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# Antibacterial and Resistance Modifying Activities of *Nigella sativa* Essential Oil and its Active Compounds Against *Listeria monocytogenes*

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**Abstract.** *Background/Aim:* *N. sativa* essential oil (EO) and its compounds (thymoquinone, carvacrol and *p*-cymene) have a broad antimicrobial spectrum. The aim of this study was to investigate the antimicrobial and resistance modifying activity of *N. sativa* EO, thymoquinone, carvacrol and *p*-cymene against *Listeria monocytogenes*. *Materials and Methods:* *N. sativa* EO, thymoquinone, carvacrol and *p*-cymene was assessed for its antimicrobial activity, modulation of antimicrobial resistance, inhibition of antimicrobial efflux and membrane integrity by broth microdilution, ethidium bromide accumulation and LIVE/DEAD BacLight™ assays. *Results:* *L. monocytogenes* showed substantial susceptibility toward *N. sativa* EO, thymoquinone, and carvacrol. A significant reduction in MIC's of EtBr and ciprofloxacin was noticed when tested in combination with *N. sativa* EO, thymoquinone, carvacrol and reserpine. In the presence of each compound the membrane integrity was disintegrated, and the EtBr accumulation increased which was comparable to positive control reserpine. *Conclusion:* *N. sativa* EO might have a potential for controlling the antibiotic resistance in *Listeria*.

More than 200 known diseases are transmitted through food by a variety of agents that include bacteria, fungi, viruses, and parasites. Food quality and safety is becoming an important public health issue due to the increased risk of

foodborne illness over the last 20 years (1) and rapid spread of multidrug resistant (MDR) bacteria. This has necessitated the discovery of new antibacterial and resistance modifying agents (2).

Bacterial multidrug efflux pumps help bacterial populations to increase their resistance and survive in the presence of antimicrobial substances. Efflux pumps (EPs) are transporter proteins found in Gram-positive and Gram-negative bacteria, which are involved in the removal of toxic substances from the interior of the cell to the external environment (3, 4). The main cause for MDR bacteria is the overexpression of EPs able to extrude two or more unrelated antibiotics, prior to reaching their intended targets (5). Ethidium bromide (EtBr) and other fluorescent molecules, are used as substrates extruded by EPs to demonstrate efflux activity (6, 7).

*Listeria monocytogenes* is one of the most important food-borne pathogen that has been isolated from a variety of food products (8). Two efflux pumps have been described in *L. monocytogenes* (9). The efflux pump designated as MdrL, can extrude antibiotics (macrolides and cefotaxime), heavy metals, and EtBr (10). Another efflux pump, termed as Lde, is associated with fluoroquinolone resistance and, in part, with resistance to acridine orange and EtBr (11).

The interest in plant products as alternative antimicrobial agents to control pathogenic microorganisms has been increased due to the big number of antibiotic resistant bacteria (12). A major group of plant antimicrobial compounds is represented by essential oils, which are complex mixtures of volatile secondary metabolites. They are used in the food industry because of their preservative potency and their antimicrobial effect against food-borne pathogens. Essential oils and their bioactive compounds, besides their antimicrobial effect, can increase antimicrobial activity of some antibiotics (13).

*Nigella sativa* essential oil contains significant amounts of phenolic compounds (*i.e.*, *p*-cymene (*p*-cy), carvacrol (Car)

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*Key Words:* *N. sativa* essential oil, *Listeria monocytogenes*, antimicrobial activity, resistance modifying activity.

Table I. Minimal inhibitory concentration in µg/mL of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene on nine *L. monocytogenes* strains.

<i>L. monocytogenes</i> strain	Source	<i>N. sativa</i> essential oil	Thymoquinone	Carvacrol	p-cymene
L2	11/4.12.t03	116	40	150	>2144
L6	E ST/10.12.2	116	40	150	>2144
L14	10/10.12.2	116	40	150	>2144
L7	E ST/10.12.3	233	40	150	>2144
L4	E ST/10.12.1	233	40	150	>2144
L1	E 12/10.12.1	233	40	150	>2144
L9	8/4.12.t0	233	40	150	>2144
LI	NCTC, serotype 4b	466	40	150	>2144
LA	CCM4699	233	40	300	>2144

and thymoquinone (Thq)), that might be the reason of the antimicrobial potential of *Nigella sativa* essential oil (14). Thymoquinone is one of its most active constituent and has different beneficial properties. Focusing on antimicrobial effects, different extracts of *N. sativa* as well as Thq, have a broad antimicrobial spectrum including Gram-negative, Gram-positive bacteria, viruses, parasites and fungi (15).

