Anti-biofilm and Virulence Factor-Reducing Activities of Essential Oils and Oil Components as a Possible Option for Bacterial Infection Control

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ABSTRACT

Pathogenic biofilm-associated bacteria that adhere to biological or nonbiological surfaces are a big challenge to the healthcare and food industries. Antibiotics or disinfectants often fail in an attempt to eliminate biofilms from those surfaces. Based on selected experimental research, this review deals with the potential biofilm-inhibiting, virulence factor-reducing, and biofilm-eradicating activities of essential oils and single essential oil compounds using *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Chromobacterium violaceum* as model organisms. In addition, for the bacteria reviewed in this overview, different essential oils and essential oil compounds were reported to be able to modulate the expression of genes that are involved in the formation of autoinducer molecules, biofilms, and virulence factors. The anti-quorum sensing activity of some essential oils and single essential oil compounds was demonstrated using the gram-negative bacterium *C. violaceum*. Reporter strains of this bacterium produce the violet-colored compound violacein whose synthesis is regulated by quorum sensing autoinducer molecules called acylhomeserinlactones. Of great interest was the discovery that enantiomeric monoterpenes affected the quorum sensing regulation system in different ways. While the (+)-enantiomers of carvone, limonene, and borneol increased violacein formation, their (−)-analogues inhibited violacein production. For the successful eradication of biofilms and the bacteria living inside them, it is absolutely necessary that the lipophilic volatile substances can penetrate into the aqueous channels of biofilms. As shown in recent work, hydrophilic nano-delivery systems encapsulating essential oils/essential oil compounds with antibacterial effects may contribute to overcome this problem.

Biofilm – A Special Way of Life of Microorganisms

Biofilms are produced wherever sufficient water, nutrients, and microorganisms are present. They can affix themselves to any available surface such as food manufacturing equipment, stones in a stream, piping, external surfaces of marine vessels, sewage treatment plants, air-conditioning units and cooling towers, prosthetic devices, medical equipment such as endoscopes and colonoscopes, and dental irrigation units [1–3]. From a medical point of view, biofilms play an outstanding role in around 80 % of microbial infections, e.g., bacterial vaginosis, cystic fibrosis pneumonia, urinary tract infections, middle ear infections, chronic wounds, dental plaque formation, dental inflammation, endocarditis, chronic rhinosinusitis as well as contact lens and catheter infections [1, 4].

ABBREVIATIONS

AgrA	response regulator protein
AgrB	transmembrane AgrD processing protein (for AIP formation)
AgrC	histidine sensor kinase
AgrC, AgrA	two-component signal transduction system
AgrD	precursor for autoinducer proteins
AHL	N-acyl homoserine lactone (autoinducer-1)
AI	autoinducer
AIP	autoinducer protein
argA	transcriptional regulator gene in *S. aureus*
CviI	N-acyl homoserine lactone synthase (e.g., for C10-homoserine lactone) in *C. violaceum*
CviR	N-acyl homoserine lactone receptor, transcriptional regulatory protein
curli fimbriae	adhesion molecule
EOs	essential oils
EOCs	essential oil compounds (single chemical compounds of an essential oil)
EPS	extracellular polymeric substances
Las and Rhl	two different quorum sensing systems in *P. aeruginosa*
LasI	N-acyl homoserine lactone synthase (e.g., for 3-oxo-C12-homoserine lactone)
LasR	*Pseudomonas* quorum sensing receptor
LuxS	AI-2 (autoinducer-2) synthase
MBEC	minimum biofilm eradication concentration
MIC	minimum inhibitory concentration
MQSIC	minimum quorum sensing inhibitory concentration
PQS	pseudomonas quinolone signal (2-heptyl-3-hydroxy-4-quinolone)
PqsR	transcriptional regulatory protein
QS	quorum sensing
RhlI	N-acyl homoserine lactone synthase (e.g., for C4-homoserine lactone)
RhlR	*Pseudomonas* quorum sensing receptor
SarA	DNA-binding protein, transcriptional regulatory protein in *S. aureus*
SdiA	autoinducer-1 receptor protein in *E. coli* strains (e.g., for C8-homoserine lactone)

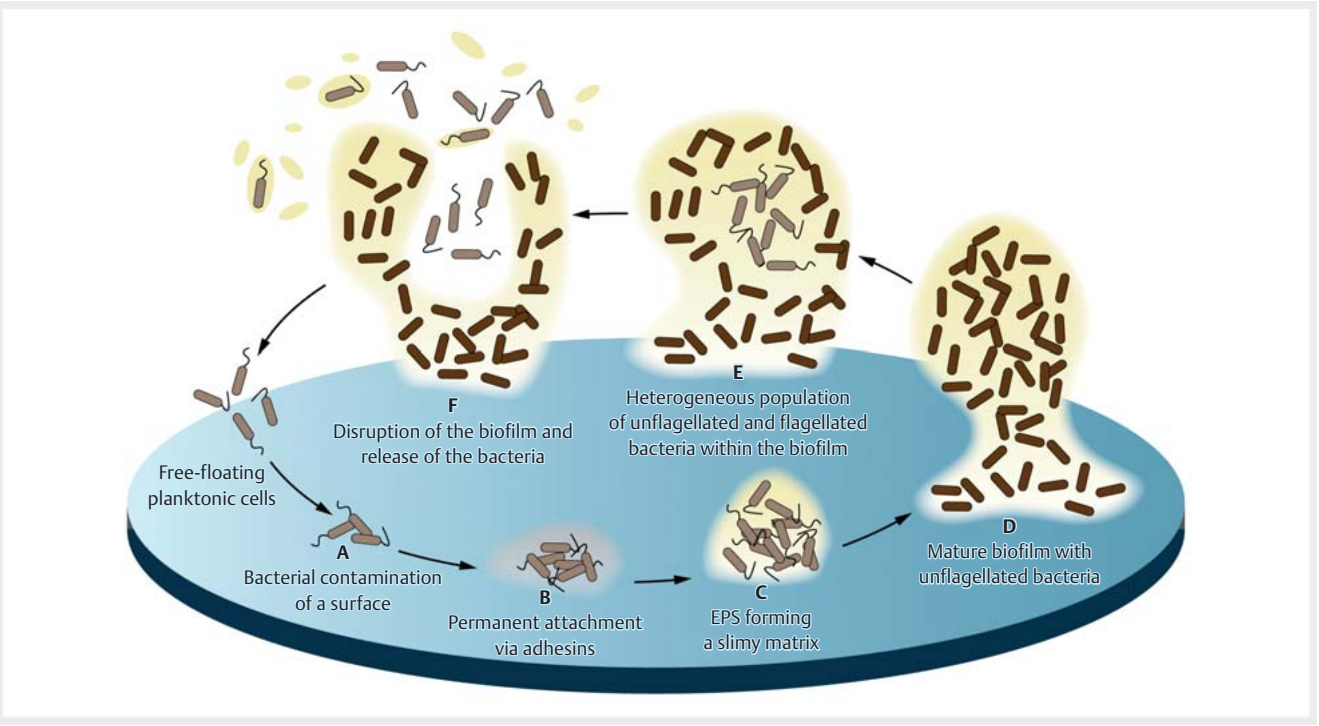
Biofilms are dynamic and complex living communities of microorganisms (such as bacteria, fungi, seaweed) that are constantly changing in space and time. In many biofilms, one often finds a microbial mixed population and, to a smaller extent, individuals of a single species [1, 5]. The microorganisms are typically surrounded by a microbially induced EPS-matrix consisting of exopolysaccharides (ePSs), lectins, exoproteins, exoenzymes, glycolipids, and bacterial extracellular DNA (eDNA), and adhere to a substrate or surface. The chemical composition and shape of biofilms are species specific [1, 6]. Lectins [7] and eDNA [8, 9] are important components of the biofilm EPS-matrix because they cross-link the extracellular polymeric substances and thus strengthen the complex overall structure. In addition, eDNA promotes the adhesion of the biofilm on to any solid surface and the bacterial aggregation [9]. Inside the biofilm there are caverns and tubes filled with water that serve the substance transport. Biofilm-forming bacteria adapt to the respective environment and have a higher resistance to environmental conditions than free-floating cells (planktonic cells). In addition, their metabolisms as well as their phenotype with respect to growth rate, expression of surface molecules, virulence factors, and nutrient utilization, clearly differ from that of planktonic cells [1, 9]. Fatally, bacteria in biofilms exchange resistance genes with each other via horizontal gene transfer, which means that antibiotic resistances can spread relatively quickly into biofilm populations, whereby species borders are also exceeded [1].

The formation of biofilms takes place in several stages and their structure depends on the nature of the organic or inorganic surfaces present and on the ambient conditions (▶ **Fig. 1**) [1, 10, 11]. First, the free-floating planktonic cells attach themselves to a suitable surface. Van der Waals forces play a role in this still reversible process. Subsequently, cell monolayers and microcolonies are developed, which are bound to the surface of the material by adhesins (special bacterial surface factors). This process is only partially reversible. Bacteria then form a slimy EPS-matrix that runs through and surrounds the entire biofilm, with the formation and secretion of ePSs playing a crucial role [1, 10, 12–14]. This stage of development is final. In this environment, bacteria are protected from unfavorable environmental conditions and can no longer be reached by immune cells, antibodies, and lipophilic antibiotics [1]. For example, the eradication of *Staphylococcus aureus* (SA) in biofilms requires 600-fold higher concentrations of sodium hypochlorite (disinfectant) and a 1000-fold higher dose of oxacillin (antibiotic) [15, 16]. Fully established and matured biofilms repel suspended bacteria, microcolonies, and biofilm fragments, which can then scatter and attach themselves elsewhere in the host organism (e.g., wounds) to form new biofilm colonies. It comes to a recurrence of the infection, which was believed to be defeated [1].

