativa* L. has quite rich active constituents. The chemical composition obtained by GC-MS was determined to have more than 20 pharmacologically specific components, such as p-cymene, α-thujene, γ-terpinene, carvacrol, α-pinene and β-pinene (Venkatachallam *et al*., 2010).

The major components were as follows: The contents of Anethole (22.97%), Thymoquinone (21.36%), α-thujene (6.22%), Longifolene (5.76%), Trans-isoeugenol (3.55%) and Carvacrol. In many studies the essential oils of *N. sativa* have been reported to have antimicrobial effects against various infectious agents. In this study, we investigated the effects of *N. sativa* L.' essential oils on multiple drug resistant *E. coli* strains; we also searched whether there would be new hope for the treatment of infections caused by these drug resistant strains.

In our study, we examined the effects of *N. sativa*'s essential oils against drug-resistant E. coli strains by both disk diffusion method and microdilution method. In the disk diffusion method, the inhibition zone diameters of *E. coli* strains treated with essential oils of *N. sativa* L. were found to be statistically significantly higher compared to those not treated with essential oils (Fig. 1).

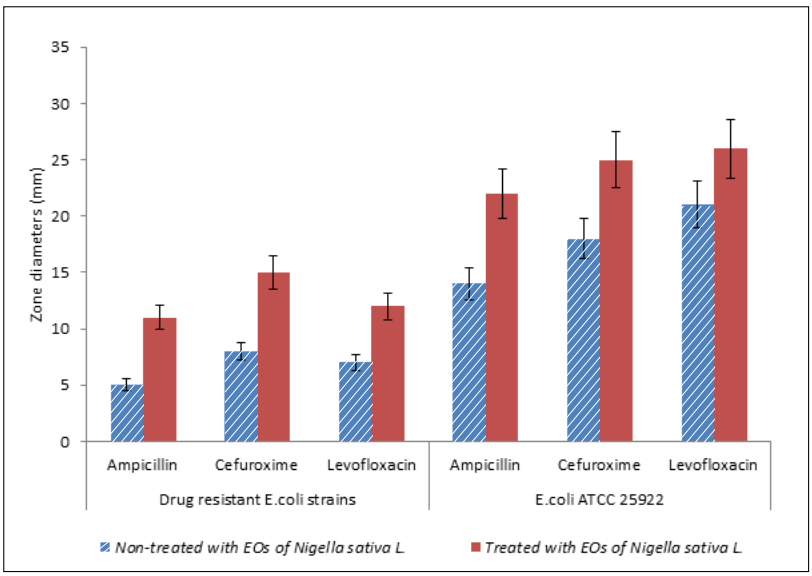

Fig. 1. Effects of EOs of *N. sativa* L. on inhibition zone diameters of antibacterial drugs against standart and drug resistant *E. coli* strains

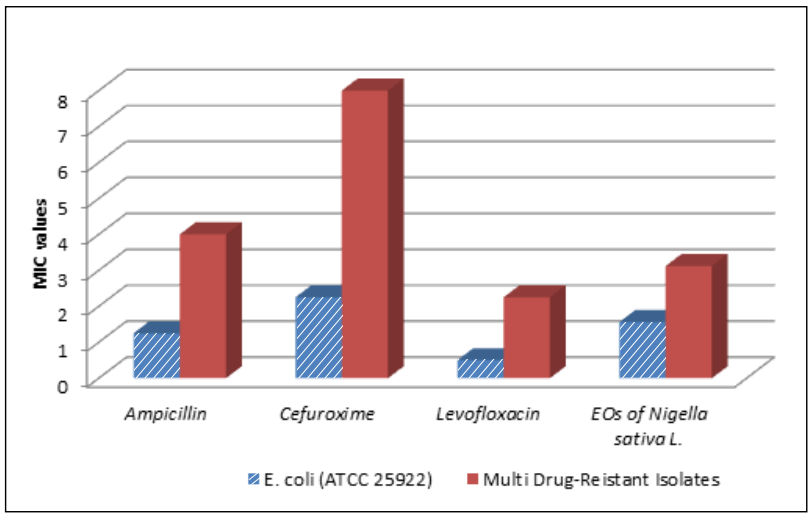

Fig. 2. MIC ranges of standart drugs and EOs of *N. sativa* L. against drug resistant *E. coli* isolates

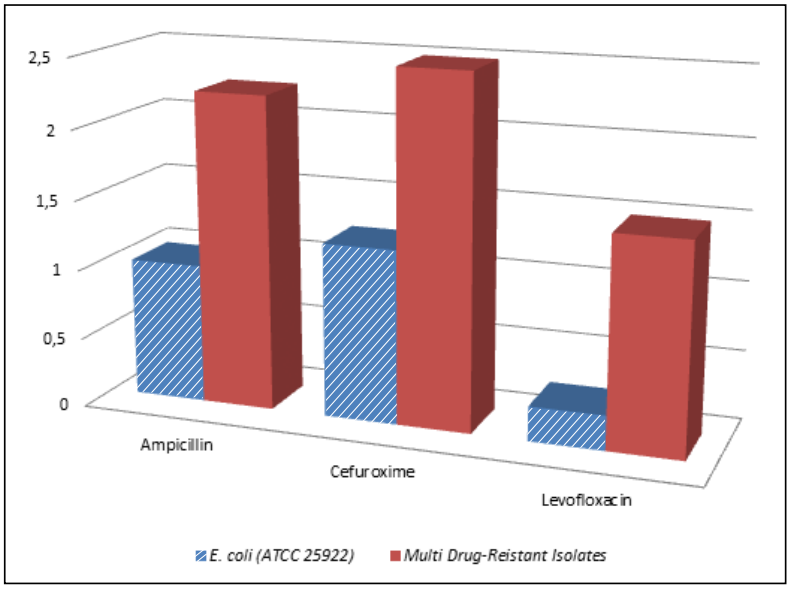

Fig. 3. After treatment with EOs of *N. sativa* L., resistance rates of drug resistant *E. coli* strains against standart drugs

N. sativa L.'s essential oils showed complete zone of inhibition of the standard *E. coli* strains ($p<0.05$). Besides this, in experiments with multi drug resistant *E. coli* isolates, it was found that the inhibition zones of *Nigella*-treated GSBL producing *E. coli* strains differed from non-treated strains. There was a statistically significant increasing in inhibition zones in drug resistant *E. coli* isolates ($p<0.01$).

In addition, in the microdilution method *E. coli* strains treated with essential oils in the MIC values of the antibacterial drugs were observed to be statistically significantly lower when compared to those not treated with the essential oils. In GSBL producing *E. coli* strains, MIC of ampicillin range of 4-32 µg/ml and MIC range of cefuroxime were found to be MIC range 2-16 µg/ml. MIC values of ampicillin range of 0.25-4 µg/ml and MIC range of levofloxacin of 1-8 µg/ml and MIC range of cefuroxime were found to be 0.25-4 µg/ml. Following treatment with essential oils of *N. sativa,* resistance rates of GSBL producing *E. coli* strains against all three antibiotics (ampicillin, levofloxacin and cefuroxime) have been found to decrease statistically significantly ($p<0.05$). (Fig. 2 and Fig. 3).

Conclusions

As can be understood from the results of the study, it is indisputable that the essential oils of *N. sativa* L. were found to be highly effective against multi-drug resistant *E. coli* strains. Even more importantly, we think that the essential oils of *N. sativa* L. may be a very important hope for the treatment of infections caused by drug-resistant *E. coli* strains. We are think that this work will be a stepping stone for further studies. This is very important for the discovery of new antimicrobial agents. More extensive clinical trials should be undertaken in this regard.

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