The effect of reserpine on efflux activity is often used as the benchmark to which the activities of novel efflux pump inhibitors are compared. Reserpine functions as an inhibitor of many MDR efflux pumps, although the exact mechanism of this activity has not been clarified.

This study reports the antibacterial and resistance modifying activities of *N. sativa* essential oil and its active component thymoquinone, carvacrol, and p-cymene alone and in combination with erythromycin, ciprofloxacin and EtBr against nine *L. monocytogenes* strains.

## Materials and Methods

**Cultures.** Nine strains of *Listeria monocytogenes* listed in Table I were used for the study. Strains were from the culture collection of the Department of Microbiology and Biotechnology, Faculty of Food Science, Szent István University, Budapest, Hungary. Each strain of *L. monocytogenes* was individually cultured in Tryptic Soy Broth (TSB).

**Chemicals.** Erythromycin (Ery), ciprofloxacin (Cip) and ethidium bromide (EtBr) were obtained from Sigma Aldrich Co. (Schnellendorf, Germany). Erythromycin and ciprofloxacin were dissolved in dimethyl sulfoxide (DMSO) in order to prepare the stock solutions, which were diluted with TSB to get the appropriate concentration. EtBr was dissolved in sterile distilled water to prepare the stock solution and then diluted with TSB to get the appropriate concentration.

**Essential oil and active compounds preparation.** *N. sativa* seeds were purchased from 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 stored finally in refrigerator for further analysis.

Thymoquinone and carvacrol were purchased from Sigma Aldrich Co. and p-cymene was purchased from Alfa Aesar (Karlsruhe, Germany). The essential oil and thymoquinone were diluted using DMSO, while ethanol (EtOH) was used as diluent for carvacrol and p-cymene to prepare the stock solutions and further on diluted with TSB to get the appropriate concentration.

**Antimicrobial activity.** The antimicrobial activity of *N. sativa* EO, thymoquinone, carvacrol, and p-cymene were determined using the broth microdilution method (16). Briefly, the stock solution of the essential oil, thymoquinone, carvacrol or p-cymene were serially half diluted in TSB in microtiter plates, and then each *L. monocytogenes* overnight culture was added at a concentration of 10<sup>6</sup> CFU/ml, to the final volume of 0.1 ml/well. After 24 h of incubation at 37°C, 10 µl of resazurin reagent, which consisted of 10 M resazurin sodium salt and 0.8 mM menadione, was added to each well. Following a 2 h incubation at 37°C, the fluorescence intensity was measured at 550 nm and 959 nm, using a microplate reader (Victor x3, PerkinElmer, Waltham, MA, USA). The minimum inhibitory concentrations (MICs) were defined as the minimal concentration at which the fluorescence signal declined to the level of the blank. All the MICs measurements were carried out in triplicates. The diluent controls were obtained by preparing culture medium with the bacterial suspension and DMSO or EtOH with the bacterial suspension corresponding to the highest concentration present in the preparation. The negative control was obtained by preparing the culture medium only or the culture medium with the given antimicrobial. The same assay was used for the determination of the MICs for the antibiotics and EtBr.

**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 essential oil and thymoquinone (1/2 MIC). The modulation factor was defined as the ratios of the MICs for the antimicrobials (Ery, Cip or EtBr) alone and for the antimicrobial agent in the presence of the EO, Thy, Carand 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

Table II. Minimum inhibitory concentration values and modulation factors for *L. monocytogenes* strains.

Antimicrobial	<i>Listeria monocytogenes</i> strain MIC ( $\mu\text{g/ml}$ )								
	L2	L6	L14	L7	L4	L1	L9	LI	LA
EtBr <sup>a</sup>	128	64	128	128	128	128	128	128	64
MF (+EO)	16	8	16	8	4	8	4	4	8
MF (+Thq)	8	8	8	8	8	8	8	4	8
MF (+Car)	16	16	8	8	16	16	8	4	2
MF (+p-cy)	2	4	4	2	2	2	2	2	4
MF (+Res)	8	8	8	8	8	8	8	8	8
Ery <sup>a</sup>	0.125	0.125	0.125	0.25	0.25	0.25	0.25	0.25	0.25
MF (+EO)	2	2	2	2	4	2	2	4	2
MF (+Thq)	2	2	2	4	2	2	2	2	2
MF (+Car)	2	2	2	4	2	2	2	2	2
MF (+p-cy)	2	2	2	1	1	2	2	2	2
MF (+Res)	1	1	1	1	1	1	1	1	1
Cip <sup>a</sup>	1	0.25	0.25	0.5	0.25	0.25	0.125	0.25	0.25
MF (+EO)	2	4	8	4	4	4	4	4	2
MF (+Thq)	4	4	2	4	2	4	4	4	2
MF (+Car)	4	4	4	4	4	4	4	2	4
MF (+p-cy)	2	2	4	4	2	2	2	2	2
MF (+Res)	4	4	4	2	2	4	4	2	2