Against this background, there is an urgent need for safe and effective biocides for biofilm prevention and/or eradication of already existing biofilms. In the last two decades, the idea has grown that antimicrobial active EOs and isolated individual oil compounds from aromatic medicinal plants and spice plants could be possible anti-biofilm agents.

Essential oils as antibacterial agents

EOs are mixtures of different lipophilic, low molecular, volatile compounds, which usually consist of monoterpenes, sesquiterpenes, and phenylpropanes and their oxygenated derivatives (alcohols, aldehydes, esters, ketones, phenols, and oxides). Over the past two decades, numerous *in vitro* and *in vivo* studies worldwide have unequivocally shown that EOs and many of their individual EOCs have antibacterial, antifungal and antiviral properties [17–22]. For example, various observational studies have shown Australian tea tree oil (TTO) is well suited for the treatment of local bacterial infections (e.g., dental caries) and fungal infections (e.g., skin mycoses) as well as for the topical treatment of uncomplicated cold sores [22]. EOs and their individual EOCs mainly affect the integrity and function of the cytoplasmic membrane. They



▶ **Fig. 1** Biofilm formation – various stages of maturity (for example: *P. aeruginosa*). **A** Contamination, reversible attachment of bacteria to biological or nonbiological surfaces. **B** Colonization, permanent attachment to surfaces. **C** Initial biofilm formation and quorum sensing. **D** Mature biofilm, bacteria lose their flagella. **E** Bacteria again form their flagella. **F** Breaking up of the mature biofilm; spread of individual bacterial cells and whole biofilm fragments in the environment; recurrence of the infection elsewhere.

disturb, for instance, their fluidity and permeability and the function of the membrane transport proteins as well as the composition of fatty acids in the cytoplasmic membrane. In addition, they influence cell division, the structure and composition of the cell wall, cell morphology, and cellular respiration as well as the ion transport and energy balance of the bacterial cell [23–33]. In ▶ **Table 1**, the most interesting antibacterial effects of EOs and EOCs are summarized. Together with its postulated "multi-target principle", this makes EOs an interesting research subject when looking for new anti-biofilm and anti-virulence agents. From a medical point of view, two therapeutic targets are being followed up with the use of those agents: (1) from the beginning, no biofilms (e.g., on catheters, in wounds) and virulence factors should be produced, and the formation of new biofilms, caused by suspended, mobile biofilm cells, should be prevented and (2) biofilms already established (e.g., in chronic wounds, catheters) should be eradicated and the bacteria inside of the biofilms should be killed.

Search Strategy, Evaluation, and Limitations of Literature

Search strategy

For the initial search in the literature, the databases PubMed and BASE (Bielefeld Academic Search Engine) were used because mainly peer reviewed articles are listed in both databases. In PubMed/BASE, under the headings "essential oils and antibiofilm activity", 63/238 articles (years 2019 to 2001/2008) were identified and using "essential oils and inhibition of quorum sensing", 16/67 articles (years 2019 to 2009/2005) were identified. Furthermore, the literature from reference lists of all papers selected were also checked for their relevance to the above topics. The google search engine was also used to find relevant articles.

Evaluation and limitation of literature

In the past two decades, numerous experimental studies on the topic of EOs and EOCs with anti-biofilm and virulence factor-reducing activities have been published, with a significant increase in publication activities from 2005 to 2019. In more recent experimental studies, it is noticeable that the mechanisms of action are being investigated increasingly through the use of modern biochemical, molecular-biological, and microscopic methods. The focus of this review article is therefore not on the endless list of relevant studies of the last 20 years. Rather, the anti-biofilm and virulence factor-reducing activities of EOs are to be demonstrated on the basis of selected experimental work. The selection of the literature is based on the following four criteria or topics: (1) prevention of biofilm formation and virulence factor production, (2) eradication of already established biofilms, (3) nanoparticles loaded with EOs and EOCs as anti-biofilm and anti-virulence active agents, and (4) *Chromobacterium violaceum* (CV), SA, *Escherichia coli* (EC), and *Pseudomonas aeruginosa* (PA) as model organisms.

▶ **Table 1** Bacterial targets for EOs and EOCs.

Targets	Bacteria	EOs and EOCs	References
Cell morphology			
Alteration of cell shape	*E. coli*	Palmrose oil, peppermint oil	[23]
Cytoplasmic membrane			
Alteration of integrity and permeability, inhibition of cell respiration, K^+ leakage	*S. aureus*, *E. coli*	TTO, farnesol, nerolidol	[24–26]
Formation of multilamellar, mesosome-like structures	*S. aureus*	TTO, terpinen-4-ol	[27, 28]
Changes in fatty acid composition	*S. aureus*	Carvacrol	[29]
	E. coli	Thyme oil, oregano oil, carvacrol, thymol, citral	[30]
Cell wall			
Desintegration of OM and LPS	*E. coli*	Thymol, carvacrol	[31]
Cell lysis	*E. coli*	Origano oil, clove oil	[32]
Cell division			
Total inhibition of cell division	*S. aureus*	TTO	[27]
R-Plasmid			
Elimination of R-plasmids	*E. coli*	Pepermint oil, rosemary oil, eucalyptus oil, menthol	[33]
Cytosol			
Formation of filamentous, electron dense material	*S. aureus*	TTO	[27]

Cellular and molecular target structures as well as physiological effects of essential oils (EOs) and their active ingredients (EOCs) on examples of *E. coli* and *S. aureus*. LPS: lipopolysaccharide; OM: outer membrane

SA, EC, and PA not only represent gram-positive and gram-negative bacteria, but some of them are well known as pathogenic hospital germs. For instance, SA causes chronic rhinosinusitis, enteropathogenic EC is responsible for recurrent urinary tract infections, and PA is associated with chronic cystic fibrosis pneumonia [3]. CV is a gram-negative bacterium that occurs in tropical and subtropical areas and lives there in the soil and in stagnant water. In rare cases, the bacterium causes systemic infections in humans, e.g., sepsis [34]. These bacteria are often multiresistant against many known antibiotics and, in addition, they are also good biofilm producers [1, 3, 34].

For anti-QS experiments, several bacterial reporter assays with QS-regulated phenotypes (e.g., color pigments, bioluminescence) have been developed to identify chemical compounds that are able to interfere with or inhibit different bacterial autoinducer QS systems (e.g., AHL, AI-2, or AIP QS systems) [35–42]. In CV-type strains ATCC 12472 and ATCC 31532, the production of the purple pigment violacein is driven by different AHL autoinducers, whereas in the type strain CV026, deficient in AHL synthase, the production of violacein requires the exogenous addition of C6-AHL [42]. The QS receptor (CviR) in CV binds to AHL at a high cell density to form a CviR-AHL complex. The receptor-AHL complex binds to DNA and activates the expression of the *vio* genes required for violacein production.

The CV strains are internationally often used as reporter bacteria to reveal possible anti-QS properties of bioactive EOs and EOCs, especially in gram-negative bacteria. Against this background, some selected experimental data obtained by these biosensor bacteria will be presented below.

Biofilm-Inhibiting and Virulence Factor-Reducing Activities of Essential Oils and Essential Oil Compounds by Disruption of the Quorum Sensing Communication Network

The basic idea of an anti-virulence strategy in fighting bacterial infections is to make pathogenic bacteria more sensitive to the host's immune system and thereby interrupt the bacterial infection process. One of the hopes is to prevent the spread of multidrug-resistant pathogenic bacteria without creating new, resistant germs. For that reason, anti-virulence agents are characterized by the fact that they stop, in sub-MICs, the bacterial infection without inhibiting the bacterial vitality or even killing the bacteria. Against this background, the bacterial communication system (QS system) appears to be a promising target for new anti-virulence agents.

Quorum sensing – the language of the bacteria

The formation of biofilms is a complex process that depends on genetic as well as environmental factors [43–45]. Gene-regulated biofilm formation is based on QS, a cell-to-cell communication system where small signaling molecules are exchanged between neighboring bacteria. These small signaling molecules are called AIs because they stimulate their own synthesis through the QS system (self-reinforcing control loop). The class of signal substances used basically differs between gram-negative and gram-

▶ **Table 2** QS system and QS-regulated virulence functions.

