Chapter

Essential Oils as an Innovative Approach against Biofilm of Multidrug-Resistant *Staphylococcus aureus*

Victor Alves Carneiro, Ramaiana Soares Melo, Antônio Mateus Gomes Pereira, Águida Maria Albuquerque Azevedo, Maria Nágila Carneiro Matos, Rafaela Mesquita Bastos Cavalcante, Renan Rhonalty Rocha, Vinícius de Queiroz Albuquerque, Jesús Alberto Pérez Guerrero and Francisco Eduardo Aragão Catunda Junior

Abstract

Staphylococcus aureus is one of the most common pathogens that cause recurrent, chronic, and biofilm-related diseases. Biofilms are the major form of bacterial structures capable of secreting polysaccharides that provide intrinsic protection against environmental stress like high concentrations of antibiotics. This, along with the emergence of multidrug-resistant strains, has made *S. aureus* infections a worldwide problem as a result of the inefficiency of the conventional medications. Plant essential oils (EOs) are an important source for drug discovery and pharmaceutical development due to their diverse biological activities, such as antimicrobial agents. The EOs' microbicide action is extensively reported at the scientific literature and frequently associated with bioactive molecules, such as aldehydes and terpenes. However, the ability of some EOs to inhibit biofilm formation has been poorly explored and it is still unclear how they could be applied in specific treatments against well-known infections. Therefore, this chapter will address virulence factors and biofilm formation of *S. aureus*, as well as bioprospecting of essential oil as a promising source in the search for new bioactive compounds employed in the fight against this microorganism.

Keywords: antibiofilm activity, biofilm-related diseases, essential oil, natural products, *Staphylococcus aureus*

1. Introduction

The emergence of multidrug-resistant (MDR) bacteria is correlated with selective pressure caused by the indiscriminate use of antibiotics, which reduces

therapeutic options available [1]. Consequently, it leads to a serious public health problem frequently associated with increase of healthcare costs and high morbimortality rates [2]. One worldwide recognized bacterial pathogen with the ability to develop severe clinical conditions such as pneumonia and septicemia is *Staphylococcus aureus* [3]. Historically, this bacterium has shown a great ability to become resistant to several antibiotics [4]. Furthermore, *S. aureus* has a highlighted ability to build surface-associated bacterial communities, called biofilm, being one of the most determinant factors for the development of chronic infections, and it is the major cause of treatment failure [5–7].

Recently, the use of natural compounds, such as EOs obtained from different parts of the plants, is receiving attention for their biological activities, including antioxidant, anti-inflammatory, and anticancer effect [8]. Moreover, EOs have been frequently mentioned on scientific literature as a promising antimicrobial agent, being effective against a wide range of pathogenic bacteria and yeast [9, 10]. Thus, this chapter will present a comprehensive overview about general features of *S. aureus*, including virulence factors, antibiotic resistance, and biofilm formation. Additionally, it will introduce the EOs used as potential therapeutic approaches against biofilm of multidrug-resistant *S. aureus*.

2. *Staphylococcus aureus*

2.1 Clinical relevance and virulence factors

Member of the *Micrococcaceae* family, *S. aureus* is Gram-positive cocci-shape arranged in a grape-like cluster. The cells are anaerobic facultative and catalase-positive with approximately 0.5–1.5 μm in diameter. Overall, 52 species have been described in the staphylococcal genus, *S. aureus* being, by far, the member most clinically relevant [11]. *S. aureus* genome has been completely sequenced and three main components were observed: conserved genes, variable genes, and mobile genetic elements (MGE). More than 97% of the *S. aureus* genome is composed of highly conserved genes found in all staphylococcal strains. More than 700 genes are variable and their distribution defines different lineages [12, 13]. Apart from the core genes, there are several numbers of MGE acquired by horizontal gene transfer by bacteriophages, transposons, and plasmids that contribute to genome plasticity and evolution, such as the antibiotics resistance and virulence gene dissemination [14].

