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Review Article

A Review on Exploring Herbal Approaches to Combat Biofilm Formation

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ABSTRACT

Biofilms, intricate communities of bacteria enclosed within a self-produced extracellular matrix, pose formidable challenges in both medical and industrial settings due to their heightened resistance to traditional antibiotics. This study explores the potential of natural treatments as alternative strategies to combat biofilm-associated issues. Natural agents derived from various sources, including plants and other organic materials, have gained attention for their antimicrobial properties and potential to disrupt biofilm formation and integrity. This research investigates the mechanisms by which these natural compounds interact with biofilms, inhibit bacterial growth, and potentially enhance susceptibility to conventional antimicrobial agents. Through in vitro experimentation and molecular analysis, the effects of selected natural agents on biofilm structure, composition, and quorum sensing mechanisms are examined. The findings shed light on the efficacy of natural treatments in preventing biofilm formation and attenuating their virulence. This study not only advances our understanding of biofilm biology but also highlights the promise of natural agents as a complementary approach in biofilm management..

INTRODUCTION

Biofilms are defined as communities of microorganisms that are attached to living or abiotic surfaces, and they are common to the growth patterns of microorganisms in nature. Biofilms offer resistance to extreme environments and can protect microorganisms from ultraviolet (UV) radiation, extreme pH, extreme temperature, high salinity, high pressure, malnutrition, antibiotics, etc., thus a biofilm acting as "protective clothing" for microorganisms [1]. The resistance of biofilms to environmental extremes allows for the creation of suitable habitats for microbial populations and facilitates material and information exchange between microorganisms; thus, biofilms are self-protective mechanisms in microbial growth [2]. In some instances, however, the growth of these mutually beneficial

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microorganisms can become uncontrolled, leading to infection [3,4]. The human body can be infected by various pathogenic agents such as viruses, fungi, and bacteria. Bacterial infections are the most common type of acute and chronic infections causing worldwide morbidity. The prevalence of untreatable bacterial infections is predicted to rise at an alarming rate due to an increase in the number of antibiotic-resistant bacteria strains. Bacteria develop biofilm on submerged surfaces such as natural aquatic systems, water pipes, living tissues, tooth surfaces, indwelling medical devices and implants [5]. Biofilm formation on indwelling medical devices and implants such catheters, mechanical heart valves, pacemakers, prosthetic joints, and contact lenses pose a critical medical problem.

Bacteria exist in two different forms, i.e. planktonic state (free floating) and sessile state (adhered to a surface) [6]. Interestingly, bacteria display very distinct characteristics between these two states, as attachment of the bacteria to a surface result in the rapid alteration in the expression of a number of genes responsible for exopolysaccharide (EPS) or "slime" production and maturation. This transformation begins almost immediately after bacterial colonization of both biotic and abiotic surfaces and results in the production of a protective barrier that protects the bacteria against the organism's endogenous defence system or from external agents such as antibiotics [7,8]. Although the first observation of surface-associated bacteria was made by **Anthony van Leeuwenhoek** in 1684, the term 'biofilm' was not used and defined until a report by Costerton et al. in 1978. Almost 15 years later, in 1993, the American Society for Microbiology recognized the significance of biofilms. In 1999, biofilms were defined by **Costerton** et al. as "a structured community of bacterial cells enclosed in a self-produced polymeric matrix, adherent to a surface" [6]. Both gram-positive and gram-negative bacteria can form biofilms, but the most common forms are ***Enterococcus faecalis***, ***Staphylococcus aureus***, ***Staphylococcus epidermidis***, ***Streptococcus viridans***, ***E. coli***, ***Klebsiella pneumoniae***, ***Proteus mirabilis*** and ***Pseudomonas aeruginosa***. Among these biofilm-forming bacteria, ***S. aureus*** and ***S. epidermidis*** are most commonly found on cardiovascular devices [9,10]. It was estimated that, ***S. aureus*** and ***S. epidermidis*** cause about 40-50% of prosthetic heart valve infections, 50-70% of catheter biofilm infections and 87% of bloodstream infections [11]. Bacterial attachment is also a well-known problem in food and dairy production. Antimicrobial agents target diverse functional hereditary compounds, enzymes, cellular respiratory system, and other. However, due to the genetic exchanges and inherent differences, such as exclusive cell envelope composition and non-susceptible proteins, various bacteria react in different ways to bactericides. Within biofilms, several mechanisms result in multi-factorial resistance to antibiotics [12]. In patients, biofilms that form are resistant to the host's endogenous defences, and as such are treated with a combination of antibacterial therapies [13,14]. Paradoxically, the large doses of antibiotics used to treat biofilms clinically have also contributed to the development of antibiotic-resistant bacteria strains [15]. Additionally, it has been seen that some bacteria within biofilms, called "persister cells," are dormant variants that exhibit antibiotic tolerance and can become active when the therapy is withdrawn [16]. In this review, the molecular mechanism of biofilm and the role of this matrix in antimicrobial resistance were discussed. Furthermore, strategies using herbal and non-herbal compounds against biofilm formation and development were described. Finally, promising natural anti-biofilm agents under clinical evaluation were introduced.