Bacteria	QS signal molecules	QS regulatory proteins	QS-regulated virulence functions	References
CV	AHLs	CviI, CviR	**Biofilm formation** **Production of virulence factors** violacein, elastase, exoproteases, hydrogen cyanide, exopolysaccharides	[34, 38, 42]
EC	AI-2 (autoinducer-2); AHLs from other bacteria species	LuxS; SdiA	**Biofilm formation** **Production of virulence factors** adhesins, flagella, type I fimbriae, curli fimbriae, capsula, α-hemolysin, expolysaccharides	[43, 52]
PA	AHLs (C4-AHL, C12-AHL); PQS	LasI/LasR, RhlI/RhlR; PqsR	**Biofilm formation** **Production of virulence factors** pyocyanin, rhamnolipids, lecA, exoproteases, hydrogen cyanide; elastase, chitinase, pyoverdine, exopolysaccharides	[53–57]
SA	AIP-I to AIP-IV	AgrC, AgrA, AgrD, AgrB	**Biofilm formation** **Production of virulence factors** cell surface proteins (e.g., protein A), exoproteins (e.g., proteases, hemolysins, enterotoxin B), capsular polysaccharides	[58–60]

Correlation between QS signal system and QS-regulated virulence functions for selected bacterial model organisms. CV: *C. violaceum*, EC: *E. coli*, PA: *P. aeruginosa*, SA: *S. aureus*

positive bacteria [46, 47]. While gram-positive bacteria use various AIPs as the signal substances (AIP-QS system), gram-negative bacteria use predominantly different AHL molecules (AI-1) as autoinducers (LuxI-type/LuxR-type QS system). AHL molecules are synthesized by LuxI-type synthases and detected by the corresponding LuxR-type receptors [47, 48]. In addition, furanosyl borate diester (AI-2) is produced by gram-negative and gram-positive bacteria (LuxS/AI-2 QS system) and is thought to be used as a universal signaling compound for intraspecies and interspecies communication. AI-2 molecules are synthesized by LuxS-type proteins [47, 48]. Once a certain concentration of autoinducer molecules in the bacterial cells is reached, AIs are bound by specific QS receptors (sensing or regulatory proteins) located either in the inner cell membrane (in gram-positive bacteria) or in the cell cytoplasm (in gram-negative bacteria). As a result, the complex of QS receptor and autoinducer triggers an intracellular QS signal response mechanism, which in a concerted action promotes the expression of various genes that are involved, on the one hand, in the synthesis of AI synthases, autoinducers, and regulatory proteins (QS signaling circuit) and, on the other hand, in the production of virulence factors and the formation of biofilms [46–51]. The correlation between the QS signal system and the QS-regulated virulence functions in the model organisms CV, EC, PA, and SA is summarized in ▶ **Table 2**. For instance, Fattahi et al. [52] described the relationship between biofilm formation and different virulence factors in EC. Several authors [53–57] highlighted the relationship between the QS signaling system and the production of different virulence factors in PA, while Li and Tian [57] provided insight into the correlation between QS and biofilm formation in gram-negative and gram-positive bacteria. Bouhrour and Bendali [58], Yadav and Gupta [59], and Novick and Geisinger [60] discussed the QS regulation of biofilm formation and virulence factor production in Staphylococci. So, QS-regulated mechanisms are the basis for the altered physiological and metabolic behavior of bacteria and other microorganisms among unfavorably developing environmental factors (e.g., nutrient deficiency, temperature, biocide treatment). This behavior makes biofilm-associated bacteria potentially dangerous pathogens [1, 61]. A better understanding of the biochemical and genetic processes controlling bacterial quorum sensing provides a good opportunity to develop novel biocides or antibiotics with novel targets.

Several *in vitro* investigations from the recent past have demonstrated that some bioactive EOs and EOCs are able to interfere with the bacterial QS system (anti-QS activity) and to inhibit the formation of biofilms and virulence factors.

A selection of scientific investigations on the anti-QS activities, anti-virulence factor effects, and anti-biofilm activities of EOs and EOCs are summarized in ▶ **Table 3**. The most interesting experiments that give insight into the mode of action of the volatile bioactive compounds will be discussed below.

Anti-quorum sensing activity of essential oils and essential oil compounds in gram-negative bacteria using *Chromobacterium violaceum* strains as reporter bacteria

In an extensive *in vitro* test, 21 different EOs were investigated for their anti-QS activities in CV CVO26 and CV ATCC 12472 at sub-MICs [62]. Of the 21 EOs tested in an agar diffusion assay, only clove, cinnamon, lavender, and peppermint oils showed significant inhibition of violacein production. Clove oil was by far the most effective EO in the test system used. In order to rule out the possibility that the observed inhibition of violacein formation was not caused by a simple antimicrobial effect of the tested EOs, the inhibiting effect of clove oil was additionally investigated in a

▶ **Table 3** EOs and EOCs with anti-QS (AQS) and anti-biofilm (AB) activities.

EOs and EOCs	Plant sources	Bacteria	AQS and AB activities	References
Clove oil, cinnamon oil, lavender oil, peppermint oil	*Syzygium aromaticum, Cinnamomum zeylanicum, Lavandula angustifolia, Mentha piperita*	CV	**VI, QS inhibition**	[62]
Monoterpenes, phenylpropanes	—	CV	**VI, QS inhibition**	[63]
Clove oil	*Syzygium aromaticum*	PA	**IBF, RVF**	[64]
Peppermint oil, menthol	*Mentha piperita*	PA	**IBF, RVF**	[65]
Eugenol	—	PA	**IBF, BE, RVF**	[66]
Bay oil, clove oil, pimento berry oil, eugenol	*Laurus nobilis, Syzygium aromaticum, Pimento dioica*	EC	**IBF, RVF, IVBGE by eugenol**	[67]
Oregano oil, thyme oil, carvacrol, thymol	*Origanum vulgare; Thymus vulgaris*	EC	**IBF; carvacrol, thymol inhibited fimbriae formation and swarming motility**	[68]
Tee tree oil	*Melaleuca alternifolia*	SA	**IBF, IVBGE**	[69]
HinokiCypress oil	*Chamaecyparis obtusa*	SA	**IBF, RVF, IVBGE**	[70]
Eugenol	—	SA, PA	**IBF, RVF**	[71]
Eugenol	—	SA	**IBF, BE, IVBGE; inhibition of colonization of rat middle ear cavity**	[72]
Carvacrol, citral, (+)-limonene	—	SA	**IBF**	[73]
Cinnamon oil, cinnamaldehyde	*Cinnamomum zeylanicum, Cinnamomum cassia*	SA, EC, PA	**IBF**	[74]
Tea tree oil	*Melaleuca alternifolia*	SA	**IBF**	[75]
MBEO	*Melaleuca bracteata*	CV	**IBF, RVF, IVBGE**	[76]
Thyme oil, clove oil, eugenol, cinnamaldehyde	*Thymus vulgaris, Syzygium aromaticum*	SA	**IBF, BE**	[77]
Oregano oil, carvacrol, thymol	*Origanum vulgare*	SA	**IBF, BE**	[78]
Tee tree oil	*Melaleuca alternifolia*	SA	**BE**	[79]
Oregano oil	*Origanum onites*	SA, EC	**IBF, BE**	[80]
Tea tree oil, lavender oil, lemon balm oil, α-terpineol, terpinen-4-ol, linalool, linalyl acetat	*Melaleuca alternifolia, Lavandula angustifolia, Melissa officinalis*	SA, EC	**BE**	[81]
Clove oil, lemongrass oil	*Syzygium aromaticum, Cymbobogon citratus*	SA	**BE**	[82]
Cassia oil, Peru balsam oil, thyme oil	*Cinnamomum aromaticum, Myroxylon balsamum, Thymus vulgaris*	PA, SA	**BE**	[83]
Menthol	–	EC	**IVBGE**	[84]
Clary sage oil, juniper oil, lemon oil, α-pinene, limonene, linalool	*Juniperus communis, Citrus lemon, Salvia sclarea*	EC	**IBF**	[85]
Cinnamon oil, tea tree oil, palm rosa oil	*Cinnamomum zeylanicum, Melaleuca alternifolia, Cymbopogon martini*	PA	**BE**	[86]
Thyme oil	*Thymus vulgaris*	EC, PA	**IBF**	[87]
Citrus oil	*Citrus reticulata*	PA	**IBF, RVF**	[88]
Tea tree oil, clove oil, cinnamon oil	*Melaleuca alternifolia, Syzygium aromaticum, Cinnamomum zeylanicum*	SA, PA	**IBF**	[89]
Eucalyptus oil, 1,8-cineole	*Eucalyptus globolus*	SA	**IBF, BE; inhibition of swarming motility**	[90]
Citronella oil, geraniol	*Cymbopogon nardus*	SA	**IBF**	[91]

continued

▶ **Table 3** *Continued*

EOs and EOCs	Plant sources	Bacteria	AQS and AB activities	References
Oregano oil	*Origanum vulgare*	SA, PA	**BE; change biofilm morphology, killing biofilm bacteria**	[92]
Cinnamon oil, eugenol	*Cinnamomum zeylanicum*	SA, PA	**IBF, BE**	[93]
T. zygis oil, rosemary oil, oregano oil	*Thymus zygis, Rosmarinus officinalis, Origanum majorana*	EC	**IBF**	[94]

Essential oils (EOs) and individual oil compounds (EOCs) with QS and biofilm-inhibiting, virulence factor-reducing, and biofilm-eradicating activities. Bacteria: *S. aureus* (SA), *E. coli* (EC), *P. aeruginosa* (PA), *C. violaceum* (CV). Abbreviations: IBF = Inhibition of biofilm formation, BE = biofilm eradication, RVF = Reduction of virulence factors, VI = Violacein inhibition, IVBGE = Inhibition of virulence factor- and biofilm-related gene expression. Anti-QS activity means the disruption of the quorum sensing signaling circuit by bioactive substances. For *in vitro* testing and testing in aqueous surrounding the lipophilic EOs and EOCs were dissolved in different emulsifiers.

quantitative test approach using CV ATCC 12472. Its violacein inhibiting property was dose dependent. In comparison to the untreated control, clove oil inhibited violacein production in the treated bacteria by 48, 58, 78.40, and 92.30% at the sub-MICs (MIC for clove oil: 0.20%) of 0.04, 0.08, 0.12, and 0.16%, respectively, with little or no significant vitality loss of bacterial cells up to 0.12% (cell viability assay at 10^5 dilution: control: 8.14 log CFU/mL; clove oil at 0.12%: 8.02 log CFU/mL; at 0.16%: 7.29 log CFU/mL). This finding ensures that, up to a concentration of 0.12%, the measurable reduction in the formation of violacein in bacteria is not due to the antimicrobial properties of clove oil, but to its anti-QS activity. Only in the higher concentration of 0.16% was the observed reduction in violacein formation not only due to the anti-QS activity of clove oil, but also based on its toxic (antibacterial) properties.

