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ORIGINAL ARTICLE

Combined effects of *Allium sativum* and *Cuminum cyminum* essential oils on planktonic and biofilm forms of *Salmonella typhimurium isolates*

Reza Hakimi Alni[1,2] · Khodayar Ghorban[2,3] · Maryam Dadmanesh[1,2]

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Abstract

Salmonella typhimurium (*S. typhimurium*) represents an important global public health problem and has the ability to survive under desiccation conditions in foods and food processing facilities for years. The aim of this study was to investigate the effects of *Allium sativum* (*A. sativum*) and *Cuminum cyminum* (*C. cyminum*) essential oils (EOs) against planktonic growth, biofilm formation and quorum sensing (QS) of *S. Typhimurium* isolates, the strong biofilm producers. The major components of EOs were determined by gas chromatography–mass spectrometry (GC–MS). Biofilm formation of *S. Typhimurium* isolates was measured by crystal violet staining. Then, the effects of the EOs on the planktonic cell growth (using determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)), measurement of the synergistic effects of EOs (using checkerboard method), biofilm formation (using microtiter-plate test and scanning electron microscope (SEM)), and expression of QS and cellulose synthesis genes (using quantitative real-time PCR) were assessed. Finally, tetrazolium-based colorimetric (MTT) assay was used to examine EOs cytotoxicity on the Vero cell line. GC–MS analysis showed that terpineol, carene and pinene in *C. cyminum* EO and sulfur compounds in *A. sativum* EO were the major components of the plant extract. The Geometric mean of MIC values of the *A. sativum* and *C. cyminum* were 0.66 and 2.62 µL mL^{-1}, respectively. The geometric means of the fractional inhibitory concentration index (FICi) for both EOs were calculated as 1.05. The qPCR results showed that MIC/2 concentrations of both EOs significantly down-regulated of QS (*sdiA* and *luxS*) and cellulose synthesis (*csgD* and *adrA*) genes. Scanning electron microscopy showed the EOs reduced the amount of *S. Typhimurium* mature biofilm. In general, we showed that *C. cyminum* and *A. sativum* EOs can be considered as the potential agents against planktonic and biofilm form of *S. Typhimurium* without any concern of cytotoxic effect at 4 MIC concentrations on the eukaryotic Vero cells.

Keywords *Salmonella typhimurium* · Biofilm · Quorum sensing · *Allium sativum* · *Cuminum cyminum*

Introduction

Salmonella enterica serovar Typhimurium can cause infections in humans and animals. Salmonellosis is one of the most important food-borne diseases in the world that has various economic and health effects on human societies. Poultry meat and its derivatives are the best sources of high-quality proteins, transmitting Salmonella infections in different communities (Dar et al. 2017).

Recently, the antibiotic resistance *Salmonella typhimurium* (*S. typhimurium*) is increasing worldwide, especially those related to β-lactams and macrolide (Shaikh et al. 2015). One of the reasons for resistance to antibiotics is biofilm formations. Biofilms are the bacterial colonies, attached to biotic or abiotic surfaces. The attachment occurs via production of extracellular matrix, called exopolysaccharide (Watnick and Kolter 2000). Due to the matrix, biofilm formation protects bacteria from antimicrobial agents and increases their resistance to disinfectants and antibiotics (Ebrahimi et al. 2016). Compared with planktonic

✉ Maryam Dadmanesh
dadmanesh@gmail.com

[1] Department of Infectious Diseases, School of Medicine, Aja University of Medical Sciences, Tehran, Iran

[2] Infectious Diseases Research Center, Aja University of Medical Sciences, Tehran, Iran

[3] Department of Immunology, School of Medicine, Aja University of Medical Sciences, Tehran, Iran

counterpart, bacteria living in the biofilm statuses are often up to 1000 times more resistant to antibiotics (Caraher et al. 2007; Ebrahimi et al. 2014).

Quorum sensing (QS) is the mechanism of cell-to-cell communication in the biofilm masses and plays an important role in biofilm formation. QS mechanisms include: (i) acylhomoserine lactone (AHL) QS system (in Gram negative bacteria), (ii) autoinducing peptide (AIP) QS system (in Gram positive bacteria) (iii) autoinducer-2(AI-2) QS system (in both Gram negative and positive bacteria). *Salmonella enterica* serovar Typhimurium harbors QS systems activated by autoinducer 2 (AI-2) (Brackman and Coenye 2015). *sdiA* and *luxS* genes, *QS* genes, play important roles in the production of fully developed biofilms in *Salmonella enterica*. The *luxS* gene encodes the AI-2 proteins (i.e., LuxS protein) (Sperandio et al. 2011; Bai and Rai 2011).