Biofilm Formation

Biofilm formation is a complex multi-step process (usually cyclic) involving multiple bacterial species [17]. Bacterial biofilms secrete a mixture of polysaccharides, proteins (composed primarily of D-amino acids), fatty acids, and a nucleic acids which is referred to as extracellular polymeric substance or EPS. Biofilms consist of about 80% EPS which plays an important role in biofilm formation [18,19]. The EPS is a sticky matrix comprised mostly of water channels that serve as a medium for the distribution of nutrients and oxygen. In addition to protecting the bacteria from the host's defences (antibodies, white blood cells, monocytes) and antibiotics, the EPS serves as a basic platform for surface attachment [18, 20, 21]. It has also been shown to facilitate the functioning of intercellular signalling molecules such as cyclic dimeric guanosine monophosphate (c-di-GMP) that is found in most bacterial species. This signalling mechanism stimulates the growth and adherence of bacterial species [22]. C-di-GMP helps in the synthesis of matrix components including polysaccharides and proteins that are part of a feed-forward loop as seen in ***P. aeruginosa***, where c-di-GMP stimulates the production of different polysaccharides including pentasaccharide (PSL), glucose-rich polysaccharide (PEL), and alginate. PSL and PEL act as signal molecules to further stimulate c-di-GMP production [18], leading to increased levels of c-di-GMP and resulting in thicker and stronger biofilms [7,8].

The proteins that promote EPS production are specific to the various species of bacteria. For example, proteases, nucleases, teichoic acids and phenol soluble modulins promote EPS production and biofilm formation in staphylococcal bacteria. Whereas, glucan binding proteins like GbpC are responsible for EPS growth in streptococcal bacteria. Furthermore, extracellular DNA is reported to be responsible for cellular communication in ***P. aeruginosa***, ***staphylococcus*** and ***streptococcus*** biofilms, especially in the early stages of biofilm development [23]. Bacterial biofilm growth is typically a result of physical, chemical, and biological events. The formation is typically classified into three stages; (i) initial attachment (reversible and irreversible), (ii) maturation of microcolonies, and (iii) dispersion/detachment [24, 25, 26, 27, 28,29]. Attachment is characterized by the production of bacterial adhesins that stick to the surface, while cell-cell adhesion mechanisms mediate maturation, and enzymes that degrade the biofilm matrix mediate dispersal [30,31,32].

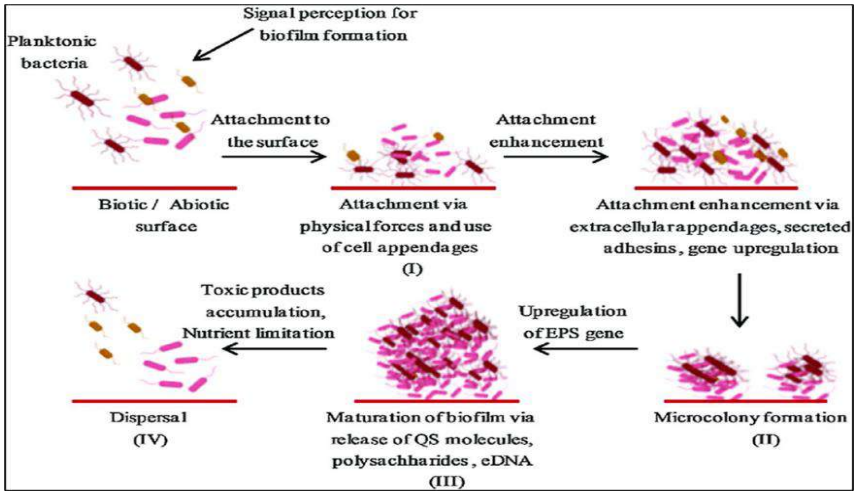

Figure 1: Schematic representation for the main stages of biofilm formation on solid surfaces.