Ahmad et al. [63] systematically investigated 29 monoterpenes and phenylpropanes commonly found in EOs for their anti-QS activity in CV ATCC 12472. There, 22 out of 29 test substances inhibited violacein production to varying degrees and at different MQSICs. The MQSICs of thymol, carvacrol, linalool, geraniol, *p*-cymene, menthone, thujone, camphor, nerol, estragole, isoeugenol, and citral were below their MICs, averaging 0.5 × MICs. This result means that the reduction of violacein was based on the anti-QS activities of these test substances and not by their toxic properties. This claim is further supported by the fact that no growth inhibition occurred when subculturing bacteria previously treated with test substances in MQSI concentrations. Of particular interest were the apparent differences in violacein inhibition of the enantiomers tested. While the (+)-enantiomers of carvone, limonene, and borneol increased violacein formation, in contrast, their (−)-analogues inhibited violacein production. From a therapeutic point of view, this observation is of particular interest, as more attention must be paid to the chemical composition of EOs containing such structural analogues.

Inhibition of quorum sensing-regulated formation of biofilms and virulence factors

In two *in vitro* studies, clove oil [64], peppermint oil (with 36.8% menthol) [65], and menthol [65] were used in sub-MICs to reduce the biofilm formation in PA PAO1. In order to ensure that the observed inhibition of biofilm formation by the test substances is a QS-regulated process, the reduction of various virulence factors (elastase, proteases, pyocyanin, chitinase) in sub-MICs was also determined. Compared to the corresponding controls, both EOs as well as menthol were able to inhibit, in sub-MICs, the QS-regulated biofilm formation in a concentration-dependent manner without inhibiting the growth of the test strains. In addition, all test compounds strongly inhibited the production of virulence factors, ePS production, and swarming motility (▶ **Table 4**) [64, 65]. Furthermore, it could be shown that the violacein production in CV CVO26 was maximally reduced by clove oil (at 0.12%), peppermint oil (at 0.1%), and menthol (at 400 µg/mL) in sub-MICs without reducing bacterial cell growth [62, 65].

Using the recombinant reporter bacterium EC MG4 (pKDT17), it was demonstrated that clove oil (at 1.6%) was able to significantly reduce the native AHL production up to 56% in PA PAO1 cells [64]. This result is an interesting finding because it is known from the literature that PA strains defective in producing the autoinducer molecule 3-oxo-C12-HSL (N-(3-oxo-dodecanoyl)-L-homoserine lactone) are limited in their ability to form full established biofilms [55].

In *in silico* molecular docking simulation experiments, menthol was found to fit very well to the AHL binding site of the LasR transcription factor [65].

Taken together, the data from the *in vitro* studies and the *in silico* analysis of menthol and LasR indicate that the production of virulence factors was substantially inhibited by clove oil [64], peppermint oil, and menthol [65] through interfering with the AHL-QS signaling cycle and inhibition of the LasR/RhlR regulation system (anti-QS activity). It is known from literature that the production of the virulence factors pyocynin, elastase, chitinase, and total proteases in PA is under control of the LasR/RhlR transcriptional regulatory proteins [13, 45, 53]. Regarding biofilm formation, one can assume a direct correlation between the QS system and biofilm formation through interfering of the test substances with the AHL-QS signaling circuit and in a more indirect way by reduction of essential components such as ePSs and swarming motility. ePSs are crucial for establishing biofilm structure, and swarming motility contributes to the initial steps of biofilm formation [13, 45].

In two *in vivo* experiments (nematode model, *Caenorhabditis elegans*), it was shown that nematodes infected with PA survived the bacterial infection by 62 and 58%, respectively, when treated with sub-MICs of clove oil (1.6%) and menthol (800 µg/mL) com-

▶ **Table 4** Reduction of biofilms and virulence factors.

EOs and EOCs	Sub-MICs	Biofilm formation	ePS production	Pyocyanin production	Elastase activity	Swarming motility
Peppermint oil (MIC: 6.4%)	**(%; v/v)**	**Reduction (%)**	**Reduction (%)**	**Reduction (%)**	**Reduction (%)**	**Reduction (%)**
	0.375	42.8	39.9	52.4	30.3	50.6
	0.750	61.5	59.2	62.2	48.2	61.3
	1.500	72.1	64.8	72.1	65.5	70.6
	3.000	84.2	76.5	85.2	80.0	81.3
Menthol (MIC: 1 mg/mL)	**(µg/mL)**	**Reduction (%)**	**Reduction (%)**	**Reduction (%)**	**Reduction (%)**	**Reduction (%)**
	100	14.7	38.6	59.7	34.7	14.7
	200	43.2	41.7	63.4	56.0	43.2
	400	54.0	48.3	74.4	75.8	54.0
	800	69.4	57.7	83.5	78.7	69.4
Clove oil (MIC: 3.2%)	**(%; v/v)**	**Reduction (%)**	**Reduction (%)**	**Reduction (%)**	**Reduction (%)**	**Reduction (%)**
	0.2	20.0	31.0	37.0	14.0	52.0
	0.4	31.0	61.0	47.0	33.0	60.0
	0.8	50.0	65.0	60.0	49.0	66.0
	1.6	65.0	77.0	75.0	69.0	80.0

Inhibitory effects of sub-MICs of clove oil [64], peppermint oil [65], and menthol [65] on the quorum sensing-regulated biofilm formation and production of selected virulence factors in *P. aeruginosa* PAO1. The reduction of biofilm formation as well as of virulence factors is stated in percent over the control (percentage decrease); ePS: expopolysaccharide; ▶ **Table 4** changed according to [64, 65].

pared to non-treated specimens. In this case, the nematode model is a very interesting *in vivo* infection model because the PA-mediated killing of the invertebrate worm is mainly based on QS-controlled virulence factors such as hydrogencyanid. Previously, it has been shown that both test substances in the mentioned sub-MICs drastically reduced the production of different virulence factors of PA, including pyocynin. It can therefore be speculated that clove oil and menthol could be responsible for the survival of PA-infected nematodes by interfering with the QS-dependent pathogenicity process [64, 65].

In a recent *in vitro* study [66], eugenol in sub-MICs (200, 400, 600 µM) was investigated for its anti-biofilm formation and anti-virulence factor production against PA PAO1 and two multidrug resistant PA clinical isolates. Compared to the control, eugenol (MIC: 1.67 mM) significantly inhibited, in a concentration of 400 µM, the biofilm formation (by 66%) as well as the production of virulence factors such as ePSs (by 65%), rhamnolipid (by 57%), elastase (by 70%), protease (by 65%), pyocyanin (by 68%), and pyoverdine (by 69%) in all PA test strains without bacterial cell growth inhibition. From the literature, it is well known that ePSs, rhamnolipids, and pyocyanin are crucial components of an intact biofilm matrix. Rhamnolipids are involved in microbial cell adhesion and biofilm formation as well as to maintaining open channels for nutrient transport in biofilms [13, 45]. In addition, the authors [66] could demonstrate with RT-qPCR analysis that the gene expression of the QS-regulated virulence factors was downregulated in PA after treatment with 400 µM eugenol. In a further experiment, it could be pointed out that eugenol in a concentration 400 µM was also able to reduce the levels of two signal molecules (N-butyryl-homoserine lactone, N-dodecanoyl homoserine lactone) in PAO1 without disruption of the signal molecule integrity. These findings were supported by the fact that the expression of the corresponding signal genes, lasI, lasR, rhlI, and rhlR, were reduced by eugenol. *In silico* molecular docking experiments also revealed a stable molecular binding between eugenol and the QS receptor LasR. This finding fits very well with comparable experiments with menthol carried out previously in an *in silico* docking assay [65]. If one evaluates the *in vitro* studies and the molecular biological experiments together, one may assume that eugenol unfolds its anti-QS properties via the deactivation of the QS signal cascade by the competitive binding of the AHL-QS signal molecules to the cognate receptor molecule LasR. LasR is the master regulator protein of the QS-system in *Pseudomonas aeruginosa*. As a result, gene expression is reduced, which results in a lower concentration of AHL molecules and virulence factors compared to the individual controls.