<sup>a</sup>MIC. <sup>b</sup>MF: Modulation factors, decrease in the MIC.

were prepared with the bacterial suspension only, the bacterial suspension and  $\frac{1}{2}$  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.

**Ethidium bromide accumulation assay.** The influence of *N. sativa* EO, Thq, Car and p-cy on EtBr accumulation in *L. monocytogenes* L14 was determined (17). Briefly, 150  $\mu\text{l}$  of overnight culture (4 ml inoculum, 24 h) was added to 9.9 ml TSB, centrifuged at 6000g for 5 min, then the cells were two times washed and resuspended in phosphate-buffered saline (PBS) (OD<sub>600</sub>, 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 incubation at 37 °C, 96.74  $\mu\text{l}$  of the untreated culture, the treated culture containing EO, thymoquinone, carvacrol or p-cymene, were pipetted in black microtiter plates followed by the addition of 3.26  $\mu\text{l}$  EtBr to a final concentration of 0.5  $\mu\text{g/ml}$ . The kinetics of intracellular EtBr accumulation were measured at 490 nm and 579 nm using a Victor x3 plate reader (PerkinElmer), at 45-sec intervals for 1 h. Additionally reserpine (100  $\mu\text{g/ml}$ ) was used as a positive control reference in the assay. Measurements were carried out in triplicates and the means of the last 10 times points of the measurements were used in the statistical analysis.

**Membrane integrity.** The influence of *N. sativa* EO, thymoquinone, carvacrol, and p-cymene on membrane integrity of *L. monocytogenes* L14 was assessed using LIVE/DEAD BacLight™ Bacterial Viability Kits (L-7012, Molecular Probes, Eugene, Oregon, OR, 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 (18). Briefly, exponential phase culture was washed two times and resuspended in phosphate-buffered saline (OD<sub>600</sub>, 0.2) 1 ml of the culture was heat treated at 80 °C for 15 min to obtain the dead culture, while the treated cultures with the EO, thymoquinone, carvacrol or p-cymene were incubated at 37 °C for 15 min. A mixture of SYTO 9TM (6  $\mu\text{l}$ ) and propidium iodide (6  $\mu\text{l}$ ) was added to 2 ml filtered (0.2  $\mu\text{m}$  pore size filter) distilled water. This dye mixture was added to 100  $\mu\text{l}$  *L. monocytogenes* culture (1:1, v/v) that was untreated or treated with *N. sativa* EO, thymoquinone, carvacrol or p-cymene or heat treated. The kinetics of propidium iodide and SYTO 9 intracellular penetration were followed by measuring the relative fluorescence units (RFU) in 60-sec intervals over 1 h, at 490 nm and 535 nm using a microplate reader (Tecan, Männedorf, Switzerland). The experiments were carried out in triplicate in black microtiter plates. The membrane disruption (%) was calculated from the kinetic measurements of the treated relatively to the untreated cultures over the last 10 min of the assay.

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

## Results

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

As presented in Table I, the essential oil of *N. sativa*, thymoquinone and carvacrol were active against all the