Widely disseminated in nature, *S. aureus* is a commensal component of human cutaneous and mucosal microbiota as well as an adaptive pathogen that leads to numerous invasive and, sometimes, fatal infections [15, 16]. This microorganism can be easily spread by the hands or expelled from the respiratory tract. About 30% of the population is colonized by *S. aureus*, and this increases to 60% when it involves the healthcare environment, implying in either cases high risk of further infection [17]. As a pathogen, this bacterium causes various suppurative diseases, such as boils, carbuncles, folliculitis, and scalded-skin syndrome [18]. Additionally, the lymphatic system and bloodstream contributed to bacterial spread to other parts of the body causing osteomyelitis, medical device infection, endocarditis, and pneumonia [19]. Furthermore, the presence of a variety of antimicrobial resistance mechanisms in some strains leads to treatment failure, increasing healthcare costs and risk of death [20].

Bacterial sepsis confirmed by blood cultures in pediatric hospitals, Gram-positive bacteria (62%) were involved in most of the infection cases. Among them, the major reported strains were *S. aureus* (15%), followed by *Staphylococcus* coagulase negative (11%) and *Streptococcus pneumoniae* (10%) [21]. In addition, serious

cases of high virulence profile community-associated *S. aureus* (CA-MRSA) infections have been reported globally in recent decades [22, 23]. In Taiwan, for instance, 423 cases of CA-MRSA infections were reported in children, and most of them were associated to bone, joint, and deep soft tissue infections and pneumonia [24]. Despite each disease profile, the staphylococcal species is frequently correlated with both community- and hospital-acquired infections, and it has steadily increased [25, 26]. Thus, it is necessary to look for new therapeutic alternatives to minimize this public health problem [27].

S. aureus can survive in its hosts as a commensal bacterium for a long time; however, it can also be considered one of the most relevant human pathogens [28]. This bacterium has mechanisms to evade the host's immune response through production of a variety of virulence factors, such as adhesins, exotoxins, and hydrolytic enzymes (e.g., coagulase, catalase, and staphylokinase), as summarized in **Figure 1** [29, 30].

The bacterial adherence to extracellular matrix cells in the host is one of the most important steps for colonization. It is mediated mainly by surface-anchored proteins classified as MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family. Among them, two fibronectin binding proteins, FnbA and FnbB, contribute considerably to epithelial tissue colonization in various pathological manifestations and medical device-related infections [31]. Other cell surface protein related in adhesion mechanisms are named clumping factor A and B (ClfA and ClfB). The first one has a highlighted ability to interact with soluble proteins, fibrinogen, and fibrin, present in blood plasma. These surface components aid the microorganism to interact with plasma protein-coated biomaterials and, consequently, make possible the colonization and biofilm formation on medical devices [32]. The ClfB is frequently associated to nasal colonization due to high affinity to cornified envelope of the nostrils, which promotes the formation of skin abscesses by binding to the protein loricrin [33]. It is worth mentioning genes capable of encoding proteins on the cell surface, *cna* (collagen adhesin), *ebp* (elastin-binding protein), *bbp* (bone sialoprotein-binding protein), and *eno* (laminin-binding protein), closely related to pathogenesis of implant infections caused by *S. aureus* [34–36].

2.2 Antibiotic resistance and biofilm formation

Historically, infections caused by MDR *S. aureus* strains have been often reported worldwide. This microorganism has a notable ability to acquire systems of antibiotics inactivation. Production of reduced-affinity penicillin binding,