Initial attachment:

This stage is termed reversible attachment as the initial interaction can be transient and reversible due to weak interactions between the bacteria and surface [33]. For biofilm formation to transpire, a bacterium must find a suitable surface for attachment. This material is usually a component of a solid- liquid interface, has a rough or textured surface, and has a desirable conditioning film [34,35,36,37]. Other factors, such as polarity, hydrophobicity, hydrodynamics, and general characteristic like pH and temperature have also been shown to affect attachment [38,39]. Free floating bacteria initially adhere to a surface by using structures such as pili, fimbriae, or flagella [40,41,42]. These cells are still reversible in their attachment to a surface due to weaker attractive forces such as Van der Waals. At some point, however, cells exhibit stronger attractive forces, leading to a greater resistance to dislodgement. They then enter an irreversible stage and start producing an EPS which signals the beginning of maturation and quorum sensing [43,44].

Maturation and Quorum sensing:

In this phase, microorganisms that form a larger network colony that begin to take on characteristics beyond those of individuals cells. The EPS is composed of DNA, proteins, cellulose and N-acetylglucosamines; however, variations can exist depending on the microorganism's present [43,44]. The EPS contributes to bacterial cell aggregation, water retention, cohesion of biofilms, provides nutrients, a protective barrier [45]. Proceeding the production of EPS, the microcolonies rapidly grow in size until they progress to a three-dimensional colony approximately 100 µm in thickness [46].

Quorum sensing (QS) is the second indication of a maturing biofilm in which individual cells demonstrate the ability to communicate as a collective and detect the presence of other cells. Their primary method of communication is through autoinducers, such as acyl-homoserine lactones in gram-negative strands and oligopeptides in gram-positive [47, 48]. As bacteria quantity increases so does the quantity of inducers, allowing the cells to detect and appropriately trigger the expression of specific genes [47, 49]. This can impact the structure of the colony, select for the growth of species, and lead to antibiotic resistance [46, 47, 48].

As disruption in the QS system can inhibit the growth of bacteria within the EPS, it has become an important research area [50]. It has been proposed that by manipulating the underlying pathways of QS, one can trigger the disassembly of pre-established biofilms through a phenomenon termed quorum quenching, thus serving as a pathway for the development of potential treatments [18]. Additionally, quorum quenching has been shown to increase biofilm susceptibility to antibiotics, as seen by administration of the quorum sensing inhibitors cinnamaldehyde and baicalin hydrate, which decreased biofilm resistance of ***P.aeruginosa*** and ***B.cepacia*** towards tobramycin [51].

Dispersal:

While the inner layer of cells is protected by the EPS and attached to the surface, the outer layer is able to detach from the colony and continue populating other surfaces within the host, leading to systemic infections and even acute events such as embolisms. Limited access to nutrients and accumulation of wastes lead to the dislodging of individual cells or groups of cells towards the periphery of the film [46]. As the biofilm matures, resources become limited and toxic products can accumulate. Thus, in order to expand, get nutrition, and eliminate stress-inducing conditions and waste, the cells disperse to other regions of the host's body or other regions [18]. The dispersion of cells occurs either as single cells or as clumps of cells which are sloughed off the biofilm. This is said to be a programmed process that is initiated

by oxygen level (in case of aerobic biofilms) or nutrient starvation. This starvation stimulates small molecules like fatty acid DSF (cis-11-methyl-2-dodecenoic acid), which triggers autophosphorylation and leads to activation of c-di-GMP phosphodiesterase that degrades c-di-GMP. Degradation of c-di-GMP leads to the tearing of clusters by shear forces or the release of planktonic cells that dissolve a portion of the EPS [18]. For instance, the bacterial cells inside the biofilm produce saccharolytic enzymes, which break the biofilm stabilizing polysaccharide, thereby releasing the surface bacteria. Once released, the bacterial cells either establish more biofilms at other regions of the body or freely float on the surface by upregulating the expression of flagella proteins to help them in motility.

Biofilms associated infection:

On the basis of National Institutes of Health (NIH) studies, most bacterial infections stem from microorganisms associated with biofilms. Common dental and oral health issues such as gingivitis, dental caries, and periodontitis may arise from biofilm-forming bacteria. Biofilm formation has been observed in a broad spectrum of infections such as chronic otitis media, chronic osteomyelitis, chronic rhinosinusitis, chronic wound infections, recurrent urinary tract infections, endocarditis, and cystic fibrosis-associated lung infections [52]. The "one disease, one infectious agent" paradigm is changing due to the fact that more than one microorganism species can grow on the same biofilm, leading to multiple infections at the same site. These types of infections, known as poly-microbial infections, could exacerbate the biofilm's persistence [53]. Healthcare costs rise substantially following biofilm infections due to the biofilms' resistance to antibiotic treatment.

