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Food Control xxx (2015) 1–9

Contents lists available at ScienceDirect

Food Control

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Antibiofilm activity of essential oils and plant extracts against *Staphylococcus aureus* and *Escherichia coli* biofilms

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ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form
18 September 2015
Accepted 26 September 2015
Available online xxx

Keywords:
Biofilm
Coriandrum sativum
Essential oil
Mentha × piperita
Pimpinella anisum
Plant extract

ABSTRACT

Bacterial biofilms pose health risks in clinical environments, food industry and drinking water systems. Here, we investigated *in vitro* antibiofilm activities of essential oils (EO) and plant extracts of peppermint (*Mentha* × *piperita* L.), coriander (*Coriandrum sativum* L.), and anise (*Pimpinella anisum* L.). Minimum inhibitory concentration assay (MIC) was carried out using two-fold serial dilution method and MTT assay against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. Biofilm growth and development were assessed using crystal violet (CV) and XTT reduction assays. Antibacterial activity was observed for almost all plant extracts and all EOs against both bacterial strains with stronger activity against *S. aureus*. All EOs (at MIC value of 0.8 to 0.63 μl/ml) and 8 out of 14 plant extracts (at MIC value of 2–4 mg/ml) inhibited bacteria cell attachment of both bacteria. CV and XTT reduction assay for the plant extracts and EOs with inhibition of bacteria attachment by at least 50%, demonstrated that coriander EO had the highest antibiofilm activity against biofilm formed by both tested bacteria (*S. aureus* and *E. coli*) at lowest MIC value 0.8 μl/ml and 1.6 μl/ml, respectively, indicating further investigations due to the oil's high antibiofilm activity potential.

1. Introduction

A biofilm is a complex matrix of microorganisms in which cells bind together and attach to biotic or abiotic surface (Costerton, Stewart, & Greenberg, 1999; Mah & O'Toole, 2001). Biofilms usually create a sticky gel composed of polysaccharides, proteins and other organic components on a wet surface, found in different environments including clinical and industrial, food processing environments, and drinking water distribution systems (Kavanaugh & Ribbeck, 2012; Oral et al. 2010). Bacteria within biofilms are more resistant to antibiotics and chemical agents than planktonic cells in suspension (Ceri et al. 1999; Stewart & Costerton, 2001). Chemical agents penetrating into the biofilm matrix are less effective, because most of the chemicals are active only against unattached microorganisms. In order to penetrate and degrade biofilms, it is necessary to hydrolyze the biofilm matrix. Restricting the growth and development of food borne and nosocomial pathogens such as *Staphylococcus aureus* and *Escherichia coli* is very important, however the eradiation of these organisms is not always successful because of their ability to form biofilms on a various range of surfaces (Nostro et al. 2007; Oral et al. 2010).

Interest in natural antimicrobial products has increased in recent years. The most important and well researched compounds originate from plants, which show many medicinal and antimicrobial properties (Rounds, Havens, Feinstein, Friedman, & Ravishankar, 2012; Tiwari et al. 2009), including potential activity against biofilm formation (Niu & Gilbert, 2004). Extracts and essential oils from a wide range of medicinal plants have attracted and encouraged research interest. The plant extracts have widespread application in the pharmaceutical industry, because they contain various bioactive compounds with antimicrobial properties. Biofilm inhibitory effect of plant extracts (solvent extracts and fractions) has been reported against *E. coli* (Agrawal, 2011; Vacheva et al. 2011), *Listeria monocytogenes* (Sandasi, Leonard, & Viljoen, 2010), *S. aureus* (Agrawal, 2011; Quave, Plano, Pantuso, & Bennett, 2008) and *Candida albicans* (Polaquini, Svidzinski, Kemmelmeier, & Gasparetto, 2006).

Plant compounds with strong antibacterial or bactericidal activity belong mostly to the group of phytoalexins including essential oils as the most important members (Gibbons, 2008). Essential oils are volatile compounds with antimicrobial properties

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http://dx.doi.org/10.1016/j.foodcont.2015.09.036
0956-7135/

constituting non-supportive media for the growth of many bacteria and fungi. Several studies have reported the antimicrobial properties of essential oils (Dorman & Deans, 2000). They constitute complex and heterogenous mixtures of substances comprising several structure classes with different biosynthetic origin: the main group includes terpenes (monoterpenes, sesquiterpenes) and terpenoids, together with aromatic (phenylpropanoids) and/or aliphatic compounds (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Gyawali & Ibrahim, 2014; Kalemba & Kunicka, 2003). Essential oils are readily isolated from plant material, exert low toxicity in mammalians, and degrade quickly and easily in water (Kavanaugh & Ribbeck, 2012). In recent years, studies on the anti-biofilm activity of essential oils have been intensified. Anti-biofilm activity of essential oils has been reported against *S. aureus* by using thymoquinone, an active principle of *Nigella sativa* L. seed oil (Chaieb, Kouidhi, Jrah, Mahdouani, & Bakhrouf, 2011), lemongrass oil (*Cymbopogon flexuosus* (Nees ex Steud.) W.Watson) (Adukwu, Allen, & Phillips, 2012), oregano oil (*Origanum vulgare* L.), carvacrol and thymol (Nostro et al. 2007, 2009), oregano oil (*Origanum onites* L.) (Oral et al. 2010), cassia (*Cinnamomum cassia* (Nees & T.Nees) J.Presl), Peru balsam (*Myroxylon balsamum (L.) Harms* (L.) Harms), and red thyme (*Thymus vulgaris* L.) essential oils (Kavanaugh & Ribbeck, 2012), tea tree oil (*Melaleuca alternifolia* (Maiden & Betche) Cheel) (Kwieciński, Eick, & Wójcik, 2009), and lavender (*Lavandula angustifolia* Mill.) and melissa oil (*Melissa officinalis* L.) (Budzyńska, Wieckowska-Szakiel, Sadowska, Kalemba, & Rózalska, 2011). Potential antibiofilm effect against *E. coli* has been shown for tea tree, lavender, and melissa oil (Budzyńska et al. 2011), cinnamon oil (*C. cassia* (Nees & T.Nees) J.Presl) and cinnamaldehyde (De Oliveira, Brugnera, Do Nascimento, Batista, & Piccoli, 2012; Niu, 2006), and eugenol and carvacrol (Pérez-Conesa, McLandsborough, & Weiss, 2006).