Enterohemorrhagic EC O157:H7 (EHEC) is a worldwide dreaded intestinal bacterium that, among other things, can cause hemorrhagic colitis associated with bloody diarrhea. In an *in vitro* study [67], bay oil (with 62% eugenol), clove oil (with 77% eugenol), and pimento berry oil (with 71% eugenol) as well as eugenol, a prominent constituent of these oils, were tested for their anti-biofilm activity. Biofilm formation was inhibited by all three EOs and eugenol (test concentrations: 0.001, 0.002, 0.005, 0.01%) in concentrations of 0.005% (MICs of test substances: > 0.1%) to 99.7% compared to the control. The effect of the EOs and eugenol

on biofilm formation was dose dependent. Up to the test concentration of 0.005%, the planktonic cells were not inhibited in their growth. Only from a concentration of 0.01% was the vitality of the bacteria reduced by 20%. Thus, one can assume that the anti-biofilm activity of the test compounds in the concentration range of 0.001 to 0.005% is due to anti-QS activity and not due to the toxicity of these substances. In the nematode model, *C. elegans*, it could be shown that in comparison to the control, clove oil and eugenol in a concentration of 0.005% significantly prolonged the survival of nematodes infected with PA.

In addition, the researchers [67] studied the genetic basis of the anti-biofilm activity of clove oil and eugenol in EHEC. It could be shown that the transcription state in EHEC cells under the influence of both test compounds changed significantly, and the expression of curli fimbriae genes and other fimbriae genes was significantly downregulated by both compounds in a concentration of 0.005%. Furthermore, the expression of several motility genes were also inhibited by clove oil, but not by eugenol. It is known from the literature that fimbriae and curli fimbriae are crucial for adhesion of bacteria to different surfaces. Bacterial motility is also involed in the early stages of surface colonization by bacteria [43].

Uropathogenic EC (UPEC) not only causes urinary tract infections but also colonizes the surfaces of urinary tract catheters. In a comprehensive *in vitro* study, 79 different EOs were screened for their anti-biofilm activity against UPEC [68]. Of the EOs tested, oregano oil (6.8% thymol, 77.8% carvacrol) and thyme oil (53.3% thymol, 14.7% carvacrol) as well as their main compounds, carvacrol and thymol, proved to be particularly active ingredients. In sub-MICs (at 0.01%), all test compounds significantly inhibited biofilm formation of UPEC. The reduction of biofilm mass for oregano oil was 88.9%, thyme oil 86.1%, carvacrol 94.5%, and thymol 94.5% over the control. The strong reduction of biofilm mass by carvacrol and thymol fits in with the observation that carvacrol and thymol significantly reduced fimbriae (adhesion molecule) formation and inhibited the swarm motility of the bacteria. Both virulence factors are of the utmost importance for the spread of bacteria throughout tissues and in producing high biofilm mass [43].

In a recent experimental study, TTO was investigated for its anti-biofilm activity in SA ATCC 29213 [69]. The most interesting focus of the work was the question of whether TTO influences the gene expression profile (transcriptome analysis) of biofilm-borne bacterial cells. To answer the question, RNA sequencing (RNA-seq) as well as real-time RT-qPCR methods were used. Transcriptome analysis allows for a broad mapping of genes that are involved in the regulation of physiological as well as pathophysiological processes in cells. In this case, the authors reported on the evaluation of differentially expressed genes (differential gene expression) in biofilm SA cells at a TTO concentration of 1 mg/mL (0.5 × the minimum biofilm inhibition concentration) and an incubation time of 60 min. Compared to the untreated control group, gene expression in the TTO-treated biofilm cells was altered by a total of 304 genes, with 104 genes downregulated and 200 genes upregulated. The results were discussed primarily with regard to those genes that can be linked to biofilm formation. For instance, the virulence factor sarA gene encodes for the DNA-binding protein SarA. SarA is a cytoplasmic transcriptional regulatory protein that activates the agr operon and independent of the QS system, controls the expression of many virulence genes in SA cells that are involved in biofilm formation, such as some matrix adhesion genes (e.g., fnbA, encoding fibronectin-binding protein A). In the present work, sarA was downregulated [69]. In a previous study, EO distilled from *Chamaecyparis obtusa* leaves (with 19% sabinene) reduced the expression of several regulator genes such as argA (at 0.2 mg/mL) and sarA (at 0.3 mg/mL), which resulted in a decreased production of virulence factors in SA [70]. The downregulation of sarA and the strong reduction of the subsequent biofilm mass in SA suggest that individual components of the EOs can directly interfere with gene regulation. So far, the mechanism of action is unknown.

According to Al-Shabib et al. [71], eugenol in sub-MICs (0.125–0.500 × MIC) resulted in a very strong reduction in biofilm mass in methicillin-resistant SA (MRSA) strains isolated from proband hands and noses without any decrease in bacterial growth. In parallel experiments, the authors investigated the interaction of eugenol with SarA using *in silico* molecular docking experiments. These experiments revealed a strong affinity of eugenol to SarA-binding sites, which may explain partially the strong reduction of biofilm formation in the MRSA test strains.

The authors [71] could also show that compared to the control, eugenol (in sub-MICs) was able to reduce the QS-regulated production of virulence factors (elastase, protease, chitinase, pyocyanin, and ePSs) in PA PAO1 in a concentration-dependent manner.

In a further *in vitro* experiment, Yadav et al. [72] could demonstrate that the biofilm formation of MRSA strains as well as of SA ATCC 29213 was significantly reduced by > 50% at a concentration of 0.02% eugenol (MIC: 0.04%). Real-time RT-qPCR tests demonstrated that 0.02% eugenol reduced the expression of biofilm-related genes such as sarA (*Staphylococcus* accessory regulator A gene), seA (*Staphylococcus* enterotoxin A gene), and icaD (intercellular adhesion gene). Both *in vitro* studies taken together shed light on the possible anti-biofilm mechanism of action of eugenol. The marked biofilm inhibition *in vitro* at sub-MICs of eugenol is in accordance with the corresponding gene expression studies. Therefore, it can be concluded that the prevention and inhibition of biofilm mass production in SA strains by sub-MICs of eugenol is based on the reduction of biofilm-related genes as well as on the inhibition of gene products such as SarA.

On the other hand, it was demonstrated by scanning electron microscopy that at a concentration of 2 × MIC, eugenol-treated biofilms were disrupted. The bacteria inside lost their normal structure and their cytoplasmic membrane was perforated. These structural changes are typical signs of a biocidal eugenol effect [72].

Yadav et al. [72] complemented their *in vitro* findings on eugenol anti-biofilm activity with an *in vivo* colonization experiment using an otitis media rat model. Pathogen-free Sprague-Dawley rats were treated solely (1) with *S. aureus* bacteria, (2) with bacteria together with 0.5 × MIC eugenol, and (3) with medium containing 0.1% DMSO by injecting the bacteria and the test substances into the middle ear cavity. Eugenol in 0.5 × MIC revealed a significant reduction of the bacterial colonization of the middle ear chamber (tympanic bulla) of about 88%, whereby no visible biofilms or cell

debris were detectable. In contrast, the tympanic bulla of rats treated only with bacteria were filled with both bacterial biofilms and cell debris.

Espina et al. [73] studied citral (MIC: 500 µL/L), carvacrol (MIC: 200 µL/L), and (+)-limonene (MIC: 5000 µL/L), three monoterpenes found in many EOs, for their anti-biofilm effects in multidrug-resistant SA strains. They could prove that these three monoterpenes in sub-MICs [carvacrol: 100 µL/L, citral: 200 µL/L, (+)-limonene: 2000 µL/L] reduced bacterial biofilm mass production in SA SC-01, the most sensitive strain, after 40 h of incubation of about 82% for carvacrol, 65% for citral, and 78% for (+)-limonene. Of the three monoterpenes, carvacrol proved to be the most potent anti-biofilm substance. In SA SC-01, carvacrol inhibited biofilm mass production at the low concentration of 10 µL/L up to 80%.

In a recent *in vitro* study, Firmino et al. [74] investigated the biofilm inhibition activity of cinnamaldehyde (Ca) as well as of two different cinnamon oils derived by hydrodistillation from the trunk barks of *Cinnamomum zeylanicum* (EOCz with 68.7% cinnamaldehyde, 71.2% cinnamoyl acetate) and *Cinnamomum cassia* (EOCc with 90.2% cinnamaldehyde). Amongst others, biofilm-forming strains of SA, EC, and PA were used as test bacteria. EC and PA were the most sensitive to EOCc. In sub-MICs (sM), EOCc inhibited the biofilm formation of EC (sM: 0.12 mg/mL) and PA (sM: 0.06 mg/mL) to 100%. In addition, PA biofilms were also completely inhibited by EOCc at the sub-MIC of 0.12 mg/mL. In both cases, the viability of bacterial cells in the biofilms were not affected. On the other hand, the SA biofilm mass was inhibited to 100% by both oils on their bacterial MICs of 0.25–0.50 mg/mL. Interestingly, all test bacteria were less sensitive to cinnamaldehyde, the main compound of both cinnamon oils. The 100% biofilm inhibitory effect of this substance was at the bacterial MIC of 0.25%. This means, that in this case, the biofilm inhibitory effect of cinnamaldehyde is not due to its anti-QS effect, but mainly to its cytotoxic effects, as confirmed by cell viability studies.