In recent years, QS inhibitors (QSI) compounds are considered as the novel prospective targets for antimicrobial therapies(Rasamiravaka et al. 2015). Natural broad QSI compounds have been found in edible vegetables, fruits, seaweeds, marine sponges (Adonizio et al. 2006; Packiavathy et al. 2012; Brackman and Coenye 2015). Some of the above-mentioned components have been extracted from aromatic plants, such as carvacrol (a natural terpene extracted from thyme or oregano) (Burt et al. 2014), casbane diterpene (ethanolic extract of Croton nepetaefolius) (Carneiro et al. 2010) and naphthalene derivative (extracted from *Trachyspermum ammi* seeds) (Chaieb et al. 2011). These components are deemed as biofilm formation inhibitors.

Allium sativum (*A. sativum*) and *Cuminum cyminum* (*C. cyminum*) are widely used as food additives in various communities. These plants have also been used as a medicine, as they comprise different antimicrobial components (Palombo 2011). The current study aimed to study to investigate the antibacterial (bacteriostatic and bactericidal activities), antibiofilm and anti-QS of these plants against *S. typhimurium* biofilms.

Materials and methods

Bacterial strains and biofilm assay

A total of 78 *S. typhimurium* strains which had been previously isolated from chicken meat (38 isolates) and human (40 isolates) between November 2015 and April 2017 (Tehran, Iran) were included in this project. All bacterial isolates were evaluated for biofilm formation by end-smooth 96-cell micro-plates as explained by Tendolkar and colleagues (Tendolkar et al. 2004). In the following, ten isolates that were strong biofilm producers were selected to determine the minimum inhibitory concentration (MIC) of EOs.

Plant materials and essential oils extraction

The bulbs of *C. cyminum* family Apiaceae and bulbs of *A. sativum* (Garlic) family Liliaceae were obtained from Shahid Beheshti Plant Institute, Tehran, Iran. 50 g of each plant was submitted to hydro-distillation in a Clevenger-type apparatus for 4 h according to the standard procedure (El Gendy et al. 2015). The obtained EOs were stored in a dark glass bottle and kept at 4 °C prior to further analysis. The amount of oil obtained from each plant material was calculated as: $\mathrm{EO}\left(\frac{\mathrm{V}}{\mathrm{W}}\%\right) = \frac{\text{volume of oil (mL)}}{\text{weight of samle (g)}} \times 100$.

Gas chromatography–mass spectrometry analysis

The EOs were analyzed by gas chromatography–mass spectrometry (GC–MS) analysis using Hewlett Packard 6890 gas chromatograph system. GC/MS analysis was done on a Thermoquest–Finnigan Trace GC–MS equipped with a DB-5 (5% phenyl) methylpolysiloxane column (60 m × 0.25 mm, film thickness 0.25 µm). Helium was used as the carrier gas at 1 mL/min, and 1 µL of the sample was injected for analysis (Al-Rubaye et al. 2017).

Determination of the MIC and MBC

To do this experiment, the MIC and minimum bactericidal concentration (MBC) of the plant essential oils were evaluated according to the guidelines of National Committee for Clinical Laboratory Standard. Briefly, 100 µL of the bacterial inocula (corresponding to 0.5 of the McFarland) and 100 µL of the twofold serial dilutions of the EOs (0.06–31.5 µL mL^{-1}) in TSB medium were distributed into each well of ELISA microplates (96 well). A negative control, which contained dimethyl sulfoxide (DMSO) and bacteria), and a positive control, which contained DMSO, bacteria and Norfloxacin (1 mg/mL), were also prepared in the last row. It should be noted that DMSO solution was previously used to increase the solubility of the EOs at the final concentration of 0.1%. In the following, ELISA microplates were aerobically incubated at 37 °C in 5% CO_2 for 24 h. MIC was recorded as the lowest concentration of the EOs that no visible growth was detected after overnight incubation (Man et al. 2019). To determine MBC, 5-µL solution from the last three wells that did not show bacterial growth were spot-inoculated on blood agar plates and incubated overnight at 37 °C. The MBC was noted as the lowest concentration at which no bacterial colonies were developed.

Measurement of the synergistic effects

The synergistic effects of *A. sativum* and *C. cyminum* EOs were assessed using the broth microdilution checkerboard method. The fractional inhibitory concentration index (FICi) for two combined antimicrobial agents was calculated as follows: $FICi = \frac{(\text{MIC of EO A in combination})}{(\text{MIC of EO A alone})} + \frac{(\text{MIC of EO B in combination})}{(\text{MIC of EO B alone})}$. The FIC was interpreted as follows: FICi ≤ 0.5: synergy; FICi > 0.5–1.0: addition; FICi < 4.0: indifference and FICi ≥ 4.0: antagonism (Cetin et al. 2013; de Medeiros et al. 2016).