Essential oil and solvent extracts from coriander (*Coriandrum sativum* L.), anise (*Pimpinella anisum* L.) and peppermint (*Mentha* × *piperita* L.) (Sandasi et al., 2010) expressed antibacterial activity against a range of bacteria including *E. coli* and *S. aureus* (Elgayyar, Draughon, Golden, & Mount, 2001; Hammer, Carson, & Riley, 1999; Silva, Ferreira, Queiroz, & Domingues, 2011). However, a comparative study on the antibiofilm activity of essential oil and solvent extracts of the mentioned species against *E. coli* and *S. aureus* has not been carried out so far. The aim of this study was to investigate the antibiofilm activity of essential oil and different solvent extracts of coriander (*C. sativum* L.), anise (*P. anisum* L.) and peppermint (*Mentha* × *piperita* L.) using *in vitro* assays.

2. Material and methods

2.1. Chemicals and reagents

Bacteria culture media (Muller Hinton Broth (MHB) and Muller Hinton Agar (MHA), ciprofloxacin, dimethyl sulfoxide (DMSO), hexane, dichloromethane (DCM), methanol, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma.

2.2. Plant material and extraction

The plant material used was seeds of coriander (*C. sativum* L.), anise (*P. anisum* L.) and leaves of peppermint (*Mentha* × *piperita* L.). The plant material of peppermint was obtaind in dried form from the Medicinal Plants and Drugs Research Institute of Shahid Beheshti University in Iran. The seeds of anise and coriander were obtaind from Pkan Bazr company in Iran. Dried plant material was ground to a fine powder, and samples (2 g) were then extracted via maceration by a series of solvents with different polarity using hexane (Hex), dichloromethane (DCM) and methanol (Met) (10:1 solvent to dry weight ratio) for two successive 24-h periods. The extracts were filtered and evaporated to dryness on a rotary evaporator (Rangasamy et al. 2007). Finally, the obtained extracts were solubilized in dimethyl sulfoxide (DMSO) at a concentration of 64 mg/ml and stored at 4 °C until use (Pandey, Singh, Sharma, & Lata, 2011). For essential oil (EO) extraction, the plant material (no grinding, except for peppermint leaves which were coarsely crushed) was subjected to hydrodistillation (100 g) by using a Clevenger apparatus for 3.5 h (British Pharmacopoeia, 1998). The recovered essential oil samples were stored in the dark at 4 °C.

2.3. Bacterial strains

The bacteria used were Gram-positive *S. aureus* (strain CCUG 4151, used as positive control for antimicrobial-resistant bacterial strain testing) and Gram-negative *E. coli* (CCUG 17620, an international standard reference strain for antibacterial disc susceptibility testing and antimicrobial agents) provided by the Laboratory Centre Collection, NTNU, Trondheim, Norway.

2.4. Determination of minimum inhibitory concentration (MIC)

MIC analysis was performed in Muller Hinton Broth (MHB) via broth micro-dilution techniques according to CLSI guideline (National Committee for Clinical Laboratory Standards) procedures for aerobic testing (CLSI., 1990) with 96-well microtiter plate. Bacteria strains were sub-cultured twice by streaking on Muller Hinton agar and incubated at 37 °C for 12 h. Following incubation in agar, 5–7 single colonies from the second plate were inoculated into individual tubes containing sterile Muller Hinton broth (10 ml) and incubated in a shaking incubator at 37 °C for a period of 8–12 h to ensure that the bacteria were in the log phase (Rangasamy et al. 2007). The bacterial suspensions ware adjusted to a concentration approximately 10^6 CFU/ml. This was done by diluting bacterial suspension 1:100 with fresh sterile broth to obtain an absorbance ($OD_{590\ nm}$) of 0.02 for all bacteria using a spectrophotometer to yield concentration of inoculums of 10^6 CFU/ml (Sandasi et al. 2010). Stock solutions of the different extracts at a concentration of 16 mg/ml were prepared in MHB (Rangsamy et al. 2007; Sarker, Nahar, & Kumarasamy, 2007). To each well of sterile 96-well microplates, 100 μl of MHB was added. Then 100 μl of each stock plant solution (16 mg/ml) was placed in the first well of a 96-well microplate and two-fold serially diluted in sterile MHB to obtain a final concentration range of 4–0.0312 mg/ml. In the case of essential oils, all tests were done in MHB supplemented with DMSO (maximum final concentration of 2% (v/v) to enhance the oil solubility (Silva et al. 2011). In this regard, each oil was serially diluted in MHB (Pandey et al. 2011; Sarker et al. 2007) with 2% (v/v) DMSO to give a final essential oil concentration in the medium ranging from 25 to 0.19 μl/ml 100 μl of bacterial suspension was inoculated to each well. Each plate was wrapped loosely with parafilm to ensure that bacteria were not dehydrated and incubated at 37 °C for 24 h (Rangasamy et al. 2007; Sarker et al. 2007). Each plate had a set of positive and negative controls. 100 μl ciprofloxacin (1 mg/ml) was included as positive control (instead of plant extract) in the first well of a column in serial dilution. 100 μl of DMSO was used as negative control (instead of plant extract) with 100 μl MHB instead of bacteria solution (Sandasi et al. 2010). The plates were prepared in three replicates.

