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The antimicrobial and resistance modifying activities of *Nigella sativa* oil

Ph.D. dissertation by

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1. INTRODUCTION

The search for alternatives to available antibiotics is becoming more and more needed in the developed countries due to the misuse of antibiotics. Alexander Fleming, in his 1945 Nobel lecture speech, warned against the resistance that develops in bacteria when under-exposed to antibiotics. The deeper knowledge of essential oils and their bioactive compounds would help to overcome an increasingly upsetting crisis of human illness from antibiotic resistance that is an international one, because the antimicrobial mechanisms of these bioactive compounds often differ from the current classes of conventional antibiotics, greater knowledge about them could help guide development of new classes of antibiotics.

Nigella sativa L. (Black cumin) is well known for its benefits in the field of traditional medicine. The aim of this study was to determine the chemical composition and investigate the antimicrobial activity of crude oil and essential oil of *Nigella sativa* L. on spoilage bacteria, foodborne and non-foodborne pathogenic bacteria, and the resistance modifying activity of *N. sativa* EO, thymoquinone, carvacrol, and p-cymene against methicillin susceptible and methicillin resistant *S. aureus* and *L. monocytogenes* strains.

N. sativa essential oil, thymoquinone, carvacrol, and p-cymene was assessed for antimicrobial activity, modulation of antimicrobial resistance, inhibition of antimicrobial efflux, membrane disrupting effect and anti-biofilm activity by broth microdilution, ethidium bromide accumulation, LIVE/DEAD BacLightTM assays and real-time reverse transcriptase quantitative polymerase chain reaction.

N. sativa essential oil was a source of biological active compounds, which contributed to its antimicrobial properties. Our findings enhance further study to use this oil as a potential food preservative and as a medical commodity. *N. sativa* EO and its bioactive compounds such as carvacrol and p-cymene could be applied as potent efflux pump inhibitors in MRSA strains. *N. sativa* EO might have a potential for controlling the antibiotic resistance in *Listeria*.

2. AIM

Foodborne pathogenic bacteria and antibiotic resistant organisms represent an increasing risk to the consumer. The overall objective of **my study was to study/investiagate** *Nigella sativa* oils (crude oil and essential oil) as active antimicrobial and resistance modifying agents.

The specific objectives of my research were:

1- to determine the fatty acids composition of the crude oil;

2- to determine the active compounds of the essential oil;

3- to study the antibacterial effect of the crude oil and essential oil against food spoilage organisms, foodborne and non-foodborne pathogens;

4- to investigate the synergistic activity between the crude oil and nisin against *Bacillus* spores;

5- to assess the resistance modifying activity of the essential oil, thymoquinone, carvacrol and p-cymene against *Listeria monocytogenes* and *Staphylococcus aureus*;

6- to assess the anti-biofilm activity of the essential oil, thymoquinone, carvacrol and p-cymene.

3. MATERIALS AND METHODS

3.1.1. *N. sativa* oil

N. sativa seeds were purchased from a local Turkish market and were cold pressed to produce the crude oil. The crude oil was then hydro-distilled at 100 °C in a Clevenger apparatus to extract the essential oil (EO). The essential oil was collected, dried over anhydrous sodium sulfate and finally stored at 4 °C for further analysis.

3.2. Fatty acids methylation and GLC analysis

The fatty acids methylation and GLC analysis was carried out by Toma and co-workers (2013) with some modifications.

3.3. GC-MS analysis of the essential oil

The composition of the EO was determined by GC-MS. The GC analysis was carried out using an Agilent Technologies 6890 N instrument equipped with HP-5MS capillary column (5% phenyl, 95% dimethyl polysiloxane, length: 30 m, film thickness: 0.25 μm, id. 0.25 mm), programmed as follows: initial temperature 60 °C, heating at a rate of 3°C/min up to 240 °C; the final temperature was kept for 5 min; injector and detector temperatures: 250 °C; carrier gas: helium (constant flow rate: 1 mL/min); split ratio: 30:1.