Effect of the EOs on the biofilm formation

The effects of the EOs on the biofilm formation of *S. typhimurium* were determined by Microtiter plate (MtP) assay (Thenmozhi et al. 2009). Briefly, 98 µL of Trypticase Soy Broth (TSB) that contained MIC/2, MIC/4, MIC/8 and MIC/16 concentrations of the EOs was allocated in four wells for each concentration and 2 µL of overnight cultures (~ 1.5 × 108 CFU/mL) added to each well. One microplate well in each row was also assigned to the control sample. The applied negative controls contained medium DMSO with *S. typhimurium*. After incubation without agitation for 24 h at 37 °C, the planktonic cells were removed. The surface-adhered cells were stained with 200 µL of 0.2% crystal violet (Hi Media, India) for 20 min and the excess dye solution was removed and the wells were washed with PBS. In the following, crystal violet in the stained cells was solubilized with 100 µL of 95% ethanol and optical density values (OD) was quantified using UV–Vis spectrophotometer (Bio-Tek, Winooski, USA). Eventually, the percentage of inhibition was obtained by the following formula: percentage of inhibition = 100 − [(OD490 nm of the treated wells)/(mean OD490 nm of the negative control wells contained no antimicrobial agent) × 100)] (Onsare and Arora 2015).

Quantitative real-time PCR

The effect of sub-MIC concentrations (MIC/2) of *C. cyminum* and *A. sativum* EOs on the expression of QS (*sdiA* and *luxS*) and cellulose synthesis (*csgD* and *adrA*) gens were measured in clinical isolates (8 strains) and reference strain (*S. typhimurium ST38*, Pasteur Institute of Iran). Total RNA was extracted using a RNA Isolation Kit (SinaClon, Iran), quantified by BioDrop (BioDrop, UK) and was stored at − 80 °C until further use. Then, complementary DNA (cDNA) was synthesized from RNA template, using the cDNA Reverse Transcription Kit and random primer oligonucleotides (SinaClon, Iran). The primers, qPCR assays and thermocycling conditions was determined as described previously (Latasa et al. 2005; Halatsi et al. 2006; Karavolos et al. 2008; Lee et al. 2009). The primer sequences used in this study are shown in Table 1. The target genes expression levels in comparison to the internal 16 s rRNA control were evaluated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), where ΔΔCt = (Ct target genes-Ct 16 s rRNA) treatment−(Ct target genes-Ct 16 s rRNA) control. Three experimental and three technical replicates were used to determine the relative fold changes. FC is presented as mean–standard deviation (SD) from at least three independent qPCR amplifications from each RNA preparation.

Electron microscopy (SEM)

SEM was employed for investigating the effect of each EO (MIC/2 concentration) on the biofilm structure of *S. typhimurium* isolate. A glass slide was placed in TSB medium with each EOs (final concentration of essential oil = MIC/2), and incubated for 24 h at 37 °C. Then, the glass slides were extracted from the medium and transported to the pathology laboratory for fixation (in 2.5% buffered glutaraldehyde) and preparation for observation by scanning electron microscope (JEOL JSM-840) at an accelerating voltage of 15 kV.

Table 1 Primers used in real-time PCR assays

Target gene	Sequence 5′–3′	References
16 s rRNA	F: AGGCCTTCGGGTTGTAAAGT R: GTTAGCCGGTGCTTCTTCTG	Karavolos et al. (2008)
bapA	F: ATGCGCCCAACATTCCTCT R: TGGATGACTGTGCCTGCG	
adrA	F: GAAGCTCGTCGCTGGAAGTC R: TTCCGCTTAATTTAATGGCCG	
sdiA	F: AATATCGCTTCGTACCAC R: GTAGGTAAACGAGGAGCAG	Onsare and Arora (2015)
luxS	F: ATGCCATTATTAGATAGCTT R:GAGATGGTCGCGCATAAAGCCAGC	Thenmozhi et al. (2009)

Cytotoxicity assay

Cytotoxicity of EOs was evaluated using 3-(4, 5-dimethylthiazol-2-yl)—2, 5-diphenyltetrazolium bromide (MTT) assay through the previously described method. Vero cells were cultured in the RPMI-1640 medium supplemented with 10% fetal calf serum, 1% (w/v) glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin as the growth medium (All materials prepared from Gibco, UK). Then, the EOs were added at different concentrations from 0.156 to 20 µL mL-1 in 96-well plates and distributed by Vero cells (cell density = 2 × 104 to 2 × 105). After incubation of plates (37 °C in humidified air containing 5% CO2 for 24 h) and removing of the MTT dye, fluorescence signals were measured at 490 nm using a microplate reader (ELx808, BioTek, USA).

Statistical analysis

Each experiment was performed at least three times, and the data are expressed as means + SD. Eventually, the results were analyzed by Student's *t* test using SPSS software and $p < 0.05$ was considered statistically significant.