Following incubation, 40 μl MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) (0.2 mg/ml) was added to each well and incubated at room temperature for a further 10–15 min. Bacterial growth was observed as a pink-red coloration of the wells. The well of lowest concentration of extract in which bacteria growth was prevented, no pink-red coloration was observed,

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and the corresponding concentration was referred to as the MIC value. Total activity value was calculated in terms of MIC by quantitative evaluation of antimicrobial activity of plant extracts (Eloff, 2000).

2.5. Determination of biofilm inhibition - inhibition of initial bacteria cell attachment

The plant extracts at MIC value concentration were evaluated for their inhibition potential against cell attachments (antiadhesion test). 100 μl of each plant extract (at MIC value) was added to each well of a 96-well microplate. An equal volume ciprofloxacin (0.00125 mg/ml) (MIC value) was added as positive control, while the negative control was containing 100 μl MHB instead of plant extract. Finally, 100 μl of bacteria culture (10^6 CFU/ml) was pipetted to each well (final volume was 200 μl in each well). 200 μl of MHB was added in blank wells without bacteria culture. The plates were wrapped loosely with parafilm and incubated at 37 °C for 8 h without shaking to allow the cells to attach to the surface. Following incubation, the contents of each well were removed. Wells were rinsed three times with sterile distilled water to remove loosely attached cells and non-adherent cells. The plates were air-dried and oven-dried at 60 °C for 45 min. This step was validated by staining the recovered wells with crystal violet (1%). The wells were stained with 200 μl of 1% crystal violet and incubated at room temperature for 15 min. The plates were then rinsed three times with sterile distilled water to remove unabsorbed stain. The wells were destained by adding 150 μl of ethanol. 100 μl of the destaining solution was then transferred to a new plate and the absorbance was measured at $OD_{590\ nm}$ using a microplate ELISA reader (Labsystems Multiskan MS, Finland). Each assay was performed in triplicate. The mean absorbance of the samples was determined, the absorbance in blank well was subtracted from absorbance reading and percentage inhibition and efficiency was determined. The percentage inhibition was then compared with the positive control (Sandasi et al. 2010):

$$\text{Percentage inhibition} = \frac{OD_{\text{Negative control}} - OD_{\text{Experimental}}}{OD_{\text{Negative control}}} \times 100$$

2.6. Inhibition of biofilm formation and development - biofilm biomass measurement

Biofilm formation was done for 4 h before addition of plant extracts at MIC value concentration. The plant extracts and essential oils which exhibited at least 50% inhibition in bacteria cell attachment were selected for biofilm formation inhibitory measurement. In brief, 100 μl of a bacteria culture (10^6 CFU/ml) was added to each well of a 96-well microtiter plate and incubated for 4 h at 37 °C to allow cell attachment and biofilm formation. Following incubation, 100 μl of each plant extract was added to yield a final concentration of (MIC value) in the wells. Equal volume ciprofloxacin (0.00125 mg/ml) (MIC value) was added as positive control, and negative control contained 100 μl MHB instead of plant extract. 200 μl of MHB was used in blank wells without bacteria culture. The plates were incubated for 24 h. Following incubation, inhibition of biofilm growth and development was determined by crystal violet staining assay, and percentage inhibition was calculated. Each assay was performed in triplicate (Sandasi et al. 2010).

2.7. Biofilm metabolic activity measurement

The metabolic (respiratory) activity of biofilm was determined by using (XTT) reduction assay. Biofilm formation was done as described above. Following incubation (24 h), the contents of each well were removed, and wells were washed three times with phosphate-buffered saline (PBS) to remove loosely attached cells. The sodium salt of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-*2H*-tetrazolium-5-carboxanilide) was dissolved in PBS to 1 mg/ml concentration, filter-sterilized and stored at −80 °C. Menadione was dissolved in acetone to 1 mM and sterilized immediately before each measurement. Working solution of XTT/menadione reagent was freshly prepared before each assay in ratio of 12.5:1. Following washing, 100 μl PBS was added to each well of a 96-well microtitre plate. 13.5 μl of XTT/menadione mixture was then added to each well; the plate was gently shaken, then covered (in darkness) and incubated at 37 °C for 2–3 h. Following incubation, the absorbance was measured at 490 nm. Blank well, negative and positive controls were performed as described above. Each assay was performed in triplicate (Chaieb et al. 2011; Pettit et al., 2005|).