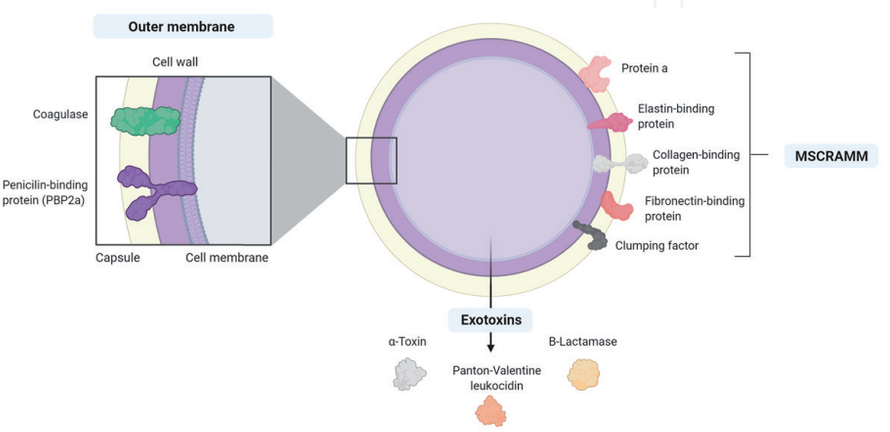

Figure 1.
Illustration of virulence factors produced by S. aureus.

ribosomal active site methylation, and efflux pumps that remove the antibiotic from the bacterial cell are the most cited mechanisms of antibiotic resistance developed by *S. aureus* cells [37]. The isolation of antibiotic-resistant strains began after the introduction of penicillin and methicillin into clinical practice, when resistant lineages, known as methicillin-resistant *S. aureus* (MRSA), were reported in 1950s and 1960s, respectively [38]. This resistance profile is mediated by mecA and mecC genes, which encode penicillin-binding protein 2a (PBP2a) in cell-wall synthesis. Those mec gene complexes are carried in an MGE known as the staphylococcal cassette chromosome mec (SCCmec), which can be acquired by horizontal gene transfer among related species [39].

Subsequently, vancomycin was used as an alternative to cases of MRSA infection [40]. However, the constant use of this antibiotic leads to the emergence of vancomycin-resistant *S. aureus* (VRSA) strains, first detected in 2002 [41]. Due to the fact that VRSA strains are generally also resistant to teicoplanin, the use of other abbreviations has been suggested: GISA (*S. aureus* of intermediate sensitivity to glycopeptides) and GRSA (*S. aureus* glycopeptide resistant) [42]. Moreover, some strains presented a relevant phenomenon known as heterogeneous resistance (heteroresistance) to vancomycin, where they have a mechanism of tolerance against this antibiotic. These strains, called hVISA, display a vancomycin-susceptible profile by microdilution assay; however, some individual cells into bacterial community might exhibit VRSA features [43].

Furthermore, the ability of some microorganisms to form cellular agglomerates, such as biofilms, contributes way more for antibiotic resistance. In summary, biofilm is a three-dimensional community of microorganisms covered and embedded in a self-produced matrix of extracellular polymeric substances (EPS) [44]. Such multicellular structure provides intrinsic protection for biofilm-embedded cells against hostile environments, for instance extreme temperature and pH, high salinity and pressure, poor nutrients, and antibiotics [45–47]. Microorganisms that grow on biofilms often exhibited different physiology profile from planktonic cells, especially in terms of their response to antibiotic treatment [48]. Although biofilm lifestyle can arise from a single cell, differential environmental conditions throughout the community can potentiate the development of distinct subpopulations. Gradients in oxygen, nutrients, and electron acceptors can cause heterogeneous gene expression throughout a biofilm. This communication between these bacterial cells, called quorum sensing, mediated the genes expression and activate virulence factors [49].

S. aureus has a great capacity to form biofilms on human body tissues and medical devices, increasing the risk of invasive infections [50]. It is estimated that *S. aureus* causes about 40–50% of prosthetic heart valve infections, 50–70% of catheter biofilm infections, and 87% of bloodstream infections [24]. The main stages of biofilm formation consist of four sequential steps: attachment, formation of microcolonies, accumulation or maturation, and detachment or dispersal (**Figure 2**) [51]. Firstly, planktonic cells adhere to biotic or abiotic surfaces and further proliferate into sticky aggregations called microcolonies. The EPS produced by bacterial cells during biofilm maturation serves as scaffold for establishing this three-dimensional architecture, also known as mushroom-like structures. Upon reaching a specific cell density, a mechanism is triggered to initiate EPS degradation that releases cells embedded into biofilm to disperse and reinitiate the biofilm formation at distal sites [7].