In persistent or frequently recurring otitis media in childhood, the insertion of a tympanostomy tube may be a therapeutic option. It allows for ventilation of the middle ear via the ear canal and drainage of fluid. After the insertion of a tympanostomy tube, MRSA-related otorrhoe is not uncommon. Thus, a colonization of the tubes with biofilm-forming MRSA strains must be avoided. In an *in vitro* study [75], it could be shown (by scanning electron microscopy) that TTO (tubes pretreated with 100% oil), in contrast to the control (tubes pretreated with saline), significantly reduced the colonization of the tympanostomy tubes with biofilm-forming MRSA strains. In addition, the biofilm formation was also significantly inhibited. Due to the high concentration of TTO used, the strong reduction in MRSA biofilm formation may be due to the direct cell toxicity (antimicrobial) effect of TTO.

Wang et al. [76] investigated the EO from *Melaleuca bracteata* (MBEO) leaves (with 90.5% methyleugenol) for its anti-QS, anti-biofilm, and anti-virulence factor activity in CV ATCC 31532. At sub-MICs of 0.625, 1.25, 2.5, and 5.0‰, MBEO (MIC: 10‰) was reported to inhibit biofilm mass production and violacein production as well as the production of different virulence factors in a concentration-dependent manner without disturbing bacterial cell growth. At 2.5‰, the production of biofilm mass and virulence factors were reduced by 75%, and at 5.0‰, MBEO reduced violacein formation by 85.4%. In addition, the swarming motility of CV cells were significantly disturbed at 5‰. In comparison to the control group, MBEO significantly reduced, in concentrations of 5.0, 2.5, and 0.625‰, the concentration of the autoinducer QS signal C6-HSL in CV cells. In the control group, the C6-HSL concentration was 0.38 mg/mL, while in the oil-treated groups (5, 2.5, and 0.625‰), the autoinducer concentrations were 0.08, 0.12, and 0.22 mg/mL.

In CV CviI (AHL synthase) and CviR (AHL receptor) are important QS regulatory proteins. Using an RT-qPCR assay, it could be demonstrated that the expression of the corresponding genes, cviI and cviR, was downregulated by MBEO in a dose-dependent manner in comparision to the control [76]. This outcome fits very well with the reduced C6-HSL concentration in CV cells after MBEO treatment. In addition, MBEO was able to reduce the gene expression of several virulence genes, such as vioA–E, hcnB, lasA, lasB, pilE1, and pilE3 in a dose-dependent manner.

The experimental outcome suggests that the reduction of biofilm formation and virulence factor production after MBEO treatment was mainly based on the reduced C6-HSL level concentration in CV cells and the subsequent downregulation of the associated regulatory and virulence genes. In addition, the reduced swarming motility of the bacterial cells may also be partly responsible for the reduction in the biofilm mass as well as for the destruction of the biofilm architecture.

Comments to the test substance concentrations used *in vitro*

The anti-virulence activities of the test compounds were mostly tested up to a maximal concentration of 0.5 × MIC, without inhibiting bacterial vitality or killing the bacteria. The authors concluded from these observations that the anti-virulence activities of the test substances were based primarily on their physiological effects (non-antibacterial) and not on their cell toxic (antibacterial) activities. At a test concentration of 0.5 × MIC, however, it cannot be completely ruled out that, in addition to physiological effects, cell toxic effects of the substances can also occur, as described by Reichling et al. [27]. The authors could demonstrate that TTO in a sub-MIC of 0.12% (0.5 × MIC) significantly disturbed the formation of the cytoplasmic membrane in SA. In the cytoplasm, lamellar-like membrane structures or mesosome-like membrane piles were seen by means of electron microscopy. In addition, the typical granulation structure of normal SA cells was disturbed and cell division of the bacteria was partially interrupted. These findings were also seen in bacteria treated with sub-MICs of antibiotics. In contrast, at the MIC of 0.25% TTO, the cell division of the bacteria was completely inhibited and condensed, and filamentous and electrondense material (probably condensed DNA and proteins) were observed in the cytosol.

Inhibition of biofilm formation at toxic (biocidal) concentrations of essential oils and essential oil compounds

Using a reduction assay for biofilm formation, a total of five EOs and four major active compounds were tested according to their anti-biofilm activities in antibiotic-resistant (vancomycin and cef-

triaxone) SA strains [77]. Of the tested agents, only eugenol (MICs for planctonic cells: 0.8–1.6%) and thyme oil (MICs for plantonic cells: 0.2–0.8%) revealed a concentration-dependent biofilm inhibition in microtiter plates. At 0.2% eugenol, the reduction of biofilm formation was found to be 19.4%, which was raised to 91.6% at 12.8% eugenol. It is noteworthy that at a concentration of 4 × MIC, eugenol inhibited biofilm formation of antibiotic-resistant SA strains by nearly 90%. In contrast to eugenol, thyme oil had slightly lower anti-biofilm activity. The maximum reduction of biofilm formation at 12.8% thyme oil (highest concentration tested) was 88.7%. Inhibition of biofilm formation by thyme oil and eugenol in the SA JSA10 strain was also observed under a light microscope. Untreated biofilms exhibited a dense network of cells with an extracellular polymeric matrix. In contrast, thyme oil and eugenol in the highest concentration tested (12.8%) efficiently reduced the number of bacterial colonies and destroyed the biofilm matrix. The microscopic control of bacterial biofilm reduction shows that the effect of both substances is mainly due to their biocidal effect. In practice, this means that the growth of biofilm-forming SA strains on surfaces (such as catheters) can definitely be inhibited or prevented by using biocidal concentrations of active EOs or oil compounds.

Eradication of already established biofilms

The antibacterial effect of antibiotics and other antimicrobial compounds is usually measured in liquid culture (free-floating cells) and expressed as MIC. However, as shown above, this method does not reflect the real situation in most cases. Many infectious bacteria form biofilms and thus escape effective antibiotic therapy. Against this background, the question arises as to whether EOs are capable of killing pathogenic bacteria such as SA, PA, and EC in biofilms that were already formed. These bacteria often cause nosocomial, biofilm-associated infections. Due to their intrinsic resistance and adaptive capacity, they are difficult to combat with antibiotics.

In some comparative *in vitro* studies, the antibacterial effects of selected EOs and individual oil compounds were investigated against the above bacteria in liquid cultures (planktonic cells) and in biofilms. As shown in ▶ **Table 5**, the tested EOs as well as single oil compounds show comparable antibacterial effects in both test systems. SA and EC were found to be relatively sensitive to the test substances compared to PA, with planktonic cells having lower MIC values than their biofilm counterparts. The MBECs were, in some cases, 2 to 8 times higher than the MICs of the corresponding planktonic cells. These results indicate that EOs can combat both planktonic bacteria as well as bacteria living in biofilms.

In a comparative *in vitro* study [83], cassia oil and the peptide antibiotic colistin (used to fight respiratory infections) were tested for their ability to kill PA in liquid culture (planktonic cells) and in biofilms. The experiments revealed that cassia oil in concentrations of 0.2–0.4% was sufficient to kill the vast majority of PA in liquid culture and in biofilm. In contrast, the MIC of colistin (3 µg/mL) was not effective against cells within the biofilm. It was only in a concentration of > 100 µg/mL that colistin killed the bacteria inside the biofilm.

To test new antimicrobial strategies against PA, three different EOs, cinnamon oil, TTO, and palmrosa oil, as well as the antibiotic ciprofloxacin were investigated for their anti-biofilm properties *in vitro* [86]. In already established biofilms (24-hour-old biofilms), all three EOs reduced the biofilm mass by about 80% at a concentration of 0.96%. In comparison to this, the number of bacterial cells living in the biofilm was reduced by 2.5 $\log_{10}$ steps (5 $\log_{10}$ CFU/cm^2 to 2.5 $\log_{10}$ CFU/cm^2). In the same experimental assay, ciprofloxacin, in the highest concentration tested (80 µg/mL), reduced the biofilm mass about 70% and the number of biofilm bacteria about 2.0 $\log_{10}$ steps (5 $\log_{10}$ CFU/cm^2 to 3 $\log_{10}$ CFU/cm^2). An interesting variation of the experiments was to combine TTO with the antibiotic ciprofloxacin. The combination of TTO (0.48%) and ciprofloxacin (10.0 µg/mL) was very effective, since in this combination a significant reduction of PA biofilms (of about 80%) and a complete eradication of the biofilm bacteria was recorded.

Lu et al. [92] demonstrated the complete killing of SA and PA in biofilms after 1 h of incubation by oregano oil (with 72.3% carvacrol) at concentrations of 0.4 and 1.0 mg/mL, respectively. In a mouse model, third-degree burn wounds were infected with PA or SA (MRSA); 24 h after bacterial inoculation, the wounds were topically treated with oregano oil in a concentration of 10 mg/mL for 3 consecutive days. The bacterial concentrations were reduced by 3 $\log_{10}$ steps without damaging the skin of the animals [92]. In parallel to these studies, experiments with transmission electron microscopy and scanning electron microscopy showed that oregano oil is able to kill bacteria in biofilms and to change their structure. Of great practical and therapeutic interest was the observation that with oregano oil in sublethal doses, over 20 passages did not lead to bacterial resistance. This observation is of high interest because in recent years it has become known that various EOs in sub-MICs can indeed lead to temporary physiological adaptations in bacteria, which may adversely affect the therapeutic effect of such oils in the short term [84, 95].