2.8. Gas chromatography-mass spectrometry

Essential oil samples were diluted in hexane (5 μl/ml) in autosampler vials and analyzed using an Agilent 6890/5975 GC-MS system (Agilent Technologies, Palo Alto, CA), equipped with a HP-5MS capillary column (30m × 0.25 mm inner diameter and film thickness 0.25 μm). Sample volumes of 1 μl were injected with a split ratio of 15:1. Injection and interface temperatures were set at 230 °C and 250 °C, respectively. He was used as carrier gas at a constant flow rate of 1 ml/min. The column oven was initially adjusted to 40 °C and ramped to 250 °C at a rate of 3.5 °C/min and finally held at 250 °C for 3 min (analysis time: 63 min). The MS source was adjusted to 230 °C, and a mass range of *m/z* 35–350 was recorded acquiring all mass spectra in EI mode. Chromatogram visualization and peak area integration were carried out using Agilent ChemStation software (Agilent Technologies, Waldbronn, Germany). Essential oil components were tentatively identified based on MS database search using NIST/EPA/NIH Mass Spectral Library (NIST 05) in combination with an *in-house* retention index library of MS spectra of volatile organic compounds, and comparison of spectra with reported MS data in literature (Adams, 2001).

2.9. Microscopic visualization of biofilm

Inhibition of biofilm formation was evaluated by microscopic technique as described by Chaieb et al. (2011) with minor modifications. Briefly, biofilm of each bacteria strain was separately performed (as described above) on round cover glass slides (diameter 1 cm) placed in 24-well polystyrene plates 4 h before addition of essential oil or plant extract (Greiner Bio-One, France), following 4 h incubation supplemented with coriander and anise oil (at MIC) with high antibiofilm activity against *E. coli* and *S. aureus*, respectively. DCM of coriander and peppermint (at MIC) with low antibiofilm activity were applied against *E. coli* and *S. aureus* respectively. Negative and positive controls were performed as described above, then incubated for 24 h at 37 °C and stained with 1/20 Giemsa (Sigma, Switzerland) solution (v/v) for 20 min at room temperature. Stained glass pieces were placed on slides with the biofilm on top of the glass slide. Biofilms were evaluated and confirmed by light microscopy at 100 × magnification.

3. Results

3.1. Determination of minimum inhibitory concentration (MIC)

In the present study, a serial extraction method was used on selected plant materials (dried and grounded), using different solvent systems in order of increasing polarity. The *in vitro*

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antimicrobial activities of essential oil and three solvent extracts of plants against selected bacteria and their activity potentials were assessed by two-fold serial dilution method and MIC values. MIC values of essential oils and different solvent extracts of the bacteria are presented in Table 1. The results showed that essential oils and DCM extracts displayed antibacterial activity against both tested Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. Antibacterial activity of hexane and methanol extracts, was only observed against Gram-positive (*S. aureus*) bacteria, except for methanol extract of peppermint which showed antibacterial activity against both tested bacteria. The coriander oil displayed the highest inhibitory activity compared to the other solvent extracts and essential oils against both tested bacteria. Recorded MIC values were 0.8 and 1.6 μl/ml against *S. aureus* and *E. coli*, respectively, showing an antibacterial activity closely similar to the tested antibiotic ciprofloxacin with MIC value of 0.00195 mg/ml against both bacteria. In comparison, peppermint and anise oil displayed higher MIC values against *S. aureus* (3.1 μl/ml) and *E. coli* recorded as 6.3 and 12.5 μl/ml for peppermint and anise oil, respectively.

3.2. Inhibition of initial bacteria cell attachment (biofilm inhibition)

Antiadhesion tests were carried out by crystal violet assay in order to evaluate essential oils and different plant solvent extracts inhibition potential against cell attachments at MIC value concentration. Results indicated that essential oil of coriander, hexane extract of anise and methanol extract of peppermint could inhibit bacteria cell attachment of *S. aureus* completely (100% inhibition activity), while the other extracts and essential oils generally displayed percentage inhibition in a range of 23–96% (Table 2). *E. coli* was more resistant than *S. aureus* as observed and proved by lower percentage inhibition values. Among the tested plant extracts and essential oils, only peppermint oil showed strong antiadhesion activity with an inhibition value of 98.4%. In total, five of seven extracts and essential oils could inhibit cell attachment of *E. coli* in the range of 48.3–98.4%.

3.3. Biofilm biomass measurement (crystal violet assay)

Inhibition of biofilm formation was conducted only on those essential oils and solvent extracts, which showed at least 50% reduction (at MIC value concentration) in cell attachment on both tested bacteria by using crystal violet assay. The results showed different effects on the growth and development of a preformed biofilm, as presented in Table 3. Essential oil of coriander and anise induced inhibition of biofilm formation against *S. aureus* by up to 91% and 88.5%, respectively. While some solvent extracts increased biofilm growth and development of *S. aureus*, no inhibition was recorded for coriander and anise extracts. In comparison, the peppermint extracts and essential oil generally displayed percentage inhibition of biofilm formation in the range of 39–70%. Our results indicated that essential oil of coriander could inhibit biofilm formation of *E. coli* completely, displaying 100% inhibition activity followed by peppermint oil with a percentage inhibition value of 81% (Table 3). In comparison, the DCM extract of coriander did not prevent biofilm formation of *E. coli*. However, our results indicated strong biofilm inhibition by coriander essential oil against *S. aureus* and *E. coli* when used at MIC value concentrations 0.8 and 1.6 μl/ml, respectively.

Table 1
MIC value concentration of different plant extracts (4 mg/ml = 25% (v/v), 2 mg/ml = 12% (v/v)) and essential oil against *Staphylococcus aureus* and *Escherichia coli*, MIC were evaluated after 24 h of incubation.

Plants	Extracts	MIC value concentration (mg/ml, μl/ml for EO)	
		S. aureus	*E. coli*
Coriander	Hex	4	–[a]
	DCM	4	4
	Met	4	–
	EO	0.8	1.6
Anise	Hex	2	–
	DCM	4	4
	Met	4	–
	EO	3.1	12.5
Peppermint	Hex	2	–
	DCM	2	2
	Met	2	2
	EO	3.1	6.3

Hex: hexane, **DCM**: dichloromethane, **Met**: methanol, **EO**: essential oil.
[a] No MIC value was observed due to lack of antibacterial effect.