Results

Biofilm assay

According to the previous study by Tendolkar and colleagues, isolates were categorized in four groups: (i) non-biofilm forming (OD ≤ ODc), (ii)weak biofilm forming (ODc < OD ≤ 2 ODc), (iii) moderate biofilm forming (2 OD < OD < 4 ODc) and (iv) strong biofilm forming (OD > 4ODc) bacteria. Overall, a total of 78 *Salmonella* isolates, 30.76% was non-biofilm producers (9 isolates from human, 15 isolates from chicken), 20.5% weak biofilm producers (5 isolates from human, 11 isolates from chicken), 21.8% moderate biofilm producers (12 isolates from human, 5 isolates from chicken) and 27% (14 isolates from human, 7 isolates from chicken) was strong biofilm producers.

GC/MS analysis of essential oils

The amount of oil obtained from *C. cyminum* and *A. sativum* was 2.7% and 1.2%, respectively. The results regarding the GC/MS analysis of essential oils are given in Table 2. In this study, the EO obtained from *C. cyminum* was mainly composed of cuminaldehyde (34.37%), followed by α,β-dihydroxyethylbenzene (22.25%), 2-caren-10-al (16.11%) and γ-terpinene (8.65%); while, the EO from *A. sativum* contained diallyl tetrasulfide (29.47%), allyl disulfide (22.49%), nitrosothymol (8.55%) and 1H-1,2,4-triazole, 3-thiol-5-methyl (7.75%).

MIC and MBC determinations

The Geometric mean of the MIC and MBC values of *A. sativum* and *C. cyminum* EOs for all isolates were 0.66, 2.18, 2.62 and 5.24 µL mL^{-1}, respectively. Further details are shown in Table 3

Measurement of the synergistic effect

A synergistic action of *A. sativum* and *C. cyminum* EOs against planktonic growth isolates was detected by the checkerboard assay and calculation of the Fractional Inhibitory Concentration (FIC) Index. There were no antagonistic effects among the 8 isolates studied, and in the two isolates an addition effect was observed. The geometric mean of the FICi for all strains for EOs was 1.05 (Table 3). A paired sample *t* test showed significant difference between ΣFIC results of multi-drug resistant (MDR) and non-MDR isolates ($p < 0.05$).

Effects of the EOs on the biofilm formations

In Table 4, the biofilm formation of the 9 strains (8 isolates and one standard isolates) in the presence of different concentrations of sub-MICs concentrations of both EOs is presented. In fact, as the concentration of the EOs decreased and the amount of optical absorption increased, indicating an increase in biofilm formation. In MtP assay, sub-MICs concentrations of both EOs (Except MIC/16 EO of *C. cyminum*) play significant roles in inhibition of biofilm formation by all of the bacteria ($p < 0.001$).

Scanning electron microscopy (SEM)

In the control slides, the bacteria were tightly clinging together and encased in a thick matrix, but in bacteria, cells treated with MIC/2 showed the predominant release of cells from biofilms (Fig. 1).

Quantification of gene expression by qPCR

The results of qPCR showed that the expression levels of QS (*sdiA* and *luxS*) and cellulose synthesis (*csgD* and *adrA*) genes were significantly ($p < 0.01$) reduced in the clinical and reference strains, treated by MIC/2 of Eos, compared to the negative control, non-treated by EOs. As shown in the Table 5, the EO of *C. Cuminum* has reduced the expression of all genes in all isolates; however, the increased FC was less than 2 in 7 cases, and they were not statistically significant. *A. sativum* EO caused a significant down-regulation of *csgD* (6 of 9 strains), *adrA* (8 of 9 strains) and all of the

Table 2 Chemical compositions of the *Cuminum cyminum* and *Allium sativum* essential oils