Data hitherto published suggest that the antimicrobial EOs may adversely affect the viability of bacteria in the biofilm but may not directly disrupt the biofilm matrix or EPS structure by chemical intervention. The observable and measurable destruction or eradication of existing biofilms by the use of EOs is most likely to be indirect, by killing the bacteria in the biofilms, which prevents further biofilm formation.

Anti-biofilm activity of essential oils and essential oil compounds against other bacteria species

Besides CV, EC, PA, and SA, the volatile EOs and EOCs revealed their biofilm-inhibiting, virulence factor-reducing, and biofilm-eradicating properties also with numerous other types of bacteria, e.g., *Aeromonas hydrophila* [64], *Staphylococcus epidermidis* [78], *Staphylococcus lugdunensis, Staphylococcus haemolyticus, Staphylococcus sciuri* [80], *Pseudomonas putida* [83, 85], *Bacillus cereus, Pichia anomala* [85], *Acinetobacter baumannii* [92], *Hafnia alvei* [96], *Pseudomonas flurescens* [97], *Klebsiella pneumoniae* and *Proteus mirabilis* [98], *Pectobacterium carotovorum*, and *Pectobacterium aroidearum* [99]. For instance, Joshi et al. [99] demonstrated that eugenol and carvacrol in both *Pectobacterium* species significantly reduced, in sub-MICs, the biofilm formation, production of

▶ **Table 5** Eradication of mature biofilms.

EOs and EOCs	Bacteria	MIC plactonic cells (%; v/v)	MBEC biofilm cells (%; v/v)	References
Eugenol	SA	0.040	0.080	[72]
Carvacrol		0.020	0.040	[72]
Carvacrol		0.015	0.250	[78]
Oregano oil		0.062	0.500	[78]
Thymol		0.031	0.250	[78]
α-Terpineol		0.190	0.380	[81]
Terpinen-4-ol		0.190	0.190	[81]
Linalool		0.190	0.780	[81]
Linalyl acetat		0.190	0.190	[81]
Cassia oil		0.300	0.600	[83]
Clove oil		1.200	1.600	[83]
Peru balsam oil		2.500	3.500	[83]
Thyme oil		0.500	1.600	[83]
Lemon balm oil		0.100	0.400	[81]
Tea tree oil		0.400	0.800	[81]
Lavender oil		0.800	1.600	[81]
Cassia oil	PA	0.200	0.400	[83]
Clove oil		> 5.000	< 5.000	[83]
Peru balsam oil		2.500	3.500	[83]
Thyme oil		> 5.000	2.000	[83]
Cinnamaldehyde		0.100	0.200	[83]
Eugenol		> 5.000	3.300	[83]
Tea tree oil	EC	0.190	0.190	[81]
Lavender oil		0.190	0.190	[81]
Lemon balm oil		0.048	0.190	[81]
α-Terpineol		0.097	0.190	[81]
Terpinen-4-ol		0.048	0.048	[81]
Linalool		0.097	0.190	[81]
Linalyl acetat		0.190	0.190	[81]

Eradication of already established biofilms. Selected essential oils and individual oil compounds with anti-biofilm activity against *S. aureus* (SA), *P. aeruginosa* (PA), and *E. coli* (EC). MIC: minimum inhibitory concentration (growth inhibition of bacteria in liquid culture); MBEC: minimum biofilm eradication concentration (elimination of biofilms from a surface indirectly detected by inhibition of bacterial growth on agar or by crystal violet staining and optical density measurement).

QS signaling molecules (AHLs) and AHL-QS-regulated gene expression. In addition, both compounds seem to exert their effects through direct binding to LuxI-type AI synthase (ExpI) and LuxR-type AI-dependent transcriptional regulator protein (ExpR), as demonstrated by *in silico* docking experiments.

Nano-encapsulated essential oils in drug delivery systems as new anti-biofilm agents

The lipophilic character of EOs and EOCs normally severely limits their experimental or therapeutic use, e.g., due to their low penetration ability in aqueous biological and nonbiological habitats (e.g., biofilm matrix, exuding wounds). In the last two decades, this limitation has been overcome by developing drug nano-delivery systems such as polymeric nanocapsules, nanoparticles, liposomes, and some other nano-carrier systems [100, 101].

Against this background, it is postulated that nano-encapsulated EOs and single oil substances could represent an optimal nano-delivery system to combat bacteria living in the aqueous biofilm medium or in other aqueous biological environments. Nano-carrier systems that contain EOs are designed in a way that makes the whole galenic formulation water soluble and, for example, allows the nano-carriers to penetrate the water-filled channels and caverns of the biofilm easily [100, 101]. Nano-encapsulated EOs are protected against oxidative influences and evaporation. Due to the increased water solubility, not only a targeted mass transport, but also a targeted and controlled release of the active EO or individual oil compound at the site of action is possi-

▶ **Table 6** Nano-encapsulated EOs as anti-virulence agents.

Essential oil	Carrier system	Bacteria	Biological effects	References
Peppermint oil, cinnamic acid	Silica (SiO_2) nano-capsules (size: 1000 nm)	SA, EC, PA	BE; kills bacteria inside biofilms	[102]
Eucalyptus oil	Silica (SiO_2) nano-particles (size: 1000 nm)	EC	Eradication of biofilms on catheter surface	[103]
Cinnamon oil	Liposomes (size: 1000 nm)	SA	Eradication of biofilms on different surfaces	[104]
Tea tree oil	Lipid carrier with acetyl palmitate as lipid substance (size: 166 nm)	PA	BE; preventing the adhesion of bacterial cells to buccal epithelial cells, reduction of bacterial motility and biofilm formation	[105]
Thyme oil, eucalyptus oil, clove oil	Bacterial cellulose polymer	SA, PA	IBF	[106]
Eucalyptus oil, cinnamon oil, orange oil	Silica (SiO_2) mesoporous nano-system	SA, EC	IBF	[107]
Thyme oil	Nano-liposomes; nano-archaeosomes	SA	IBF, BE	[108]

Anti-biofilm activities of nano-encapsulated EOs. SA = *S. aureus*, PA = *P. aeruginosa*,; EC = *E. coli*, BE = biofilm eradication, IBF = inhibition of biofilm formation. Nano-liposomes: designed of soybean phosphatidylcholine and polysorbate 80; nano-archaeosomes: designed of soybean phosphatidylcholine, polysorbate 80 and total polar archaeolipids (from archaebacterium *H. tebenquichense*)

ble [100, 101]. In two most informative reviews, the chemical and biopharmaceutical requirements for the preparation and therapeutic use of EOs encapsulated in nano-carrier systems have been described [100, 101]. Over the past 10 years, numerous publications on different nano-carrier systems with encapsulated EOs have appeared. Below are a few selected examples (▶ **Table 6**) to highlight the growing importance of this research area for the development of special EO loaded nano-delivery systems as new anti-infective agents to fight bacteria inside of mature biofilms as well as to prevent biofilm formation on biological and nonbiological sufaces.

Infections with biofilm-forming bacteria such as EC, PA, and MSRA germs are particularly problematic in immunocompromised patients as well as for patients with bad healing wounds, urinary catheters, intravenous catheters, or joint prostheses. As has been demonstrated recently in an experimental study [102], a mixture of peppermint oil and cinnamic acid, both oils loaded in silica nano-capsules (average size: 1000 nm), penetrated relatively easily into the biofilm matrix and killed the bacteria therein (EC, PA, MSRA). It has been shown that the antimicrobial activity of the nano-encapsulated EOs increased in comparison to non-encapsulated oils. This significant increase in the activity of the delivery vehicle over the control was explained by a higher bioavailability of the nano-encapsulated EOs in the bacteria-populated aqueous channels and cavern. The higher bioavailability of EOs in the biofilm was possible because the pH in the biofilm matrix changed (to a more acidic pH), which dissolved the structure of the capsules and released the EO near the bacteria [102].

In a similar experiment [103], eucalyptus oil was embedded into silica nano-particles (average size: 1000 nm; oil concentration: 50 µL/mL) and tested against EC biofilms on glass surfaces. The test result exhibited an 81 % reduction of biofilm formation by encapsulated eucalptus oil in contrast to the untreated control experiment. The oil-encapsulated nanoparticles were also significantly more active than eucalyptus oil (50 µL/mL) tested in an agar well diffusion assay and dissolved in Tween 20. In this case, the biofilm reduction only reached a value of about 62 %. A check with a light microscope revealed that the complex biofilm matrix was completely destroyed and only a few bacteria were recognizable in the image. The authors explained the relatively strong anti-biofilm effect of the nano-encapsulated eucalyptus oil with the fact that the drug delivery system used released an optimal amount of eucalyptus oil into the cells and in the biofilm of EC [103].

Cui et al. [104] studied the anti-biofilm activity of cinnamon oil encapsulated in liposomes against biofilm-forming MRSA strains on different surfaces such as gauze, non-woven fabrics, nylon membrane, and stainless steel. The morphological changes in the biofilm matrix after treatment with liposome-encapsulated cinnamon oil were followed using modern microscopic methods (e.g., scanning electron microscopy). Cinnamon oil encapsulated in liposomes was reported to eradicate established biofilms on different surfaces at a concentration of 1 mg/mL.