The GC-MS analyses were carried out using an Agilent Technologies 6890 N GC equipped with an Agilent Technologies MS 5975 detector and ionization energy of 70 eV. The MS were recorded in full scan mode that revealed the total ion current (TIC) chromatograms. The linear retention indices (LRI) were calculated using the generalized equation of Van den Dool and Kratz (1963). The MS and LRI were compared with those of commercial (NIST, Wiley) and home-made libraries. The proportions of the individual compounds were expressed as total fatty acids (TFA) area percentages.

3.4. Evaluation of antimicrobial activity of *N. sativa* crude oil by agar well method

Antibacterial activity of *N. sativa* crude oil was assessed against 27 Gram-positive strains and 2 Gram-negative strains (clinical isolates) using agar well method.

3.5. Determination of minimum inhibitory concentration (MIC)

The antimicrobial activity of *N. sativa* CO, EO, Thq, Car and p-cy were determined using broth microdilution method.

3.6. Spores preparation

B. cereus T1 and *subtilis* T1 cultures prepared on solid medium, the spores were separated and kept at 4 °C until use.

3.7. Combination of *N. sativa* crude oil and nisin against spores

The antimicrobial activity of *N. sativa* CO and nisin were tested against *B. cereus* and *B. subtilis* spores measuring the optical density using Multiskan machine. Briefly, the stock solution of the crude oil, or nisin were serially half diluted in TSB in microtiter plates, and then *B. cereus* or *B. subtilis* spores were added at a concentration of 10^6 CFU/mL, to the final volume of 300 µL/well.

Following 24-h incubation at 30 ºC, the optical density was measured at 595 nm, using a microplate reader (Multiskan plate

reader, MTX Lab Systems, USA). The minimum inhibitory concentrations (MICs) were defined as the minimal concentration at which there was no growth detected after 24 h of incubation. All the MICs measurements were carried out in triplicates.

3.8. Anti-chlamydial assay

Elementary bodies of *Chlamydia trachomatis* D ($1x10^3$ IFU/ml) were incubated with *N. sativa* EO, Thq, Car and p-cy at various concentrations (50, 25 μM; 0,005%, 0,0025% EO in DMSO) in sucrose-phosphate-glutamic acid buffer (SPG) for 1 h at 37 °C. As a control, *C. trachomatis* D was also incubated in SPG only. To quantify the anti-chlamydial effects of compounds, HeLa cells were seeded in 24-well tissue culture plates with 13-mm cover glasses. After 24 h, the confluent cells were infected with compounds-treated *C. trachomatis* D or the non-treated controls. After 48 h, the cells were fixed with acetone at -20°C for 10 min and stained with murine monoclonal anti-Chlamydia LPS antibody (AbD Serotec, Oxford, UK) and FITC-labelled secondary anti-mouse IgG (Merck KGaA, Darmstadt, Germany). The number of *C. trachomatis* D inclusions was counted under a UV microscope, and the titer was expressed in inclusion forming units/ml (IFU/ml).

3.9. Application of *N. sativa* crude oil in food matrix

Minced pork chop (1000 g) was used for the experiment, which was purchased from a local shop in Budapest on the first day of the experiment.

Different samples were prepared for the experiment (from each type of samples 3-3 parallel for each sampling day):

- MP – control minced pork,
- MP-Lm – minced pork inoculated with *L. monocytogenes*,
- MP-CO+Lm – minced pork with 2% Nigella sative CO and inoculated with *L. monocytogenes*,

Samples that were inoculated with the pathogen had 10^5 CFU/g initial cell count of *L. monocytogenes*. Samples (10 g) were measured into sterile stomacher bags, sealed and stored in a refrigerator during the experiment. Microbiological examinations were performed on the day of sample preparation and after 6 day refrigerated storage.