No	*Cuminum cyminum*			*Allium sativum*		
	Compound	%	RT	Compound	%	RT
1	δ-3-Carene	0.04	9.754	1,2-Dithiolane	0.08	4.557
2	trans-p-Menth-2-ene	0.06	9.376	Diallyl sulfide	0.23	13.896
3	1-Phenylethanol	0.08	7.143	5-Chloro-beznofurazan oxide	0.34	14.096
4	α-Terpinene	0.08	9.478	Methyl 4-methoxy-4,8,12,16-tetramethylheptadecanoate	0.42	25.843
5	trans-Sabinene hydrate	0.09	9.479	Pyridine, 2-(1,2,4-oxadiazol-3-yl)	0.56	25.408
6	α-Terpinene	0.12	14.832	Allyloxy-butyldimethylsilane	0.67	20.281
7	Phellandrene	0.13	8.546	*cis*-Propenyl methyl disulfide	0.85	4.77
8	α-Thujene	0.16	6.274	Diallyl hexasulfide	0.87	1.23
9	Terpineol, Z-.beta	0.16	13.321	Benzimidazole, 2-amino-1-methyl-	0.87	27.497
10	Sabinene	0.18	8.346	Diallyl pentasulfide	0.98	1.32
11	β-phellandrene	0.19	10.405	Semioxamazide (Thiourea, *N*,*N*′-dimethyl-)	1.02	7.046
12	Cumene	0.24	8.232	2,3-Dicarboxythiophene	1.06	1.75
13	p-Menth-1-en-8-ol	0.42	18.354	3-vinyl-[4H]-1,2-dithiin	1.11	12.459
14	β-Myrcene	1.42	6.731	2(1H)-Quinolinone, 3-fluoro-4-hydroxy-	1.33	30.461
15	1,3-Benzenediol, 4-ethyl	1.61	22.432	Arachidonic acid, trimethylsilyl ester	1.34	23.915
17	4-Isopropyl-1,3-cyclohexadien-1-yl	1.76	17.556	Diallyl disulfide	1.79	18.685
18	α-Pinene	2.21	6.757	Silane, butyltrimethoxy-	2.16	25.094
19	UNKNOWN FROM LIME OIL	2.53	27.621	M-Dithiane (Catecholborane)	2.18	6.079
20	4-Terpineol	3.73	13.485	Thiopropionamide	2.59	18.284
21	p-Mentha-1,4-dien-7-ol	4.21	29.226	1,3,5-Trithiane	3.07	12.065
22	β-Pinene	5.43	8.216	Allyl sulfide	5.32	4.866
23	Benzene, 1-methyl-4-(1-methylethyl)	6.33	8.892	1H-1,2,4-Triazole, 3-thiol-5-methyl-	7.75	22.805
24	γ-Terpinene	9.39	10.125	Nitrosothymol	8.55	28.87
25	2-Caren-10-al	12.14	23.793	Diallyl disulfid	22.49	10.583
26	α,β-Dihydroxyethylbenzene	15.16	24.543	Diallyl tetra sulfide	29.47	16.79
27	p-Cumic aldehyde	28.67	21.423	–	–	–

Table 3 MIC, MBC and FICi of *Allium sativum* and *Cuminum cyminum* EOs against the isolates

Isolates	Source	EOs (μL mL^{-1})				
		Allium sativum		*Cuminum cyminum*		FICi
		MIC	MBC	MIC	MBC	EOs
ST38	–	0.98	1.96	1.96	3.93	0.77
MDR	Human	0.98	1.96	3.93	7.86	0.64
MDR	Human	0.98	3.93	3.93	7.86	0.64
Non-MDR	Human	0.5	1.96	1.96	3.93	1.26
Non-MDR	Human	0.5	1.96	1.96	3.93	1.26
MDR	Chicken	0.5	1.96	3.93	7.86	1.13
Non-MDR	Chicken	0.5	1.96	1.96	3.93	1.26
Non-MDR	Chicken	0.5	1.96	1.96	3.93	1.26
Non-MDR	Chicken	0.5	1.96	1.96	3.93	1.26
Geometric mean		0.66	2.18	2.62	5.24	1.05

MDR Multidrug resistant

Table 4 Effects of the EOs in different concentrations on the biofilm formations

Isolates	Optical density/*Allium sativum*					Optical density/*Cuminum cyminum*				
	MIC/2	MIC/4	MIC/8	MIC/16	Control	MIC/2	MIC/4	MIC/8	MIC/16	Control
ST38	1.52	1.73	1.82	2.13	2.83	1.48	1.65	1.91	2.32	2.74
MDR	1.74	1.92	2.03	2.52	2.71	1.76	1.94	2.41	2.73	2.77
MDR	1.67	1.84	1.91	2.37	2.67	1.78	1.95	2.48	2.78	2.85
Non-MDR	1.58	1.81	1.89	2.34	2.7	1.63	1.82	2.39	2.64	2.71
Non-MDR	1.53	1.75	1.84	2.25	2.55	1.71	1.87	2.34	2.66	2.68
MDR	1.67	1.81	1.86	2.26	2.68	1.69	1.82	2.29	2.58	2.63
Non-MDR	1.55	1.77	1.82	2.28	2.55	1.65	1.85	2.44	2.5	2.57
Non-MDR	1.46	1.63	1.72	2.23	2.63	1.68	1.87	2.23	2.49	2.58
Non-MDR	1.51	1.72	1.81	2.31	2.73	1.55	1.71	2.17	2.55	2.65

sdiA and *luxS* genes in the clinical isolates. In general, the expression of QS genes was further inhibited in comparison with the cellulose genes.

Cytotoxicity assay

IC50 values for EO *C. cyminum* and *A. sativum* were calculated to be 39.5 µL mL^{-1} and 33.7 µL mL^{-1}, respectively. Detailed data are presented in Fig. 2.

Discussion

The formation of biofilms is a dynamic and complex process that plays important roles in bacterial resistance to antimicrobial agents. Biofilm formation by *S. typhimurium* was considered as a chronicity factor for diseases caused by these bacteria. Also, biofilm formation by salmonella SPP is of critical importance in food industries, and may induce massive outbreaks of the infection (Galiè et al. 2018).