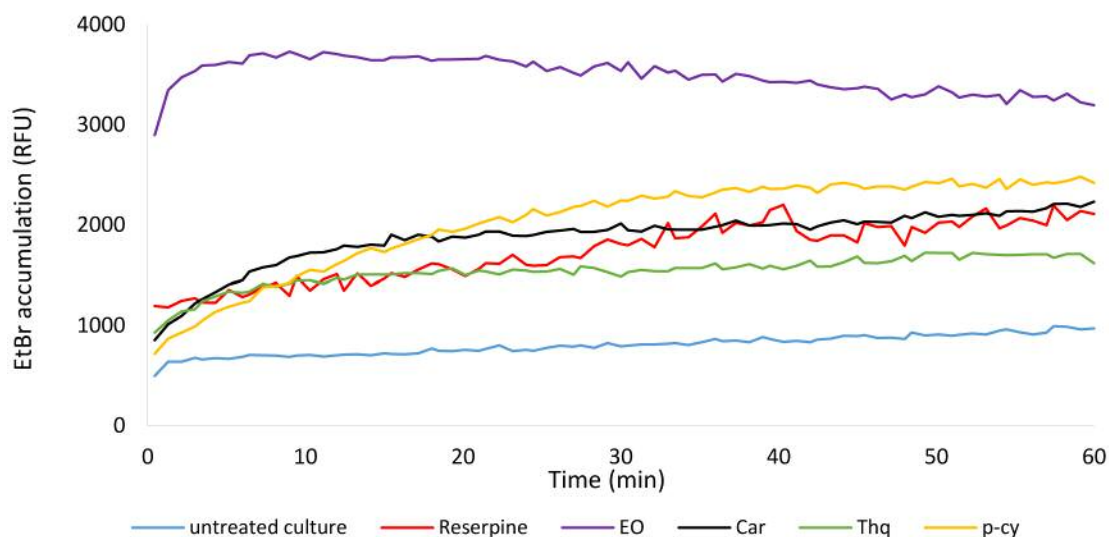


Figure 1. Ethidium bromide accumulation in *L. monocytogenes* L14 in the presence of sub-inhibitory concentration (1/2 MIC) of *N. sativa* essential oil, thymoquinone, carvacrol, and *p*-cymene, and reserpine as positive control. RFU: Relative fluorescence units; EtBr: ethidium bromide; EO: essential oil; Thq: thymoquinone; Car: carvacrol; p-cy=*p*-cymene.

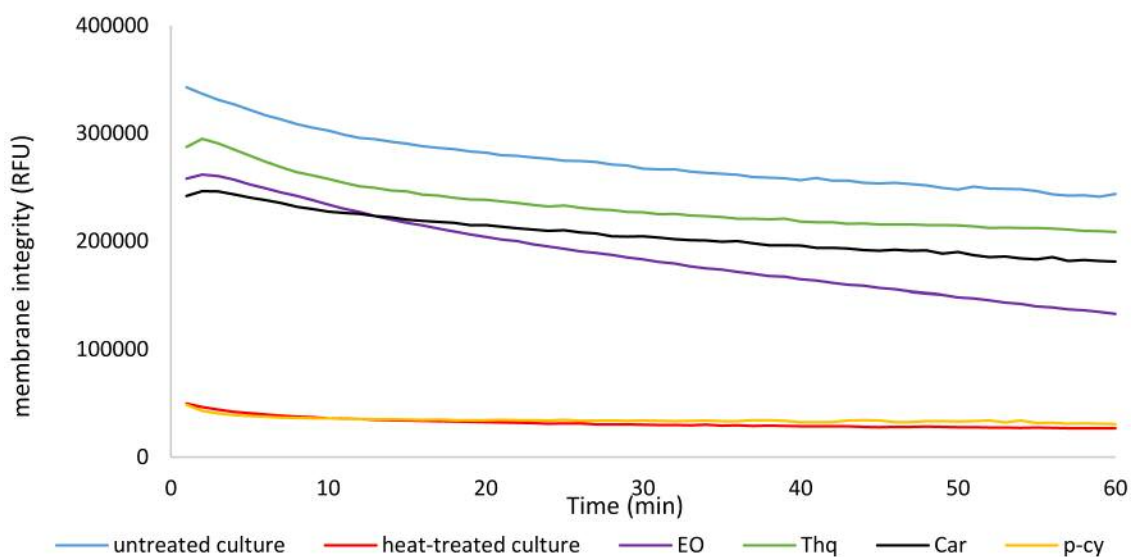


Figure 2. Influence of sub-inhibitory concentration (1/2 MIC) of *N. sativa* essential oil, thymoquinone, carvacrol and *p*-cymene on membrane integrity of *L. monocytogenes* L14. RFU: Relative fluorescent unit; EO: essential oil; Thq: thymoquinone; Car: carvacrol and p-cy: *p*-cymene.

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 value 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 (2,144 µg/ml) against any strain.

As a potential modulator of antimicrobial resistance, *N. sativa* EO, thymoquinone, carvacrol and *p*-cymene were tested at 1/2 MIC (58 µg/ml, 20 µg/ml, 75 µg/ml and 1,072 µg/ml, respectively) in combination with the antibiotics erythromycin, ciprofloxacin, and the antimicrobial efflux pump substrate EtBr, on nine *L. monocytogenes* test strains (Table II).