40 ml maximum recovery diluent was added to each sample and homogenised for 2 minutes in stomacher homogenizer and decimal serial dilutions were prepared. *L. monocytogenes* counts were determined by spreadplating the appropriate dilutions on

Palcam agar (BIOKAR BK145 with Palcam Selective Supplement BIOKAR BS004). Plates were incubated at 37 °C for 24-48h and evaluated.

3.10. Resistance-modulation assay

Modulation of antimicrobial resistance for the antibiotics, erythromycin, ciprofloxacin, and EtBr was evaluated using the same microdilution method, except that the medium was supplemented with sub-inhibitory concentration of the EO or Thq (1/2 MIC). The modulation factor was defined as the ratio of the MICs for the antimicrobials (Ery, Cip or EtBr) alone and for the antimicrobial agent in the presence of the EO, Thy, Car and p-cy. All the MICs measurements were carried out in triplicates. The chemical EPI, Reserpine (20 μg/mL) was used as positive control reference. The positive control wells were prepared with the bacterial suspension only, the bacterial suspension and ½ MIC of the EO, Thq, Car or p-cy and with DMSO or EtOH with the bacterial suspension corresponding to the highest concentration present in the preparation. A modulation factor >2 was set as the cut-off for biologically significant resistance modulation.

3.11. EtBr accumulation assay

The influence of *N. sativa* EO, Thq, Car and p-cy on EtBr accumulation in *L. monocytogenes* L14 and *S. aureus* (ATCC

25923 and MRSA 272123) were determined. Briefly, 150 μL of overnight culture (4 mL inoculum, 24 h) was added to 9.9 mL TSB, centrifuged at 6000 g for 5 minutes, then the cells were washed twice and resuspended in phosphate-buffered saline (PBS) (OD_{600}, 0.2). *N. sativa* EO, Thq, Car or p-cy were added to the culture to the appropriate concentration (1/2 MIC). After 15 min of incubation at 37 ºC, 96.74 μL of the untreated culture, the treated culture containing EO, Thq, Car or p-cy, were pipetted in black microtiter plates followed by addition of 3.26 μL EtBr to a final concentration of 0.5 µg/mL. The kinetics of intracellular EtBr accumulation was measured at 490 nm and 579 nm using a Victor x3 plate reader (PerkinElmer/USA), at 45 s intervals for 1 h. Additionally, reserpine (100 µg/mL) was used as a positive control reference in the assay. Measurements were carried out in triplicate and the means of the last 10 times points of the measurements were used in the statistical analysis.

3.12. Membrane integrity assay

The influence of *N. sativa* EO, Thq, Car and p-cy on membrane integrity of *L. monocytogenes* L14 and *S. aureus* (ATCC 25923 and MRSA 272123) were assessed using LIVE/DEAD Baclight™ Bacterial Viability Kits (L-7012, Molecular Probes, Eugene, Oregon, USA). The BacLight™ kit is composed of two nucleic acid-binding stains: SYTO 9TM and propidium iodide

(PI). SYTO 9TM penetrates all bacterial membranes and stains the cells green, while PI only penetrates cells with damaged membranes, and the combination of the two stains produces red fluorescing cells.

3.13. Expression analysis of genes by real-time reverse transcriptase quantitative polymerase chain reaction

S. aureus ATCC 25923 and *S. aureus* 272123 strains were cultured in TSB broth and were incubated overnight at 37°C with shaking at 140 rpm. On the day of RNA isolation, the bacterial suspensions (OD of 0.6 at 600 nm) were transferred to 10 mL tubes in 3 mL aliquots, and 5 µM of *N. sativa* EO, or 0.5 µM of compounds (Thq, Car, p-cy) were added to the tubes, which were incubated at 37ºC. After 4 hours of incubation, the tubes were centrifuged at 12,000 × *g* for 2 min. Pellets were suspended in 100 µL TE (Tris-EDTA) buffer containing 1 mg/mL lysozyme by vigorous vortexing, and they were incubated at 37°C for 10 min. The total RNA was isolated in a RNase-free environment using the NucleoSpin RNA kit (Macherey Nagel, Germany) according to the manufacturer's instructions. Purified RNA was stored in RNase-free water in nuclease-free collection tubes at −20ºC until quantification was performed. The concentration of the extracted RNA templates was assessed by spectrophotometry

at 260 nm. Expression of the efflux pump genes *mepA* was studied by reverse transcription of the total RNA. The data obtained for gene targets were normalized against the *S. aureus* 16S ribosomal RNA measured in the same sample. The primers used in the assay were the following:

1. Sequence (5'-3') of *mepA* (198 bp)

TGCTGCTGCTCTGTTCTTTA (Fw)

GCGAAGTTTCCATAATGTGC (Rv)

2. Sequence (5'-3') of *16S rRNA* (492 bp)

AGAGTTTGATCMTGGCTCAG (Fw)

GWATTACCGCGGCKGCTG (Rv)

Real-time quantification of the RNA templates by real-time one-step RT-qPCR was performed in a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA), and the manufacturer's recommendations of the SensiFAST^{TM} SYBR No-ROX One-Step Kit (Bioline GmbH, Luckenwalde, Germany) were applied.

3.14. Biofilm formation assay

Biofilm formation of *S. aureus* (ATCC 25923 and MRSA 272123) was studied under defined growth conditions that experimentally approximate different stress conditions. M9 minimal media (containing NH_4Cl [1.9 mM], Na_2HPO_4 [42.3 mM], KH_2PO_4 [22 mM], NaCl [8.56 mM], $MgSO_4$ [2 mM],

$CaCl_2$ [0.1 mM], and TSB broth were used for this purpose to enable an assessment of the inhibition of biofilm formation capacity using *N. sativa* EO, Thq, Car and p-cy at different concentration. Initially, overnight cultures were adjusted to 1 McFarland with M9 minimal media, and 200µL of the bacterial cell suspension was dispensed across a 96-well microtiter plate. Plates were incubated for 72 h at 37 ºC. Crystal violet was used to detect total biofilm biomass formed.

3.15. Statistical analysis

The results were statistically analyzed using Microsoft Excel program (2016). Comparisons of the group mean values and the significance of the differences between the groups were verified by one-way ANOVA. The results were considered significant when $p \leq 0.05$.

4. RESULTS

4.1. Chemical composition of *N. sativa* oils

The results of fatty acids analysis of *N. sativa* crude oil showed ten saturated and unsaturated fatty acids. The major fatty acid of CO was linoleic acid (18:2n-6) with a level of 55.03g/100g total fatty acids (TFA), followed by oleic (18:1n-9) with a level of

22.53 g/100g TFA and palmitic (16:0) with a level of 14.39 g/100g TFA, respectively.

The results showed that 10 components were identified in the EO (seven monoterpenes hydrocarbon (MH), two oxygenated monoterpenes (MO) and one sesquiterpene hydrocarbon (SH), representing 94.03% of the total amount. The oil consisted mainly of MH with 71.69 % followed by lower contents of MO with 20.51 % and very low amounts of SH (1.83 %). Therefore, among the monoterpenes hydrocarbons, p-cymene represent the major constituent (52.24%) while thymoquinone was dominating the oxygenated monoterpenes (18.76%).

4.2. Evaluation of antimicrobial activity of *N. sativa* oils

The crude oil of *Nigella sativa* seeds was evaluated for its antimicrobial activity. Antibacterial activity was evaluated against different microbial strains, including Gram-positive and Gram-negative bacteria, using agar well method and the diameters of inhibition zones was measured. For Gram-positive bacteria, CO showed the best activity against *Micrococcus* at 0,33 % and the lowest activity was for *Enterococcus* at 20 %. The oil was less active against *Listeria* strains. In this study, CO inhibited especially Gram-positive bacteria.