In this study, the ability of biofilm formation by *S. typhimurium* isolated from food and clinical samples was measured using phenotypic and molecular methods. According to our results, 69.2% of Salmonella isolates were capable of forming biofilm while 39% of these isolates formed strong biofilms. However, 67% of strong biofilm producer strains belonged to human isolates. These findings indicated that the human isolates have higher probability of pathogenicity compared to chicken isolates. Sereno et al., (2017) reported that 72% of *Salmonella* sp. strains isolated from frozen poultry carcasses in Brazil were able to produce biofilm, but none of the isolates showed strong biofilm (Sereno et al. 2017).

Due to the increased resistance to antimicrobial agents, more studies have been performed for controlling biofilm formation by new chemical component. Various natural components such as EOs of *C. cyminum* and *A. sativum* have been used to control of bacterial biofilms.

The MIC results demonstrated that the EOs of *C. cyminum* and *A. sativum* had strong antimicrobial activities against *S. typhimurium* isolates. The antibacterial properties of *A. sativum* are mainly due to the presence of sulfur compounds (Ikram et al. 2019). Also, *C. cyminum* EO has some compounds, such as pinene, carene, α and β terpineols, Menthol, which have antimicrobial effects. In the present study, the results of the GC analysis showed that the sulfur compounds and phenolic compounds were at an acceptable level in the EOs of *A. sativum* and *C. cyminum,* respectively. This is in agreement with the previously published reports, wherein, the EOs containing sulfur and phenolic compounds have strong antimicrobial and antibiofilm activities (Packiavathy et al. 2012; Li et al. 2018).

The synergistic inhibitory effects of EOs on planktonic cell growth were evaluated, using the FICi value with the checkerboard method. We found that the EOs did not have a synergistic effect against *S. typhimurium* isolates. However, in some of the isolates, the addition effect was observed, which was probably due to the same antimicrobial mechanism.

The anti-biofilm properties of the EOs were examined, using MtP assay. To do this, we used sub-MIC concentrations of the EOs which were insufficient to inhibit the bacterial growth. The results indicated that these EOs can inhibit *Salmonella* biofilm formation at a sub-MIC concentration. In this regard, Nidadavolu et al. reported that garlic ointment (GarO) could be used as a biofilm inhibitor by wound pathogens including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains (Nidadavolu et al. 2012). Researchers have shown that the garlic component allicin is one of the main factors in inhibiting of biofilm formation by pathogens (Jakobsen et al. 2012, Nidadavolu et al. 2012, Wu et al. 2015). In the present study, allicin was considered as the second most common substance and included 22% of garlic EO, and this can be considered as a reason for