Table 1:Major pathogens involved in biofilm formation [54,55,56,57,58]

Mode of action	Substrate/support for biofilm formation	Bacteria
Persister cells	Urinary tract Urethral catheters	Escherichia coli
AHL molecules Persister cells eDNA	Ventricular assist devices Endotracheal tubes Coronary stents Cochlear implants	Pseudomonas aeruginosa
Poly-β (1–6)-Nacetylglucosamine (PNAG)	Coronary stents Peritoneal dialysis catheters Cochlear implants	Staphylococcus aureus
Polysaccharide intercellular adhesion (PIA)	Central venous catheters Orthopaedic prostheses	Staphylococcus epidermidis
LuxS	Endotracheal tubes Nasopharynx	Streptococcus pneumonia

Biofilm resistance to antimicrobial agents:

The nature of biofilms' structure and the characteristics of the cells within it result in an environment that protects against unfavourable conditions [59]. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for biofilm-growing bacteria are generally 100–1000 times greater than for planktonic bacteria, which suggests that they are probably 150–3000 times more resistant to disinfectants [60]. Although some studies have found that antibiotics like penicillin G, ampicillin, cloxacillin, ceftiofur, tetracycline, oxytetracycline, streptomycin, gentamicin, erythromycin,

tilmicosin, enrofloxacin, and trimethoprim-sulphadoxine are active against cultures like ***Corynebacterium renale***, ***Corynebacterium pseudotuberculosis***, ***Staphylococcus hyicus***, ***S.aureus***, ***Streptococcus agalactiae***, and ***Actinomyces pyogenes***, biofilms developed by all these pathogens were found to be resistant. For instance, a 600-fold increase in sodium hypochlorite concentration is the most effective oxidizing antimicrobial agent for treating the biofilm formed by ***S. aureus*** [61].

Despite decades of research, there is little information about the molecular mechanisms of antibiotic resistance in biofilms. Nevertheless, resistance factors can be intrinsic (or innate) and extrinsic (or induced) [12]. The role of oxygen penetration in the resistance of biofilm bacteria is crucial. The susceptibility of agar-entrapped ***E.coli*** to β-lactam (latamoxef) and aminoglycoside (tobramycin) antibiotics under various aeration levels. In moderate aeration conditions, the bacteria cells displayed higher resistance to both antibiotics. Under anaerobic incubation, the free organisms were highly resistant to the antibiotics. The researchers concluded that in oxygen-deficient conditions, the agar-entrapped bacteria had noticeably lower susceptibility than the suspended cells and suggested that the effect was relevant to the limited uptake of the antibiotics by the oxygen-deprived cells, due in particular to the greater thickness of the biofilms [62]. Moreover, slower-growing bacterial cells are especially resistant to antibiotics. For instance, in slow-growing ***E. coli***, penicillin-binding proteins (PBPs) are expressed insignificantly. Therefore, antibiotics such as ceftazidime and ceftriaxone have a poor effect, regardless of the presence of growth-limiting nutrients [63].

Herbal anti-biofilm compounds:

Researchers' efforts to introduce substances with anti-biofilm properties have led to identifying plant-derived compounds that are naturally made to protect themselves against bacterial attack [64]. These compounds, which have a molecular weight of less than 1 kDa, are called "Parvome" and consist of various herbal complexes, including alkaloids, terpenoids, flavonoids, coumarins, peptides, glycosides, nucleosides, and polyphenols [65]. It is generally believed that herbal compounds are safer than synthetic products because of their both biocompatibility and biodegradability [66]. Plants whose extracts have bactericidal or growth inhibitory properties inhibit or reduce the formation of bacterial biofilms. However, some plant-derived compounds affect the biofilm without killing or inhibiting bacterial growth. The advantage of these agents is that the bacteria do not become resistant to them. For example, some of them interfere with the QS network. As described earlier, the first step in establishing a bacterial biofilm is adhesion to the surface, so any compound that interferes with the bacteria's adhesion can potentially act as an anti-biofilm constructing agent [67]. **Chaperon–pilin complex**, known as a synthetic peptide mimicking the structure of pilus protein, inhibits bacterial assembly [68]. Various types of adhesion analogues or anti-adhesion antibodies are among novel treatments aiming to prevent and treat bacterial infectious diseases that could be used as an anti-biofilm adhesion agent in the future [69,70].