Minimal inhibitory concentration (MIC) of the *N. sativa* crude and essential oils was evaluated by microdilution method against 7 bacteria (spoilage and/or pathogenic). The results of the antimicrobial activity showed that both oils of *N. sativa* had a good activity on the tested strains. The activity of essential oil was 10 times higher than the crude oil. In addition, the MIC of essential and crude oils varied between 0.003-0.46% and 0.06-0.25% for all bacteria tested, respectively.

The MIC of *N. sativa* crude oil and nisin were determined by measuring the optical density using Multiskan machine. Both compounds were found to inhibit *Bacillus* spores. Based on these MICs values, two combinations between *N. sativa* crude oil and nisin (1/2 MIC + 1/2 MIC or 1/4 MIC + 1/4 MIC) were used to check if *N. sativa* crude oil is able to enhance the activity of nisin against *B. cereus* and *B. subtilis* spores.

In case of *B. cereus* spores the first combination (1/2 MIC + 1/2 MIC) resulted in full inhibition of the strain. As for the second combination (1/4 MIC + 1/4 MIC) it was less effective than the first one, yet the optical density was much lower and the time needed to detect growth was much longer than the positive control. In case of *B. subtilis* spores the first combination resulted in full inhibition of the strain, while as for the second combination after 15 hour the optical density increased and

reached optical density values close to the positive control. Interestingly, combining nisin with *N. sativa* crude oil resulted in extra reduction of the OD. This clearly indicates that *N.sativa* crude oil enhance the activity of nisin.

The IC50 of *N. sativa* EO, Thq, Car and p-cy on HeLa cells were determined, where the inhibitory concentration (IC50) is defined as the inhibitory dose that reduces the growth of the compound-exposed cells by 50%. The IC50 of *N. sativa* EO was indicated to be 0.009%, while the IC50 of Thq, Car and p-cy was higher than 100 μM.

Counting of the number of viable *C. trachomatis* inclusions demonstrated that all the compounds were highly effective killers of *C. trachomatis*. *N. sativa* EO, Thq and Car (at ½ IC50) exhibited 100% reduction in the viability of *C. trachomatis*, while p-cymene showed lower reduction by 83 %.

The control, oily and bacterial inoculated food samples were subjected to a storage experiment to investigate the ability of the crude oil to inhibit *L. monocytogenes* proliferation and deterioration of the meat product. For each type of sample, the results were derived from 3 parallel measurements.

For the oily sample, the oil inhibited the growth of *Listeria monocytogenes* by 2 log. It is clear that *N. sativa* crude oil has an antimicrobial effect on the *L. monocytogenes* pathogenic

bacterium and is therefore an alternative natural preservative. In further experiments, it would be beneficial to investigate the inactivation potential of *Nigella sativa* oil in other foods.

4.3. Resistance modifying activity of *N. sativa* essential oil and its active compounds against *L. monocytogenes*

The antibacterial activity of *N. sativa* EO and its active compounds were determined by broth microdilution method.

The essential oil of *N. sativa*, thymoquinone and carvacrol were active against all the tested strains of *L. monocytogenes*. The MIC values of *N. sativa* EO ranged from 116 to 466 μg/mL, thymoquinone exhibited a significant antibacterial activity with a MIC values of 40 μg/mL, and carvacrol exhibited the MIC of 150 μg/mL, while p-cymene showed no inhibitory activity at the tested maximum concentration (2144 μg/mL) against any strain. As a potential modulator of antimicrobial resistance, *N. sativa* essential oil, thymoquinone, carvacrol and p-cymene were tested at 1/2 MIC (58 μg/mL, 20 μg/mL, 75 μg/mL and 1072 μg/mL, respectively) in combination with the antibiotics erythromycin, ciprofloxacin, and the antimicrobial efflux pump substrate EtBr, on nine *L. monocytogenes* test strains. A modulation factor >2 was set as the cut-off for biologically significant resistance modulation.