S. aureus shows a variety of adhesins that mediate attachment to host factors, essential for biofilm formation [48]. These proteins are surface-associated by different means, such as ionic or hydrophobic interactions, such as autolysin, SERAM (secretable expanded repertoire adhesive molecules) proteins, membrane-spanning proteins, and the polysaccharide intercellular adhesin (PIA) [52]. It is worth to

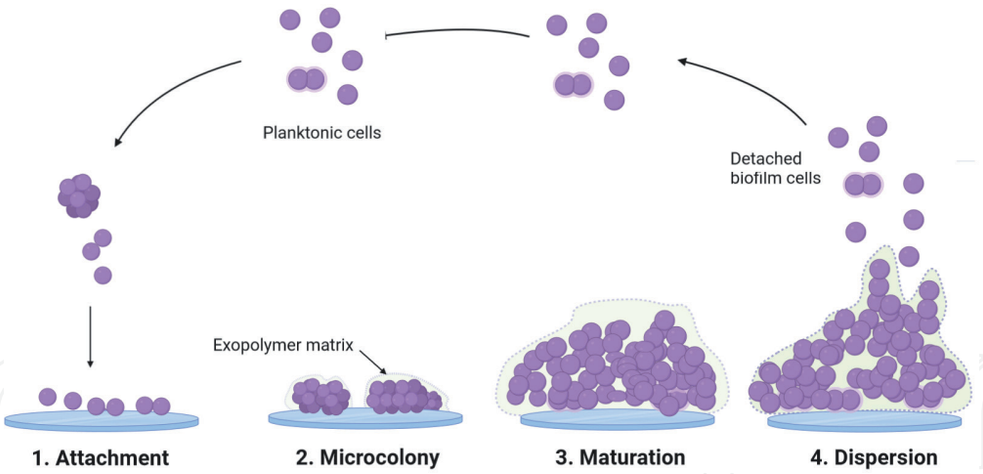

Figure 2.
General steps of S. aureus *biofilm formation.*

highlight that the presence of the ica gene located in the icaADBC (intercellular adhesion [ica]) operon works like a genetic determinant that contributes for biofilm establishment [53]. This genetic element can mediate the production of an extracellular mucopolysaccharide composed mainly of N-acetylglucosamine, which is associated to adhesion and colonization of several surfaces [54, 55].

Thus, several steps regarding biofilm formation of *S. aureus* might be considered as target to antibiofilm approaches. As many conventional antimicrobial agents have no satisfactory effect against mature biofilms, EOs already used for hundreds of years as a natural medicine to combat a variety of infections became a great antimicrobial alternative. The EOs are made up of various compounds, and it is further believed that this makes it difficult to develop bacterial resistance compared to antibiotics that have only one target action, making it attractive to fight MDR biofilm-forming bacteria [56]. Then, such attributes qualify the EOs as an important product from natural source to be explored by pharmaceutical industry [57, 58].

3. Essential oils

3.1 General aspect

Essential oils are compounds obtained from the secondary metabolism of the plants. They are characterized as complex mixtures of volatile compounds abundant in aromatic plants found in different parts of the plant, including leaves, flowers, stem, roots, seeds, and fruits [59]. There is a diversity of these substances described in the literature in commercial use, such as in perfumes, pharmaceuticals, cosmetics, insecticides, and food additives [60].

Generally, they are oily-looking liquids at room temperature of complex mixtures of volatile lipophilic substances, usually with pleasant scent. In water, EOs have a limited solubility, which allows their separation by steam or water distillation. Other methods to obtain EOs include cold-press extraction used for citrus peels, separation by solubility using organic solvents, and through supercritical fluid extraction [61]. They are usually unstable against environmental factors such as light, temperature, water activity, and salinity, affecting their constitution, contributing to the appearance of chemotypes with particular compositions. Depending on the technique used in the course of a separation, reactions such as ester hydrolysis, autoxidation, and rearrangements may occur, leading to the formation of artifacts and modifying their biological activity [62].