3.4. Biofilm oxidative activity (XTT assay)

The metabolic (respiratory) activity of cells in biofilm after 24 h was evaluated by using XTT reduction assay. Our result indicated that most solvent extracts and essential oils reduced metabolic activity of cells in biofilm of *S. aureus* and *E. coli*, showing an inhibition percentage range of 38.3–72.6% and 57.4–86%, respectively (Table 3). In contrast, DCM and methanol extracts of coriander did not inhibit metabolic activity of biofilm cells of *S. aureus* at all (0%). When comparing all extracts and oils, essential oil of anise and coriander were the most effective in inhibiting formation and growth of *S. aureus* biofilm by 72.6% and 71.5%. Our data also provided evidence that coriander oil had the highest inhibitory potential with 86% and 71.5% reduction in metabolic activity of *E. coli* and *S. aureus*, respectively, as it affected the oxidative activity of both tested bacteria. In summary, results from antiadhesion testing, crystal violet and XTT assays (Tables 2 and 3) indicated that essential oils of coriander, anise and peppermint and its methanol extract were effective in reducing biofilm biomass, and impaired metabolic activity of cells adherent in biofilm formed by *E. coli* and *S. aureus*.

3.5. GC-MS analysis

Essential oil analysis of coriander, anise and peppermint was performed by GC-MS, and the results are presented in Table 4. The results of GC-MS showed that the main components of coriander oil are linalool, γ-terpinene, α-pinene, geranyl acetate, octanol and *p*-cymene. Menthol, menthone, 1,8-cineole, menthyl acetate and isomenthone, and (*E*)-anethole, estragole and carvone were the main constituents of the essential oil of peppermint and anise, respectively (Table 4). The total amount of terpenes in essential oil of coriander, anise and peppermint was 89.73%, 8.3% and 97.99%, respectively.

3.6. Microscopic visualization of biofilm formation

The inhibition of biofilm formation by coriander and anise essential oils with high antibiofilm activity against *E. coli* and *S. aureus*, respectively, was confirmed by microscopic visualization (Fig. 1). The relatively lower antibiofilm activity of DCM extracts of coriander and peppermint against *E. coli* and *S. aureus*, respectively, could be demonstrated (Fig 1, C1 and C2). The inhibition pattern of biofilm formation by ciprofloxacin (positive control) (Fig. 1, A1 and A2) was similar to the inhibition effect of essential oils of coriander and anise (Fig. 1, B1 and B2).

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Table 2
Antiadhesion effect of different plant extracts (4 mg/ml = 25% (v/v), 2 mg/ml = 12% (v/v)) and essential oil on initial bacteria cell attachment.

Plant	Strain	Extract	Concentration (mg/ml, μl/ml for EO)	% Inhibition of bacteria attachment
				Crystal violet
Coriander	*S. aureus*	Hex	4	33.5[a]
		DCM	4	72.3
		Met	4	96
		EO	0.8	100
Anise	*S. aureus*	Hex	2	100
		DCM	4	23.5[a]
		Met	4	93.6
		EO	3.1	90.3
Peppermint	*S. aureus*	Hex	2	95.6
		DCM	2	67.8
		Met	2	100
		EO	3.1	74.7
Coriander	*E. coli*	DCM	4	76.1
		EO	1.6	72.3
Anise	*E. coli*	DCM	4	0[a]
		EO	12.5	56.2
Peppermint	*E. coli*	DCM	2	0[a]
		Met	2	48.3[a]
		EO	6.3	98.4

Hex: hexane, **DCM**: dichloromethane, **Met**: methanol, **EO**: essential oil.
[a] Plant extracts which did not show at least 50% reduction (at MIC value concentration) in cell attachment on both tested bacteria by using crystal violet assay, were not included in inhibition of biofilm formation assay.

Table 3
Effect of different plant extracts (4 mg/ml = 25% (v/v), 2 mg/ml = 12% (v/v)) and essential oil on biofilm formation (growth and development).

Plant	Strain	Extract	Concentration (mg/ml, μl/ml for EO)	% Inhibition of biofilm development	
				Crystal violet	XTT assay
Coriander	*S. aureus*	DCM	4	0	0
		Met	4	0	0
		EO	0.8	91	71.5
Anise	*S. aureus*	Hex	2	0	55.7
		Met	4	0	19.9
		EO	3.1	88.5	72.6
Peppermint	*S. aureus*	Hex	2	39.2	55.6
		DCM	2	51.3	38.3
		Met	2	70	61.7
		EO	3.1	67.5	52.7
Coriander	*E. coli*	DCM	4	0	57.4
		EO	1.6	100.0	86.0
Anise	*E. coli*	EO	12.5	17.4	63.2
Peppermint	*E. coli*	EO	6.3	81.0	68.5

Hex: hexane, **DCM**: dichloromethane, **Met**: methanol, **EO**: essential oil.