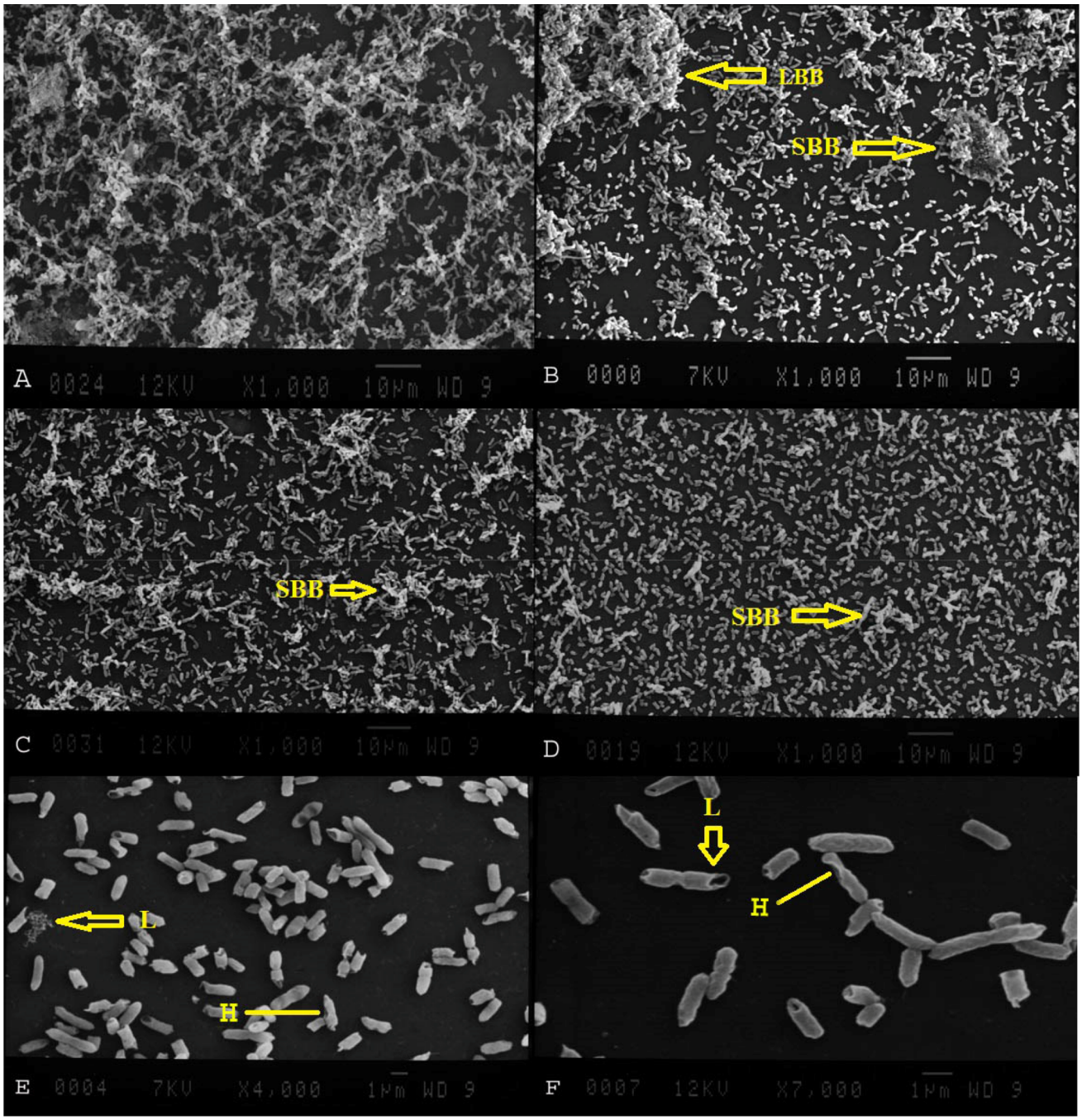


Fig. 1 Effect of EOs on biofilm of *S. typhimurium*. **a** Untreated group. **b**, **e** *A. sativum* at MIC/2, **c**–**f** *C. cyminum* at MIC/2. *LBB* Large biofilm biomass, *SBB* small biofilm biomass, *H* holes, *L* lysis

controlling *Salmonella* biofilms. The phenolic compounds in *C. cyminum* are also known as biofilm inhibitors of bacteria. They interfere with flagella motility and exopolysaccharides (EPS) production by bacteria, and promote the loosening of biofilm architecture (Packiavathy et al. 2012).

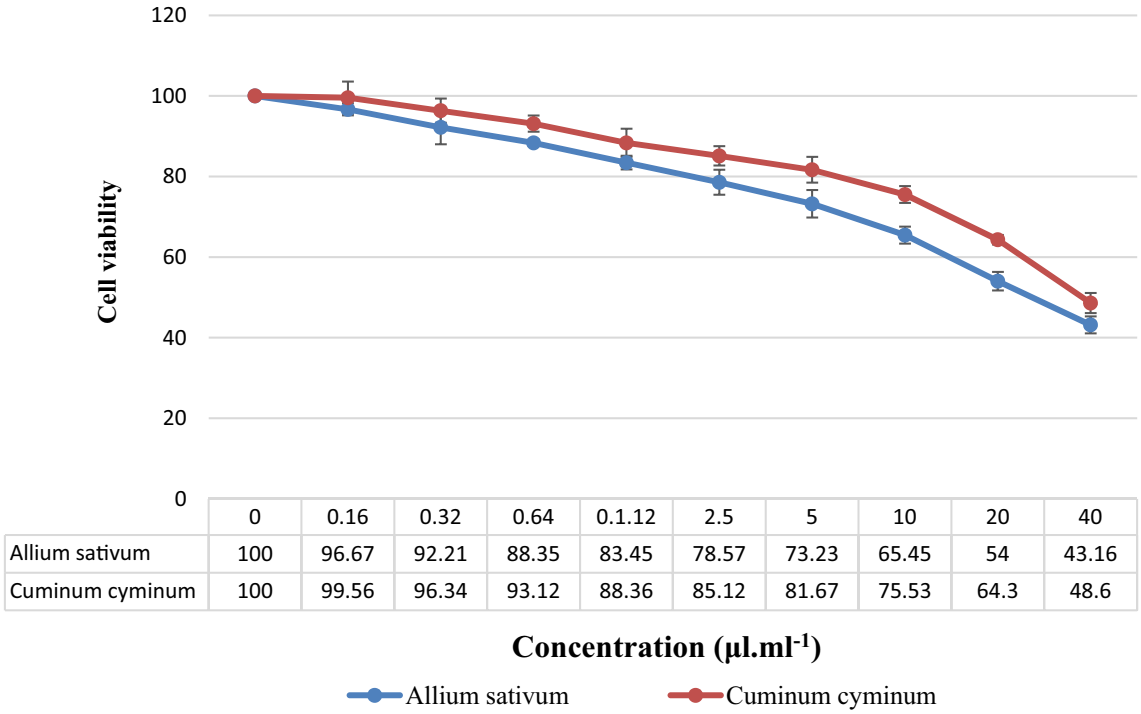
SEM is the most commonly used method for analysis of biofilm morphology, which assists in a greater understanding of formation and persistence (Wilson et al. 2017). In the current study, using SEM images demonstrated that MIC/2 concentration of *C. cyminum* and *A. sativum* EOs could inhibit formation of biofilm by *S. typhimurium*. The mechanism of this EOs impact on biofilm formation has not been completely clear though some of the studies have shown that this formation might be connected with inhibition of bacterial adhesion (Nakamoto et al. 2020). Researchers have shown that EO of *A. sativum* inhibits biofilm formation by