Data presented in Table II show that the supplementation of *N. sativa* EO (at 1/2 MIC) decreased the MICs of erythromycin from two-fold to four-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 a decrease in the MIC of erythromycin from two-fold up to four-fold against seven *L. monocytogenes* strains and had no activity against the other three strains. Furthermore, a four-fold to eight-fold reduction of EtBr was noted against *L. monocytogenes* strains (Table II).

Carvacrol supplementation (at 1/2 MIC) decreased the MIC of erythromycin: 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 (Table II).

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 four strains. P-cymene induced decrease in the MIC of EtBr from two-fold up to four-fold against all *L. monocytogenes* strains (Table II).

In order to elucidate the mechanism of the modulatory activity of *N. sativa* essential oil and its active compounds, thymoquinone, carvacrol, and p-cymene in *L. monocytogenes* L14, the potency of the compounds was evaluated 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* EO, p-cymene, carvacrol, and thymoquinone, respectively, compared to the untreated culture of *L. monocytogenes* (Figure 1). *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, carvacrol or p-cymene decreased by 43%, 14%, 25% and 87% respectively (Figure 2). 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 1 h assay, and they were statistically significant ( $p < 0.00001$ ). The disruptive impact of p-cymene on membrane integrity is not surprising, as it is known that monoterpenes can cause alteration in membrane permeability (19).

## Discussion

In general, antimicrobial resistance alters the activity of antibiotics through one of the following mechanisms: antimicrobial target modification (decreasing drug affinity), a decrease in drug absorption, activation of efflux mechanisms to expel the toxic molecules (overexpression of efflux pumps) or global changes in important metabolic pathways through the modulation of regulation networks (20). The search of new efflux pump inhibitors is necessary to combat the emergence of antibacterial resistant strains (21).

Data presented in Table I show that all tested strains were sensitive to *N. sativa* essential oil, thymoquinone and carvacrol, while p-cymene did not show any activity against the tested *L. monocytogenes* strains. These results support previous studies which reported an effective antibacterial activity of *N. sativa* essential oil (22), thymoquinone (23) and carvacrol (24) against Gram-positive bacteria. DMSO and EtOH as diluents did not show any inhibitory effect on the growth of the strains.

Efflux is an important mechanism of resistance in *L. monocytogenes* (10). The efflux pumps are proteins of bacterial membranes which extrude and limit the intracellular accumulation of antibiotics and other antimicrobial agents (5). It was previously identified that the increased resistance to ciprofloxacin (a fluoroquinolone) confers also increased resistance to EtBr, and an increased expression of the Lde transporter (11). Our data showed the potential of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene to decrease the MIC of ciprofloxacin at least two-fold and up to eight-fold.

Additionally, in a previous study, increased susceptibility to macrolides resulted in the inactivation of the MdrL in *L. monocytogenes* (10). The MIC of erythromycin (a macrolide) decreased in the presence of *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene at least two-fold up to four-fold, while reserpine had no activity with erythromycin.

EtBr is a substrate of many MDR pumps and causes fluorescence when bound to DNA (25). Multidrug efflux pumps are known to cause resistance to this agent, thus the

effects of inhibition of efflux can be assessed fluorometrically (26, 27). As found previously, the MICs of EtBr were also lower in combination with *N. sativa* essential oil, thymoquinone, carvacrol, and p-cymene (Table II).

*Nigella sativa* essential oil, thymoquinone, carvacrol, and p-cymene induced the increase of EtBr accumulation in the treated strain and was comparable to the chemical EPI, reserpine. The inhibition of EtBr via a number of efflux pumps has been already reported for *L. monocytogenes* (10, 11).

Since plant essential oils and their components are hydrophobic in nature, their primary target is the bacterial membrane to make it more permeable, which could permit an increased uptake of the antimicrobial agent by the bacterial cell (28).

These observations presume the modulating activity of *N. sativa* essential oil and thymoquinone through efflux pumps inhibition leading to increased accumulation of antibiotics in the cells enhancing their effects at lower doses. So, the inhibition of EtBr efflux supports the hypothesis of antibacterial activity of *N. sativa* essential oil and its active components thymoquinone, carvacrol, and p-cymene through pump efflux inhibition.

Therefore, the results presented in this study suggest that the essential oil of *N. sativa* and its major components thymoquinone and carvacrol act as putative efflux pump inhibitors in *Listeria*, modulating the bacterial resistance to antibiotics. To the best of our knowledge, this is the first report on resistance modifying activity of *N. sativa* essential oil against *L. monocytogenes*.

In conclusion, *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 antimicrobials, showing increased EtBr accumulation. Additionally, by 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 have the potential to be promising modifiers of antimicrobial resistance in *L. monocytogenes*.

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