The obtained data showed that the supplementation of *N. sativa* EO (at 1/2 MIC) decreased the MICs of erythromycin from two-fold to four-fold and decreased the MIC of ciprofloxacin from two-fold up tp eight-fold against all *L. monocytogenes* strains. Additionally, a four-fold up to sixteen-fold reduction of EtBr was observed against *L. monocytogenes* strains in the presence of the EO.

Whereas, the supplementation of thymoquinone (at 1/2 MIC) induced decrease in the MIC of ciprofloxacin four-fold against seven *L. monocytogenes* strains and two-fold decrease against the other three strains. Furthermore, eight-fold reduction of EtBr was noted against eight *L. monocytogenes* strains and four-fold against 1 strain.

Carvacrol supplementation (at 1/2 MIC) decreased the MIC of ciprofloxacin: two-fold reduction was observed for all the tested strains of *L. monocytogenes.* Additionally, a two-fold up to sixty-four-fold reduction of EtBr MIC was detected against *L. monocytogenes* strains in the presence of carvacrol.

p-cymene supplementation (at 1/2 MIC) showed two- fold reduction in the MIC of erythromycin against six *L. monocytogenes* strains and had no activity against the other three strains. P-cymene induced decrease in the MIC of EtBr from two-fold up to four-fold against all *L. monocytogenes* strains.

In order to elucidate the mechanism of modulatory activity of *N. sativa* essential oil and its active compounds, thymoquinone, carvacrol, and p-cymene in *L. monocytogenes* L14, we have evaluated the potency of the compounds to increase the accumulation of the common efflux pump substrate EtBr indicating efflux inhibition.

The level of EtBr accumulation in cultures treated with half of the MIC values of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene were compared, relatively to the untreated culture, to evaluate whether it can potentiate intracellular EtBr accumulation. The known efflux pump inhibitor reserpine was included in the study as a positive control. The results showed significant ($p<0.0001$) increase in the EtBr accumulation in the presence of *N. sativa* essential oil, p-cymene, carvacrol, and thymoquinone, respectively, compared to the untreated culture of *L. monocytogenes*. *N. sativa* essential oil increased the EtBr accumulation significantly compared to reserpine. p-cymene and carvacrol activity were comparable to reserpine, while thymoquinone activity was lower than reserpine.

Again, 1/2 MIC of *N. sativa* EO, Thq, Car, and p-cymene were tested for their influence on membrane integrity in *L. monocytogenes* L14, to determine whether membrane

permeability is the main mechanism of its modulation of antimicrobial resistance.

The membrane integrity of cultures treated with 1/2 MIC of *N. sativa* essential oil, thymoquinone or carvacrol decreased by 43%, 14% and 25% respectively. Hence, at this half MIC concentration of the essential oil and its active compounds, the disruption of the membranes is likely to have contributed to their antimicrobial resistance modifying effect. Cultures incubated at 80°C for 15 min were used as positive controls for disrupted membranes, and these showed 89% decreased membrane integrity, compared to the untreated control cultures. These differences were calculated based on the kinetics measurements over the last 10 min of the 1h assay, and they were statistically significant ($p<0.00001$).

4.4. Resistance modifying activity of *N. sativa* essential oil and its active compounds against *S. aureus*

The antimicrobial activity of *N. sativa* EO, Thq, Car and p-cy against the tested strains were as follows: 30 μg/ml for *N. sativa* EO, 10 μg/ml for Thq and 75 to 150 μg/ml for Car, while p-cy showed no inhibitory activity at the maximum tested concentration (2144 μg/ml) against both strains.

Data showed that the presence of Thq at half MIC, in combination with Tet resulted in decrease of MIC up to 16 fold in the MRSA strain. An 8-fold reduction was noted in combination with Cip in the MRSA strain. Whereas, the supplementation of *N. sativa* EO at half MIC induced a 2-fold decrease in the MIC of EtBr in both strains. A modulation factor >2 was set as the cut-off for biologically significant resistance modulation.