Another mechanism is to inhibit or remove the biofilm. Different pump systems are applied by bacteria to expel toxic substances and waste metabolites. The presence and activity of such pumps cause resistance to chemical compounds, including antibiotics, and the development of resistant strains. Infact, waste products result from bacterial metabolism during the biofilm phase; thus, bacteria activate their efflux pumps to throw away these wastes. Studies show that efflux pumps inhibitors (EPIs) can destroy the biofilm formed and increase the susceptibility of biofilm bacteria to antibiotics [71]. Experiments were carried out

utilizing mutants and wild-type strains to validate the efflux pumps' participation in the biofilm development [72]. The antimicrobial agents directly conducted to deletions of efflux pumps and/or their regulators of biofilm mass may be a good predictor of the antimicrobial agent's role in the biofilm breakup [73].

On the other hand, it should be noted the essential oils (EOs) of plants. EO is a naturally plant-derived volatile substance and due to their preservative and antimicrobial effects, they are promising and effective natural ingredients in the food industry. In particular, the availability of many EOs, low mammalian toxicity, and quick degradation in the environment make them safe anti-biofilm agents. These compounds inhibit biofilm formation through various mechanisms. The most important mechanisms include inhibition of bacterial adhesion and biofilm formation, especially in polystyrene and stainless-steel surfaces [74,75], or biofilm control on painted surfaces [76]. Further studies should be performed to introduce these plant compounds as well as their active ingredients along with their mechanism of action.

Table 2: Natural compounds with anti-biofilm properties. [77-87]

Plant species	Compound	Mechanism of action	Pathogen strains
Teucrium polium L. (Lamiaceae)	4α,5β-Epoxy 7α Hgermacr-10(14) en,1βhydroperoxyl, 6α-ol	Inhibition of *S. aureus* biofilm.	*S. aureus*
Allium sativum	Ajoene	Reduced QS-controlled virulence factors, including rhamnolipid.	*P. aeruginosa*
Whole of *Emericella variecolor* Berk & Broome	6-epi-Ophiobolin G (3) 6-epi-Ophiobolin K (2)	Inhibition of biofilm formation	*Mycobacterium smegmatis* *Mycobacterium amegmatis*
Rootes of *S. sclarea*	Ferruginol Salvipisone Aethiopinone 1-Oxoaethiopinone	Inhibition of biofilm formation in a dose-dependent manner (46.8±2.74% to 65.6±2.0%)	*S. aureus*
Rootes of *S. sclarea*	Salvipisone	Inhibited biofilm formation by bacterial adhesion prevention	*S. aureus* *S. epidermidis* *Enterococcus faecalis*
Rhizome of *Kaempferia pandurata* Roxb	Panduratin A	Inhibition of biofilm formation < 70% at 10 µg/mL	*Streptococcus sanguinis* and *Streptococcus mutans*
Rhodiola rosea L	Phenylpropanoid glycosides	Inhibition of biofilm formation about 45%	Urinary clinical isolate PU-1 from a woman with pyelonephritis
Vaccinium oxycoccos	Proanthocyanidin myricetin	Inhibition of biofilm formation by disrupting the biochemical	*S. mutans*

		function and production of EPS by > 80%	
Streptomyces sp.	Nahuoic acid A, B, C, E	Inhibition of biofilm formation (63–98%)	*Shewanella onedensis* MR-1
Mare colostrum	Colostrum hexasaccharide	Inhibited QS-regulated secretion, hemolysis, protease, and lipase	*S. aureus*
Root bark of *Swartzia simplex* (Sw.) Spreng	Simplexene A, B, E, D	Inhibition of biofilm	*C. albicans*
Leaves of *Scutellaria oblonga* Benth	Quercitin-3-glucoside Negletein Techtochrysin	Inhibition of biofilm formation (66.7–98%)	*S. aureus*, *B. subtilis*, *P. aeruginosa*, and *E. coli*
Rauwolfia serpentina	Reserpine	Inhibition of biofilm formation	*S. aureus*
Piper longum	Piperine	Inhibition of biofilm formation	*S. mutans*