4. Discussion

Retardation and inhibition of biofilm growth and development in a preformed biofilm of both bacteria tested was successful for most of the essential oils except for anise oil which exerted low antibiofilm activity against *E. coli* (Table 3). In general, the extent and the amount of inhibitory biofilm formation was less pronounced compared to inhibition of initial attachment except for coriander oil, which showed 100% inhibitory activity at 1.6 μl/ml against *E. coli* (Tables 2 and 3). The reduced inhibition of biofilm development as shown in Tables 2 and 3 demonstrated that the bacteria cells in a biofilm are more resistant to antimicrobial agents compared to planktonic cells. In fact, inhibition of biofilm growth and development is more difficult to achieve than inhibition of cell attachment. These results were consistent with those found previously (Frank & Koffi, 1990; Krysinski, Brown, & Marchisello, 1992; Sandasi et al. 2010). Despite the activity of some of the plant solvent extracts (DCM of coriander and peppermint, methanol of coriander and anise, and hexane of anise and peppermint), the results demonstrated limited or low activity potential against biofilm formation and growth. In this regard, Adukwu et al. (2012) suggested that biofilm formation could induce protection against plant extracts used. Based on findings of the present study with plant extracts and essential oils, coriander oil exhibited the highest antibiofilm activity against both tested bacteria (Gram-positive and Gram-negative strains) with lowest MIC values (Table 3) against *S. aureus* (0.8 μl/ml). In the case of *S. aureus*, lemongrass EO inhibited biofilm formation at inhibitory concentration of 1.25 μl/ml (Adukwu et al. 2012). Similar biofilm inhibitory concentrations have been reported at 1.25, 0.31 and 1.25 μl/ml for oregano oil, carvacrol and thymol, respectively (Nostro et al. 2007). Oral et al. (2010) even reported biofilm inhibitory concentration against *S. aureus* for oregano oil at MIC value as low as 0.5 μl/ml. According to our findings (Table 3), coriander oil was also effective on biofilm formed by *E. coli* at 1.6 μl/ml. Previous studies have reported biofilm formation inhibition against *E. coli* by oregano oil at 1.0 μl/ml (Oral

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Table 4
Chemical composition (%) of the essential oils of coriander (*Coriandrum sativum* L.), anise (*Pimpinella anisum* L.) and peppermint (*Mentha* × *piperita* L.). Levels of major compounds (≥3%) are marked in bold.

Compound	RI[a]	Coriander	Anise	Peppermint
α-thujene	931	0.07	–[b]	0.08
α-pinene	939	**7.67**	–	1.17
camphene	953	0.05	–	–
sabinene	976	0.26	–	0.56
1-octen-3-ol	978	–	–	0.11
β-pinene	980	0.71	–	1.58
myrcene	991	0.29	–	0.12
3-octanol	994	–	–	0.14
α-phellandrene	1005	–	0.07	–
α-terpinene	1018	0.07	–	0.38
p-cymene	1026	**3.00**	0.06	0.22
limonene	1031	0.17	2.99	0.84
1,8-cineole	1032	0.10	–	**7.18**
(*Z*)-β-ocimene	1040	–	–	0.12
γ-terpinene	1062	**9.80**	–	0.99
(*E*)-sabinene hydrate	1068	0.05	–	1.53
octanol	1080	**3.02**	–	–
terpinolene	1088	0.07	–	0.13
(*Z*)-sabinene hydrate	1090	–	–	0.12
linalool	1098	**56.79**	–	0.39
nonanal	1102	0.29	–	–
amyl isovalerate	1108	–	–	0.16
camphor	1143	0.29	–	–
menthone	1145	–	–	**23.69**
menthofuran	1146	–	–	0.59
isomenthone	1149	–	–	**4.11**
neomenthol	1155	–	–	2.91
borneol	1165	0.06	–	–
menthol	1171	**3.24**	–	**33.19**
4-terpineol	1177	0.51	–	2.07
isomenthol	1182	–	–	0.83
α-terpineol	1193	0.22	–	0.41
neoisomenthol	1199	–	–	0.24
estragole	1200	–	**3.92**	–
decanal	1209	0.43	0.59	–
(*E*)-dihydrocarvone	1210	–	0.48	–
linalyl formate	1219	0.14	0.66	–
citronellol	1228	0.25	–	–
carvone	1242	–	**3.83**	–
piperitone	1252	0.11	–	1.32
geraniol	1255	0.89	–	0.34
(*E*)-2-decenal	1264	0.42	–	–
(*Z*)-anethole	1269	–	0.22	–
neomenthyl acetate	1274	–		0.22
(*E*)-anethole	1283	0.54	**86.77**	–
dihydroedulan I	1292	–	–	0.22
menthyl acetate	1294	–	–	**5.04**
undecanol	1371	–	0.04	–
geranyl acetate	1383	**7.75**	–	–
β-bourbonene	1384	–	–	0.62
dodecanal	1386	0.09	–	–
(*E*)-β-caryophyllene	1418	0.11	–	1.37
α-caryophyllene	1454	–	–	0.10
(*E*)-β-farnesene	1458	–	–	0.21
(*E*)-2-dodecenal	1462	0.55	–	–
γ-himachalene	1476		0.20	–
germacrene D	1480	0.06	0.04	2.61
β-selinene	1485	–	0.04	1.48
bicyclogermacrene	1494	–	–	0.35
spathulenol	1576	–	–	0.09
caryophyllene oxide	1581	–	–	0.16
globulol	1593	–	–	0.65
TOTAL IDENTIFIED (%)		**98.07**	**99.91**	**98.62**
Monoterpenes (MT), total		**89.56**	**8.02**	**90.35**
- *oxygenated MT*		70.39	4.49	84.41
Sesquiterpenes (ST), total		**0.17**	**0.28**	**7.64**
- *oxygenated ST*		–	–	0.90
Aromatic compounds		**3.54**	**90.98**	**0.22**
Aliphatic compounds, total		**4.80**	**0.63**	**0.41**
- *aldehydes*		1.78	0.59	–
- *alcohols*		3.02	0.04	0.25
- *esters*		–	–	0.16

[a] Kovats retention index.
[b] Below threshold level of <0.01, or not detected.

et al. 2010). The enhanced antibiofilm activity observed for essential oils, especially coriander oil, may be closely related to the action and the presence of certain or principal EO compounds.