In order to elucidate the mechanism of modulatory activity of *N. sativa* EO and its active compounds, Thq, Car, and p-cy on *S. aureus (*ATCC 25923 *and* MRSA 272123), the potency of the compounds was evaluated to increase the accumulation of the common efflux pump substrate EtBr indicating efflux pump inhibition.

The level of EtBr accumulation in cultures treated with half of the MIC of *N. sativa* EO, Thq, Car, and p-cy (at 1072 μg/ml) was compared, relatively to the untreated culture, in order to evaluate whether they can potentiate the intracellular accumulation of EtBr. The known efflux pump inhibitor CCCP was included in the study as a positive control. The results showed significant ($p<0.0001$) increase in the EtBr accumulation in the presence of *N. sativa* EO and Car in the ATCC strain and elevated intracellular EtBr concentration was observed in the presence of

N. sativa EO in the MRSA strain. Compound p-cy had no effect on the EtBr accumulation in the ATCC strain, while the activity of Thq was lower than the EtBr accumulated by the untreated ATCC and MRSA strains. CCCP showed activity on the ATCC strain while marginal effect was detected in case of the MRSA strain. Car was the most active compound on both strains.

With the aim of detecting the influence of these bioactive compounds on membrane integrity, the membrane damaging effect was evaluated in the presence of half of the MIC of *N. sativa* EO, Thq, and Car in *S. aureus* (ATCC 25923 and MRSA 272123) to determine whether the compounds affect the membrane permeability, thus contributing to the modulation of antimicrobial resistance.

Cultures incubated at 80°C for 15 min were used as positive controls for disrupted membranes, and these controls showed decreased membrane integrity by 81% in both strains, compared to the untreated control cultures. These differences were calculated based on the kinetics measurements over the last 10 min of the 1h assay, and they were statistically significant ($p<0.00001$).

The membrane integrity of *S. aureus* ATCC 25923 treated with Car decreased by 50%, while *N. sativa* EO and Thq increased the membrane integrity by 24% and 11%, respectively.

The membrane integrity of *S. aureus* MRSA 272123 treated with 1/2 MIC values of *N. sativa* EO, Thq or Car decreased by 25%, 31% and 6%, respectively for *S. aureus* MRSA 272123. Hence, at half of MIC the essential oil and its bioactive constituents, the disruption of the membranes could have contributed to their antimicrobial resistance modifying effect.

In real-time quantitative RT-PCR assay, the gene of MepA transporter was investigated to evaluate the effect of compounds on the relative expression of efflux pump gene in both *S. aureus* strains.

Gene expression data showed that Thq significantly down-regulated the expression of *mepA* after 4 hours of exposure, while p-cy up-regulated the expression of *mepA* in the ATCC strain. In the case of MRSA strain, *N. sativa* EO significantly up-regulated the expression of *mepA* after 4 hours of exposure, and p-cy down-regulated the expression of *mepA* after 4 hours of exposure.

In this study, *N. sativa* EO, Thq and Car could reduce effectively the development of bacterial performed biofilm of *S. aureus* (ATCC and MRSA). The reduction ranged from 30 to 40 % after EO treatment, furthermore 18 to 22 % and 11 to 35 % when the medium was supplemented with Thq and Car, respectively.

5. NEW SCIENTIFIC RESULTS

1- The MIC of *N. sativa* crude oil (0.125 % for *B. cereus* spores and 0.25 % for *B. subtilis*) and MIC of nisin (0.3 mg/ml) were determined against *B. cereus* and *B. subtilis* spores. I proved that the combination of crude oil with nisin enhanced the activity of nisin against *Bacillus* spores.

2- I demonstrated that *N. sativa* essential oil and its active compounds carvacrol and p-cymene possess anti-chlamydial activity against *Chlamydia trachomatis.*

3- I proved that *N. sativa* essential oil modulate antimicrobial resistance of *L. monocytogenes* and methicillin resistant *S. aureus* by targeting the efflux pump and the membrane integrity. Furthermore, I confirmed p-cymene downregulates the expression level of efflux pump gene *mepA* in MRSA.