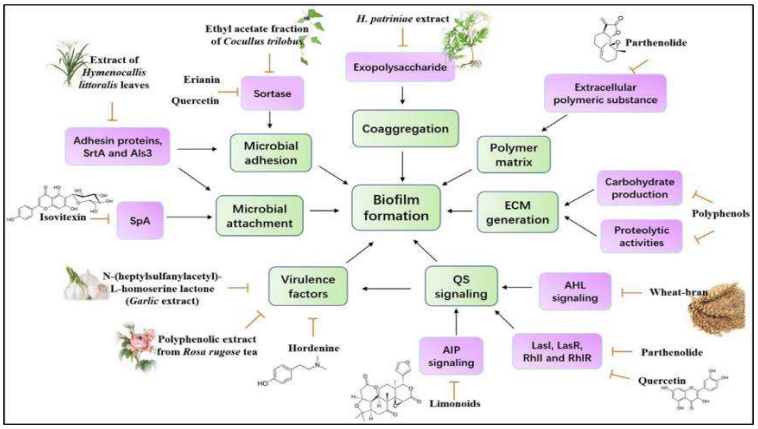

Fig. 2: Developing natural products as potential anti-biofilm agents.[88]

Natural anti-biofilms agents under clinical evaluation:

During the recent decades, the extent of biofilm effects on inanimate substances and the development of various persistent infectious diseases in humans and animals. Recent studies have reported the antibiofilm activity of several compounds, no definitive agent has been reported so far. Therefore, extensive studies have been conducted systematically and clinically. Some clinical trials of herbal compounds have been reported to achieve the antimicrobial, anti-biofilm, and antifouling effect in patients with artificial tooth transplants or oral inflammation ***Ricinus communis*** is one of the most important herbs showing antimicrobial and antibiofilm effects against ***S. mutans*** and ***Candida* spp.** in patients with stomatitis and dentures [89,90]. Moreover, some clinical trials have revealed that mouthwashes containing ***Salvadora persica*** and ***Matricaria chamomilla* L.** can significantly reduce bacterial biofilm caused by dental plaque and suppress biofilm development in chronic periodontitis cases [91,92]. Controlled clinical trials in patients with chronic periodontitis have also shown that mouthwash has EOs (e.g., ***Cymbopogon flexuosus***, ***Rosmarinus officinalis***, and ***Thymus zygis***) which is effective in reducing bacterial biofilm construction in the gums [93]. Additionally, it was indicated that antibacterial and anti-biofilm activity of lemongrass oil against ***Aggregatibacter actinomycetemcomitans* ATCC 43,718** and ***Porphyromonas gingivalis* W50** can significantly diminish oral malodour [94]. On the other hand, ***Melaleuca alternifolia***, cranberry extracts (PAC-A, proanthocyanidin-A), and garlic or olive oil were reported as potential anti-biofilm in orthodontic,

subclinical recurrent urinary tract infection (UTI), and lung cystic fibrosis patients, respectively [95,96]. There have been several clinical trials, including natural compounds that have achieved positive antimicrobial and anti-biofilm activity results such as Nujol, Alpha Care, and CISTIMEV PLUS that have been extensively studied. The results show the potential dental anti-biofilm and antibacterial effects of these compounds in a randomized pilot study [97,98]. Some natural anti-biofilm medicines have been studied via clinical trials and have shown to be effective. Various phases of clinical studies (I, II, III, and IV) are now being investigated in patients to evaluate the anti-biofilm agents as a single therapeutic compound. However, the outcomes of the trials have not yet been published completely. According to the inclusion of these natural products in the clinical trial studies to investigate their inhibitory effects on biofilm formation, it can be concluded that these compounds have the potential to replace the other bacterial infection treatments. Based on the data of these researches, such compounds can provide encouraging results for the other studies of this field. The anti-biofilm mechanism of these agents is interference in any stages of biofilm construction or inhibition of the QS network. Here, we introduced the present studies of the herbal compounds involved in phases I–IV of clinical trials regarding periodontitis, dental plaque, and gingivitis to provide a background for more extensive researches in this area.

Table 3: Natural anti-biofilm factors with clinical assessment [99]

Intervention	Outcome	Clinical trials	Studies
Mouthwash containing green and black tea with 0.12% chlorhexidine against gingivitis	Anti-biofilm properties of green and black tea	Completed	Mouthwash (orally against biofilm
Toothpaste with curcumin	Anti-biofilm properties of curcumin	Phase I	Effects of curcumin-containing toothpaste on dental biofilm and associated oral halitosis
Mineral and vegetable oil considerably decrease dental biofilm formation	Anti-biofilm properties of Nujol in periodontal diseases	Completed	Anti-biofilm properties of dentifrice containing mineral and vegetable oil

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