Table 5 The expression of quorum sensing (QS) and cellulose synthesis gens in the bacteria that were treated with 1/2MIC of *Cuminum cyminum* and *Allium sativum* essential oils

Strains	*Cuminum cyminum*				*Allium sativum*			
	sdiA	*luxS*	*csgD*	*adrA*	*sdiA*	*luxS*	*csgD*	*adrA*
Standard strain (ST38)	−4.04*	−3.41*	−3.83*	−2.75*	−3.46*	−3.31*	−4.78*	−3.96*
MDR	−2.82*	−3.05*	−2.72*	−3.56*	−4.31*	−2.56*	−1.73#	−2.27*
MDR	−1.7#	−1.56#	−2.41*	−2.46*	−5.63*	−4.24*	−2.32*	−4.63*
Non-MDR	−2.49*	−5.37*	−4.14*	−4.54*	−3.23*	−2.58*	−3.23*	−3.16*
Non-MDR	−3.27*	−3.45*	−1.18#	−1.83#	−4.23*	−3.64*	−1.43#	−3.69*
MDR	−2.16*	−3.83*	−4.51*	−2.36*	−2.24*	−4.52*	−1.42#	−1.74#
Non-MDR	−2.73*	−3.49*	−1.07#	−3.61*	−2.87*	−5.72*	−4.29*	−3.18*
Non-MDR	−4.16*	−2.63*	−3.42*	−1.29#	−3.81*	−3.77*	−2.86*	−3.46*
Non-MDR	−4.11*	−3.42*	−1.21#	−4.72*	−4.13*	−3.31*	−2.63*	−4.14*

*Significant downregulation

#Non-significant downregulation



	0	0.16	0.32	0.64	0.1.12	2.5	5	10	20	40
Allium sativum	100	96.67	92.21	88.35	83.45	78.57	73.23	65.45	54	43.16
Cuminum cyminum	100	99.56	96.34	93.12	88.36	85.12	81.67	75.53	64.3	48.6

Fig. 2 Viability percentage of Vero cells after treatment with *Cuminum cyminum* and *Allium sativum* essential oil

reducing metabolic activity (Mohsenipour and Hassanshahian 2015). As presented in the Fig. 1, untreated bacterial cells were well connected to each other and formed microcolonies, but these structures were rarely seen in EOs-treated *S. typhimurium* cells. Decrease of adhesion and biofilm formation by *S. typhimurium* cells may be due to anti-adhesive properties of the EOs compounds on production of agents which play essential roles in the biofilm formation (Koo et al. 2013).

Though *C. cyminum* and *A. sativum* are well known for their antimicrobial activities against several important pathogens, including enterobacteriaceae, the mechanism of their effects on the biofilm formations has not been fully explained (Jakobsen et al. 2012; Nidadavolu et al. 2012; Packiavathy et al. 2012). In this study, the effect of sub-MIC concentrations of EOs on the expression of biofilm-related genes was measured and the results showed that the MIC/2 concentrations of EOs caused a significant down-regulation of QS and cellulose synthesis gens ($p < 0.05$) in most of strains, which accounts for the formations of biofilm. Lamas et al. (2016) reported that there was a relationship between the expression of biofilm and QS-related genes in *S. typhimurium* (Lamas et al. 2016).

Toxicity potential of selected plants in this study has not been demonstrated, but we assessed cytotoxic effects of various concentrations of *C. cyminum* and *A. sativum* EOs on the eukaryotic Vero cells using MTT test. Cytotoxicity results demonstrated that *C. cyminum* and *A. sativum* EOs at MIC to 4 MIC concentrations had not significant cytotoxic effects.

Conclusion

According to the results, the EOs of *C. cyminum* and *A. sativum* are efficient for control and elimination of the planktonic and biofilm forms of *S. typhimurium* isolates by down-regulation of QS (*sdiA* and *luxS*) and cellulose synthesis (*csgD* and *adrA*) gens. These results show that *A. sativum* and *C. cyminum* essences may be suitable as the natural preservatives for controlling the growth of food microorganisms; however, these EOs did not have a synergistic effect against *S. typhimurium* isolates.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest.

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