4- The formation of *S. aureus* biofilm was proved to be partially inhibited by *N. sativa* essential oil, thymoquinone and carvacrol.

6. CONCLUSIONS AND SUGGESTIONS

Multidrug resistant bacteria are a growing threat to human health and welfare. The aim of this study was to investigate antimicrobial properties and resistance modifying activities of *N. sativa* oils. In particular their antibacterial effect against food spoilage organisms, foodborne and non-foodborne pathogens, their anti-spore and anti-biofilm activity.

In our study the major fatty acids of *N. sativa* crude oil were linoleic (18:2n-6), oleic (18:1n-9) and palmitic (16:0) acids. While, the major compounds of its essential oil were p-cymene and thymoquinone. The essential oil of the *N. sativa* showed 10 times higher antimicrobial activity than the crude oil, however, the crude oil still showed good inhibitory effect against Gram-positive bacteria.

The present study showed that *N. sativa* essential oil was a source of biological active compounds, which contributed to its antimicrobial properties. *N. sativa* and its examined active compouds were highly effective killers of *C. trachomatis*. The crude oil was also effective against endspores of *Bacillus* strains and showed good activity against *Listeria monocytogenes* not only in laboratory media buti in food matrix, as well. Our

findings enhance further study to use this oil as a potential food preservative and as a medical commodity.

Nigella sativa essential oil and its active compounds, thymoquinone and carvacrol are confirmed as efficient modulators of antimicrobial resistance in *L. monocytogenes*, with at least two different mechanisms that contribute synergistically to their activity. Half MIC of *N. sativa* essential oil, thymoquinone, and carvacrol modulates antibiotic resistance in *L. monocytogenes* against various antimicrobial, showing increased EtBr accumulation. Additionally, targeting the membrane, they caused increased permeability, thereby promoting the influx of antimicrobials. P-cymene had no antimicrobial activity; however, it increased the membrane permeability. Due to the modulation of the antimicrobial resistance in *L. monocytogenes*, *N. sativa* essential oil, thymoquinone, and carvacrol has the potential to be promising modifiers of antimicrobial resistance in *L. monocytogenes*.

According to the results obtained in our study, *N. sativa* essential oil and its bioactive compounds such as carvacrol and p-cymene could be applied as potent efflux pump inhibitors in MRSA strains, furthermore p-cymene downregulates the expression level of efflux pump gene *mepA* in MRSA. Influencing efflux pumps and their genes, the resistant isolates can lose their

virulence and we can provide better perspectives to treat MRSA related infections. In addition, essential oil, thymoquinone, and carvacrol might be used as adjuvants in combination with antibiotics enabling a better therapeutic efficacy in *S. aureus* related infections.

These findings prove that *Nigella sativa* oils represent a potential source of bioactive compounds to use in food preservation and different fields of medicine.

Further studies are needed to apply the oil as food preservative, and to check the mechanism of action between the crude oil and nisin whether it's synergistic or additive effect.

Additional research is required to determine precisely which efflux pump in *L. monocytogenes* is inhibited by *N. sativa* essential oil

Due to the promising effect of p-cymene on *mepA* gene expression, more genes included in the resistance to antibiotics should be tested.

Further examination are needed to assess the effect of *N. Sativa* essential oil and its bioactive compounds against *S. aureus* quorum sensing in biofilm.

7. LIST OF PUBLICATIONS

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Mouwakeh, A., Telbisz, A., Spengler, G., Mohácsiné, F. Cs., & Kiskó, G., (2018). Antibacterial and resistance modifying activites of *Nigella sativa* essential oil and its active compounds against *Listeria monocytogenes*. *in Vivo, 32*: 737-743. DOI:10.21873/invivo.11302

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