Compounds included in the EOs are produced in the cytoplasm and plastids of plant cells through the action of terpene synthase enzymes (TPSs), in which they use substrates from two pathways involved in the synthesis of terpenes: the mevalonate (MVA) and the methyl-eritritol phosphate (MEP) pathways [63]. They are localized and stored in complex secretory structures, such as glands, secretory cavities, hairs or trichomes, epidermal cells, internal secretory cells, and the secretory pockets [64]. Many of these substances are now known to be directly involved in the defense or attraction mechanisms of plants and often show interesting biological activities [65].

Despite containing two or three main components at a level of 20–70%, EOs are very complex mixtures of substances. In general, the majority components are formed by terpenes and phenylpropanoids [66]. In the very first definitions of EOs, these were frequently identified with terpenes, principally mono- and sesquiterpenes. Other substances have also been identified as alcohols, aldehydes, ketones, phenols, esters, ethers, oxides, peroxides, furans, organic acids, lactones, coumarins, sulfur compounds, anthraquinones, and alkaloids [61]. In the mixture, such compounds come in different concentrations. Usually, one of them is the majority compound, with others in lower grades and some in very low quantities (trace). EOs are composed of volatile hydrocarbons, and they may contain oxygen, sulfur, and halogens (rare) in their chemical structure [67]. In a reduced number of species, the predominant components are aromatic molecules, and these include thyme (thymol and carvacrol), peppermint (menthol), and anise (anethol) [68].

3.2 Antimicrobial and antibiofilm potential

Humans have used EOs for thousands of years, not only as aromatic extracts and for beauty care and culinary uses, but also in folk medicine, due to their many different pharmacological activities, such as antiseptic, anti-inflammatory, and analgesic properties [65]. Some of the EOs, and their components, have demonstrated the relevant antimicrobial potential against a wide range of microbial pathogens [69]. Additionally, Gram-positive bacteria, such as *S. aureus*, seem to be much more susceptible to EOs than Gram-negative cells, probably due to cellular surface constitution. Gram-positive has only the inner membrane and a cell wall that allows hydrophobic molecules to easily penetrate into the cells. For instance, phenolic compounds have a dose-dependent effect, at low concentrations they interfere with enzymes involved in energy production, and at high amounts they can denature proteins [70, 71].

The broad-spectrum activity of EOs is related to the diverse chemical reactions of aldehydes, phenolic compounds, and terpenes, synthesized from secondary metabolism by different plant parts [10]. The EO action is attributed to the ability of their constituents to interact with the cell membrane and consequently disturb the microbial integrity, leading to cell death [72]. However, EO bioactive components can have several cellular targets, and they are mainly associated with cytoplasmic coagulation, inhibition of ATP-production enzymes, alteration in ion transport, cell-wall damage, and bacterial membrane destruction (**Figure 3**) [73].

The emergence of MRD pathogens has caused an interesting shift from the onerous development of novel classes of antibiotics to the more straightforward application of synergism or combinatory therapy in the hope of reviving the efficacy and effectiveness of existing antibiotics [74]. Several studies have demonstrated that there was synergetic effect when two or more EOs are mixed together. Moreover, there are also reports of synergistic activity of EOs when used in combination with well-known antibiotics. When blended with other antimicrobial agents, the