Comin et al. [105] investigated the influence of TTO nanoparticles on the adhesion of PA cells to buccal epithelial cells. It was shown that TTO nanoparticles significantly decreased the bacterial adhesion to buccal epithelial cells by about 70 % and inhibited bacterial cell motility about 63 %, both at a TTO nanoparticle concentration of 0.78 %. In addition, the oil-loaded nanoparticles also inhibited the biofilm formation about 40 % at the oil concentration of 12.5 %.

Junka et al. [106] impregnated bacterial cellulose (different biopolymers) with various EOs (0.98–1.03 µL oil/mg of dry bacterial cellulose), such as thyme oil, clove oil, and eucalyptus oil and tested these oil-impregnated particles against the biofilms of SA and PA attached to hydroxyapaptite. Thyme oil was reported to exhibit the best eradication effect against SA biofilms, whereas clove oil was more active against PA biofilms.

Balaure et al. [107] embedded various EOs (eucalyptus oil, orange oil, cinnamon oil) in a nano-carrier system consisting of mesoporous SiO_2 nanosystems (MSN). First, the physical and chemical data (morphology, porosity, chemical composition, loading capacity) of the designed nano-delivery systems were well characterized and then tested in sub-MICs for their biofilm inhibition activities using clinically relevant SA and PA strains. In an EO nanoparticle concentration of 0.62 µg/mL (and higher), the biofilm formation of both bacteria was significantly inhibited by all test compounds.

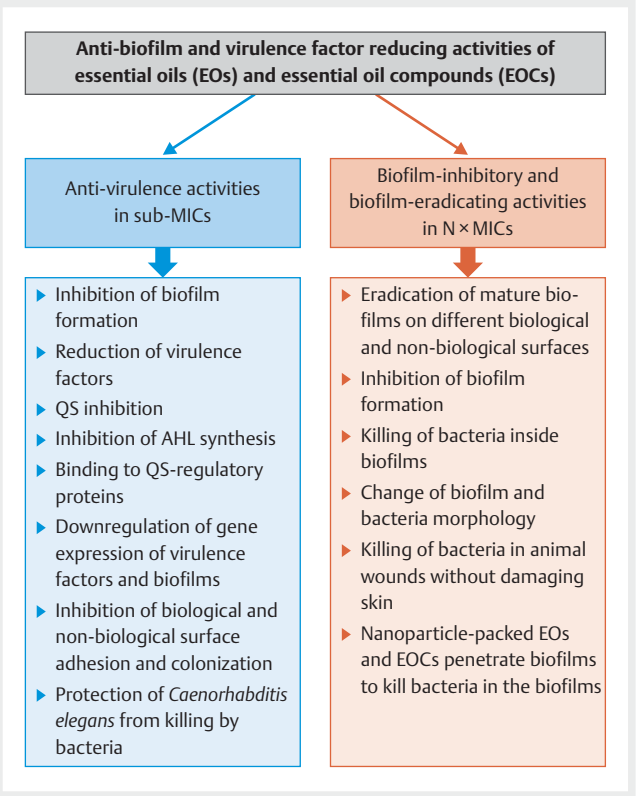
In a recent experimental study [108], two different nano-vesicles, nano-liposomes and nano-archaeosomes, were loaded with thyme oil (with 30% thymol and 23% *p*-cymene) and tested for their anti-biofilm activity in SA ATCC 25923 and four clinical MRSA strains. Nano-liposomes were designed of soybean phosphatidylcholine and polysorbate 80, while the nano-archaeosomes were made of soybean phosphatidylcholine, polysorbate 80, and total polar archaeolipids derived from the archaebacteria *Halorubrum tebenquichense*. Thyme oil (MIC_{90} for all SA strains: 2 mg/mL) encapsulated in nano-archaesomes exhibited a maximal inhibition of SA/MRSA biofilm formation (for SA, about 50–70% over the control) at sub-MICs of 0.5–1.0 mg/mL, while thyme oil containing nano-liposomes did not inhibit biofilm formation compared to the untreated control. In addition, thyme oil containing nano-archaeosomes also significantly decreased the biofilm mass of already established SA/MRSA biofilms (for SA, about 80%) at a concentration of 4 mg/mL thyme oil [108].

The results of the studies presented above show that nano-encapsulated EOs ideally combine the special properties of nano-carrier systems with the antibacterial activity of EOs. Thus, these special drug delivery systems provide an interesting opportunity in medicine to prevent the formation of biofilms as well as to eradicate any remaining biofilms and to fight bacteria within the biofilms.

Summary and Conclusion

In this review, an attempt was made to confront the interested reader with the problem of biofilm formation and virulence factor production of pathogenic bacteria. Biofilms protect bacteria living inside against adverse environmental influences and conditions. Antibiotics or disinfectants often fail in attempting to remove biofilms from biological or nonbiological surfaces, which can then serve as a source of recurrent infections. Against this background, efforts are currently being made worldwide to identify antibiotic active agents with new principles of action using plant-based natural products as a possible option [50, 51].

EOs and individual EOCs were reported in this overview to inhibit biofilm formation and reduce the production of virulence factors in sub-MICs. In higher concentrations (N × MICs), the volatile compounds were able to prevent biofilm formation from the very beginning on different sufaces and to kill bacteria inside the biofilm and subsequently reduce mature biofilms. In particular, cassia oil, cinnamon oil, clove oil, eucalyptus oil, oregano oil, TTO, thyme oil, and the oil components carvacrol, eugenol, thymol, limonene, and 1,8-cineol proved to be particularly effective



▶ **Fig. 2** Anti-biofilm and anti-virulence activities of essential oils (EOs) and individual essential oil compounds (EOCs) in biofilm forming bacteria such as *S. aureus, P. aeruginosa*, and *E. coli*. Summary of the physiological effects; N = ≥ 1.

substances. The most interesting anti-virulence effects of these compounds are put together compactly in ▶ **Fig. 2**.

The *in vitro* studies published so far and the limited *in vivo* data show that in sub-MICs, various bioactive EOs and EOCs are able to interrupt the QS-regulated bacterial signal transmission, which leads to the reduction of biofilms, virulence factors, and autoinducer synthesis. In order to better understand their mode of action, more studies have recently been performed at the genome and molecular levels using recombinant bacterial reporter strains, RT-qPCR assays, and *in silico* molecular docking studies. Summarizing the key findings of these studies, one can conclude that the active EOs and some of their ingredients can interfere with the expression of genes that are directly or indirectly responsible for the formation of biofilms, virulence factors, and the synthesis of autoinducer molecules. Most of the biofilm-related genes as well as virulence genes were downregulated. Thus, it is also known from studies on eukaryotic cells (e.g., fibroblast cells) that EOs are able to modulate the genome-wide expression of numerous genes in a variety of ways [109].

In silico analysis are used to discover anti-QS compounds and to study their bioactive mechnisms. This approach is also useful to estimate the binding affinity of EOCs to QS regulatory proteins to demonstrate and explain the anti-QS activity of EOCs and EOs in pathogenic bacteria. With this technique it could be shown that

eugenol has a strong and stable bond to LasR (in PA) [66] and SarA (in SA) [71], menthol to LasR (in PA) [65], L-carvone to HalI and HalR (in *H. alvei)* [96], and carvacrol and eugenol to ExpI and ExpR (in *Pectobacteria* spp.) [99]. LasR, Sar A, HalR, and ExpR are important master regulatory proteins that control the transcription and expression of genes that are involved in the production of virulence factors and biofilm formation. HalI and ExpI are autoinducer synthases. The inhibition of these QS regulatory proteins fits very well with the downregulated virulence genes and the reduced formation of virulence factors and biofilms in the bacteria named above. The *in silico* molecular docking analysis revealed a plausible molecular mechanism for the inhibition of the bacterial QS signalling system by eugenol, menthol, carvacrol, and L-carvone. In order to put these results on a broader basis, further *in silico* studies are required, which will analyze the interactions of EOCs with QS regulatory proteins or other transcription factors in the biofilm and virulence factor pathway in more detail. It is also a suitable method to find new anti-QS agents based on bioactive EOCs.

Nano-encapsulated bioactive EOs could represent a feasible and efficient approach to fight bacteria inside biofilms. Such a drug delivery system would modulate drug release, increase the physical stability of the active EOs/individual oil compounds, protect them from interactions with the environment, decrease their volatility, enhance their bioactivity, reduce toxicity, and improve patient compliance. The studies presented in this review show that nano-encapsulated EOs ideally combine the special properties of nano-carrier systems with the antibacterial activity of EOs. Thus, EO-loaded nano-carrier systems are therefore interesting antimicrobial agents to eradicate biofilms on catheters, prosthesis, and other medical devices [110] and for managing acute and chronic wounds [111].

In addition, combinations of EOs or individual EOCs and antibiotics both encapsulated in nano-carrier systems may be another way to successfully combat biofilm-associated bacteria and overcome existing antibiotic resistance.

Biofilm-associated pathogenic bacteria are a serious problem not only in medicine but also in the food industry. For instance, the formation of biofilms on food and food packaging can lead to food spoilage and foodborne illness [112]. Therefore, it is not surprising that in recent years EOs or single EOCs packaged in nanoparticles are also discussed in the food industry as possible active agents for combating biofilm-forming bacteria [112].

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Conflict of Interest

The authors declare that they have no conflict of interest.

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