The results as shown in Table 3 were almost similar between the biomass and metabolic activity assay. Moreover, the inhibition of biofilm formation by coriander oil was also confirmed by using XTT reduction assay. With the exception of anise oil and DCM of coriander used against *E. coli*, also hexane and methanol extracts of anise used against *S. aureus* showed antibiofilm activity, however results by XTT reduction assay were not correlated with crystal violet assay (Table 3). In spite of an increase in biofilm formation, metabolic activity had been decreased. Several studies concluded that biofilms have reduced metabolic activity mainly because of decreased nutrient and oxygen supply. Such reduction in metabolic activity as a physiological change can account for the resistance of biofilms to antimicrobial agents (Costerton et al. 1999; Mah & O'Toole, 2001; Sandasi, Leonard, & Viljoen, 2008). Also other reports indicated an inverse correlation or no correlation between biomass and metabolic activity for plant extract or essential oil (Budzyńska et al. 2011; Kwieciński et al. 2009; Sandasi et al. 2008). However our results showed that the selected essential oils in general had better results in inhibition of biofilm growth and formation. Particularly in relation to *S. aureus* biofilms, essential oil of coriander showed the strongest effect, followed by anise oil, methanol extract and oil of peppermint in subsequent ranking in inhibition of biofilm growth. In *E. coli* the essential oils of coriander and peppermint showed the strongest effect in the inhibition of biofilm growth and development, while anise oil proved to be least effective. Overall, the essential oil of coriander and peppermint had the highest activity in the inhibition of biofilm growth against both tested bacteria (Gram-positive and Gram-negative). Furthermore, our results showed that Gram-negative *E. coli* showed higher MIC values for the tested EOs compared to Gram-positive *S. aureus*, due to the characteristic structure of the outer membrane of Gram-negative bacteria which makes them more resistant to lipophilic molecules (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013).

The total amount of terpenes in essential oil of coriander, anise and peppermint was 89.73%, 8.3% and 97.99%, respectively. In general, essential oils containing terpenes are reported to exhibit antimicrobial activity (Dorman & Deans, 2000; Van Vuuren, 2008). In addition, numerous studies have shown that terpenes (e.g., citral, geraniol, linalool, menthol, and thymol), which are the main components of distinct essential oils, alter the permeability of the cell by penetrating through fatty acyl chains of membrane lipid bilayers, disrupt lipid packing and change the fluidity of the cell membrane (Di Pasqua, Hoskins, Betts, & Mauriello, 2006; Serio, Chiarini, Tettamanti, & Paparella, 2010). Kotan et al. (Kotan, Cordali, & Cakir, 2007) reported that oxygenated monoterpenes show antibacterial activity, among them linalool, nerol, α-terpineol, fenchol, terpinen-4-ol, against a wide range of bacteria. Moreover, several studies have shown that the antimicrobial effect of essential oil are a result of the interaction between all the components of the oil, and not only due to single compound effects (Delaquis & Stanich, 2004; Lis-Balchin & Deans, 1997; Mourey & Canillac, 2002). The use of a specific EO compound alone is not effective enough for inhibition of biofilm growth (Sandasi et al. 2008). The higher inhibitory effecton biofilm formation against both *S. aureus* and *E. coli* by coriander and peppermint oil, respectively, as compared to anise might be due to the presence of terpenes such as linalool, γ-terpinene, α-pinene, geranyl acetate, octanol and *p*-cymene, also including potential synergistic effects between compounds. Furthermore, the total amount of terpenes in essential oil of coriander was 89.73%, of which 70.39% were oxygenated monoterpenes. Levels of other constituents of coriander oil