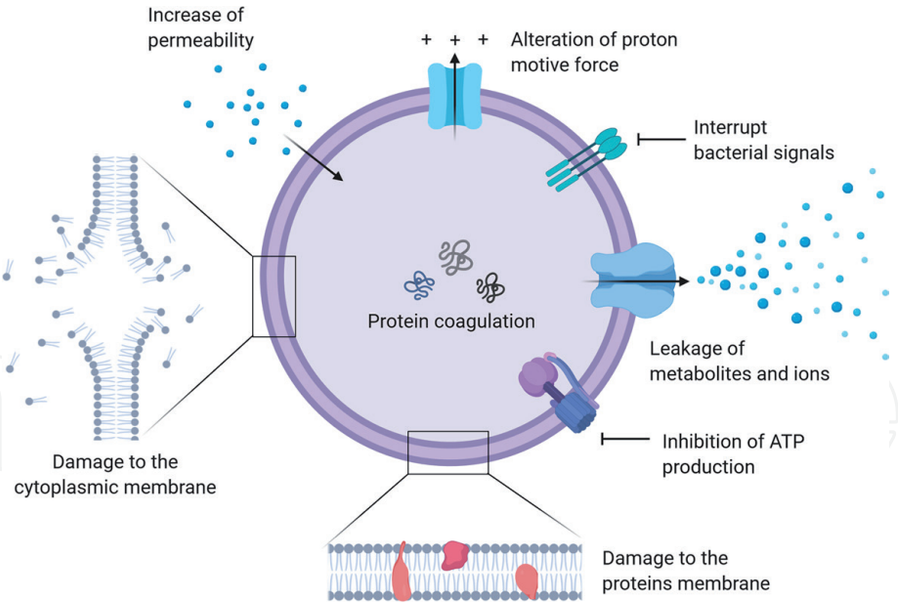

Figure 3.
General antibacterial mechanisms of essential oils.

constituents of EOs can unlock the cell membrane channels, thus opening the passage of antimicrobial agents to reach their target sites [75].

The capacity of some EOs to inhibit biofilm formation has been less explored; however, some reports suggested their utilization as potent inhibitor of virulence factors and biofilm formation [76]. So far, a plethora of potential antibiofilm agents, mainly inspired by natural products, has been developed and shown great promise in either facilitating the dispersion of preformed biofilms or inhibiting the formation of new biofilms *in vitro* [77]. In contrast to conventional antibiotics, the recently developed antibiofilm molecules do not directly affect bacterial survival and thus the expectation is that resistance to these molecules will not readily occur [77].

Table 1 shows some studies based on the *S. aureus* antibiofilm activity of OEs extracted from several plant sources. The EO action on biofilm inhibition and dispersal can be related to reactivity, hydrophobicity, and the diffusion rate of the EO in the matrix, as well as the biofilm composition and structure [78]. Same studies correlated sublethal concentrations of EOs with their capability to inhibit the first steps of biofilm formation. The main constituents of OE can act by several ways to disturb the biofilm development, such as blockage of the quorum-sense system, inhibition of the flagellar gene transcription, or through interference with bacterial motility [71].

Antibiofilm agents can have different therapeutic applications depending on their effects on the biofilm: compounds that interfere with biofilm formation could be exploited in the prophylaxis of implant surgery or for the coatings in medical devices, whereas agents able to disperse biofilm structure could be administered in combination with conventional antibiotics for the treatment of biofilm-associated infections [96]. Despite the growing number of new potent EO-based antibiofilm compounds described, there is still a great challenge in the development of antibiofilm drugs. Once the EO compounds, which has such activity, discovered so far need further optimizations to improve potency for it become one clinical candidate for such approach. Other EO features such as stability, volatility, encapsulation, and optimal dosage should be considered for the development of EO-based antibiofilm drugs. However, it is expected that in the coming years some of these compounds would be translated into antibiofilm drugs.

Scientific name	Plant part	Major chemical compounds	Resistant phenotype	Ref.
Artemisia absinthium	Aerial	1,8 Cineole, methyl chavicol, camphor	MRSA, MRSAMupR	[79]
Artemisia dracunculus	Aerial	Methyl chavicol, methyl eugenol	MRSA, MRSAMupR	[79]
Artemisia longifolia	Aerial	Camphor, 1,8-cineole	MRSA, MRSAMupR	[79]
Artemisia frigida	Aerial	1,8-cineole, methyl chavicol, camphor	MRSA, MRSAMupR	[79]
Cinnamomum zeylancium	Bark and leaves	Cinnamaldehyde	MRSA	[80]
Cymbopogon citratus	Fruit	Ethanolic compounds	MSSA MRSAMupR	[80]
Cymbopogon nardus	Leaves	Eugenol, cinnamaldehyde, citral, geraniol	MSSA, MRSA	[80, 81]
Eucalyptus globulus	Aerial	Eucalyptol, [+] spathulenol, α-pinene	MRSA, MSSA	[82]
Lippia alba	Aerial	Geranial, neral	MSSA	[83]
Mentha piperita	Aerial	Menthol, menthone, menthyl acetate	MRSA, MSSA	[84]
Melaleuca alternifolia	Aerial	α-Terpineol, terpinen-4-ol	MSSA	[85]
Myrtus communis	Leaves	Eugenol, α-terpineol, γ-terpinene	MSSA	[86]
Ocimum gratissimum	Leaves	Eugenol, 1,8-cineole	MRSA, MSSA	[87]
Origanum vulgare	Leaves and Arial	1-Terpineol, sabinene, γ-terpinene	MRSA, MSSA	[88, 89]
Piper nigrum	Leaves	Limonene, sabinene, β-pinene	MSSA	[90]
Rosmarinus officinalis	Leaves and flower	1,8-cineol, camphor, α-pinene	MSSA	[91]
Satureja hortensis	Leaves	β-cubebene, limonene, α-pinene	MSSA, MRSA	[92]
Satureja montana	Leaves	Carvacrol, p-cymene, δ-terpinene	MRSA	[93]
Syzygium aromaticum	Aerial	Eugenol, caryophyllene	MSSA	[90]
Thymus vulgaris	Aerial	p-Cymene, γ-terpinene	MRSA, MSSA	[82, 94]
Thymus daenensis	Aerial	Carvacrol, γ-terpinene	MSSA	[92]
Zataria multiflora	Leaves	Thymol, carvacrol, rho-cymene	MRSA, MSSA	[95]

Table 1.
Summarized antibiofilm activity of EOs against MDR S. aureus.

4. Conclusion

Due to the emergence of multiresistant strains and biofilm formation, there is an urgent need to find effective alternatives against *S. aureus*. Thus, EOs became a promising alternative for treatment and prophylaxis of infections caused by *S. aureus*. Many EOs have proven to be effective antimicrobials and antibiofilm,

opening the possibility of using EOs in clinical formulations alone or in synergy with already known antibiotics. However, further research is needed to better understand the interactions between the steps of biofilms formation with the EOs and their constituents separately, as well. In addition, more acute studies in relation to volatility and solubility should be done in order to increase the essential oils' antimicrobial potential as a pharmacological product.

Conflict of interest

The authors declare no conflict of interest.

Author details

Victor Alves Carneiro[1,2*], Ramaiana Soares Melo[1], Antônio Mateus Gomes Pereira[1], Águida Maria Albuquerque Azevedo[1], Maria Nágila Carneiro Matos[2], Rafaela Mesquita Bastos Cavalcante[2], Renan Rhonalty Rocha[2], Vinícius de Queiroz Albuquerque[1], Jesús Alberto Pérez Guerrero[2] and Francisco Eduardo Aragão Catunda Junior[3]

1 Center of Bioprospection and Experimentation Molecular Applied [NUBEM], University Center INTA—UNINTA, Sobral, Ceará, Brazil

2 Laboratory of Biofilms and Antimicrobial Agents [LaBAM], Federal University of Ceará, Sobral, Ceará, Brazil

3 Center for Exact, Natural and Technological Sciences, State University of the Tocantina Region of Maranhão, Imperatriz, Maranhão, Brazil

*Address all correspondence to: viktorcarneiro@gmail.com

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