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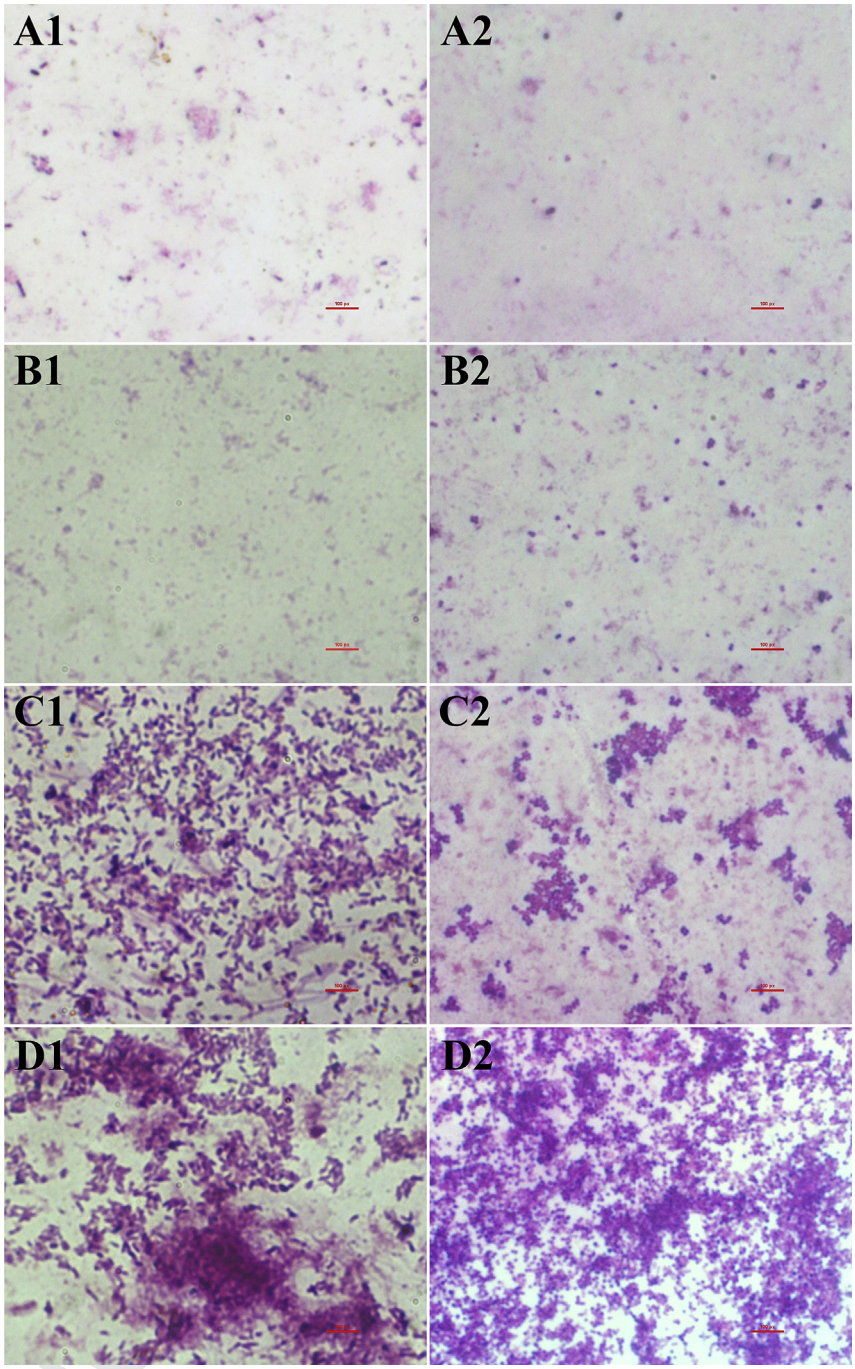

Fig. 1. Light Microscopy assay. Effect of plant extracts on inhibition of biofilm formation (growth and development) was as follows: *Escherichia coli*, A1: Positive control (bacteria supplemented with antibiotic ciprofloxacin at MIC), B1: Bacteria supplemented with coriander essential oil (EO) at MIC (high activity), C1: Bacteria supplemented with coriander dichloromethane (DCM) extract at MIC (poor activity), D1: Negative control (non-treated slides). *Staphylococcus aureus*, A2: Positive control (bacteria supplemented with antibiotic ciprofloxacin at MIC), B2: Bacteria supplemented with EO of anise at MIC (high activity), C2: Bacteria supplemented with DCM of peppermint at MIC (poor activity), D2: Negative control (non-treated slides).

(aliphatics 4.8%) were also higher compared to the oils of peppermint and anise. The total amount of terpenes in peppermint oil accounted for 97.99%, of which 84.41% were oxygenated monoterpenes. According to studies on oxygenated monoterpenes, linalool exhibits a broad spectrum of antibacterial activity, representing one of the most important compounds of this structural group (Kotan et al. 2007). In our study, linalool levels of 56.79% were determined in coriander oil. Minor levels were found in peppermint oil, while linalool was not detected in anise oil. The high level of oxygenated monoterpenes, particularly linalool, and potential interactions between the compounds might be responsible for high antibiofilm activity of coriander essential oil, which is followed by peppermint oil. Low level of monoterpenic complexity and lack of synergistic effects in anise might be the reason for low antibiofilm activity against *E. coli*, despite of reduced metabolic activity of biofilm. Therefore, activity of anise oil against *S. aureus* bacteria biofilm was mainly due to high levels of (*E*)-anethole and estragol. *S. aureus* is generally less resistant to antimicrobial

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compounds in comparison with *E. coli* (Budzyńska et al. 2011). Finally, linalool and other monoterpenic alcohols represent compounds with quorum-sensing inhibitory activity by affecting bacterial cross-talk (Mukherji & Prabhune, 2015). Such EO compounds might play a key role with regard to bacteria cell attachment and biofilm inhibition as observed in our study, and suggests further investigations of their activity on the molecular level.

5. Conclusions

In the present study, essential oil and solvent extracts derived from coriander (*C. sativum*), anise (*P. anisum*) and peppermint (*Mentha* × *piperita*) showed *in vitro* antibiofilm activity through inhibition of bacteria cell attachment of *E. coli* and *S. aureus*. Compared to earlier findings, our study revealed the potential role of coriander oil as a new antibiofilm agent with inhibitory concentration at 0.8 and 1.6 μl/ml against *S. aureus* and *E. coli*, respectively. To our knowledge, this is the first time the antibiofilm activity of coriander essential oil has been reported against biofilm formed by *S. aureus* and *E. coli.*

Acknowledgments

This project was supported by a grant (No.692) from Invited Collaborative Research Program (ICRP), Center for International Scientific Studies and Collaboration, Ministry of Science, Research and Technology of Iran to MMB. Scientific and technical support by the host institution, the Department of Biology, Norwegian University of Science and Technology (NTNU), Trondheim (Norway), is greatly acknowledged.

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