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Effect of Indian spices on *lasR* mutant of *Pseudomonas aeruginosa* PAO1 biofilm

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Abstract
The study was undertaken to analyse the impact of bark of cinnamon (*Cinnamomum zeylanicum*), seeds of mustard (*Brassica nigra*) and nutmeg fruit (*Myristica fragrans*) on the biofilm by *lasR* mutant of *Pseudomonas aeruginosa* PAO1. The analysis of the phytoextracts showed that the test culture is inhibited to varying degrees by different phytoextracts. Aqueous and methanolic extracts were prepared and its phenolic content and antioxidant properties were assessed. The effect of the extract was further studied for the biofilm formation by the *lasR* mutant. Different phytoextract cause varying degree of structural changes in biofilm architecture of wild type and *lasR* mutant. The finding indicates involvement of *lasR* during biofilm development in presence any antimicrobial compound of plant origin.

Keywords: Antimicrobial, antioxidant, biofilm, phenolic, quorum sensing

1. Introduction

Spices are the dried part of a plant used for seasoning and flavouring a recipe and include various seeds, roots, fruits, barks, and vegetables. Their main use is to add colour and flavour to food as well as to help in remain preserved and good to eat. Apart from these uses, they also find application in control of various microorganisms. The antimicrobial activity has been reported since very long and is still a topic of interest amount the researchers [1-5]. The antimicrobial activity of spices varies with; spice variety, phytochemical, organism and its occurrence level and processing conditions and storage [6, 7]. The alternates to chemical preservatives are being searched for food preservation so as to partially or completely replace the chemical preservatives [8, 9]. Consumers prefer foods free or with lower chemical preservatives because of the toxicity they possess [10]. Thus, plants with antimicrobial activity can be an alternate to these preservatives in food.

Food contamination by microorganisms is mainly mediated by biofilm formation. Biofilms are formed by several pathogenic bacterial species e.g. of human pathogens forming biofilms in natural aquatic environments include *Pseudomonas aeruginosa, Vibrio cholera* and certain species of nontuberculous mycobacteria [11]. Most oligotrophic bacteria in environment grow in biofilms rather than as single planktonic cells as biofilms provides a protective degree of homeostasis and stability in changing environment, are structured, specialised communities of adherent microorganisms encased in a complex extra polymeric substance (EPS) matrix [12]. Therefore, biofilm formation represents a facile microbial survival strategy where microorganisms [13].

Biofilm formation is a result of bacterial cell to cell communication called quorum sensing (QS). It allows cells to communicate and share information about the cell density and gene expression adjustment accordingly. The biofilm formation and bio fouling are the result QS molecules produced by the microorganisms in their natural environments. For instance, in *P. aeruginosa lasI/lasR* and *rhlI/rhlR* are QS genes regulating many features including biofilm development [14]. Thus, interfering with the quorum sensing mechanism of the bacteria could be useful to control the biofilm growth and thus, prevent the food spoilage. The attachment of different bacteria with subsequent development of biofilms on food surface can lead food spoilage or transmission of diseases [15-17]. The most common foodborne biofilm producers belong to the genera *Pseudomonas, Listeria, Enterobacter, Flavobacterium, Alcaligenes, Staphylococcus* and *Bacillus* [18, 19].

The aim of present study is to evaluate the effect of phytochemicals extracted from spices used in food preservation on the *lasR* mutant of *Pseudomonas aeruginosa* PAO1 biofilm.

2. Materials and Methods

2.1 Culture media and chemicals

Nutrient agar containing (g per litre) peptone 10.0, meat / beef extract 3.0, NaCl 5.0, agar-agar15.0 and pH 7.6 in distilled water.

Luria Bertani (LB) broth containing (g per litre) peptone 10.0, NaCl 10.0, yeast-extract 5.0 in distilled water, pH 7.2.

Acridine orange (0.02%) dissolving 0.02 g of acridine orange in 100 mL distilled water.

Gallic acid: Stock prepared by dissolving 1 mg gallic acid in 1 mL distilled water, 5% (w/v) sodium carbonate in distilled water.

0.1mM 1,1-diphenyl-2-picryl-hydrazil (DPPH) was prepared by dissolving 1.9 mg of DPPH in methanol and allowed to react for 30 min in dark.

2.2 Strain

Pseudomonas aeruginosa PAO1 wild type and *lasR* mutant was obtained from Biofouling and biofilm processing section, BARCF, Kalpakkam- Tamil Nadu

2.3 Extraction of phytochemicals

Cinnamomum zeylanicum (bark of cinnamon), *Brassica nigra* (mustard seeds of mustard) and *Myristica fragrans* (nutmeg fruit) were obtained from the local market. The spices were washed thoroughly, dried and ground to fine powder. Aqueous (CA- Cinnamon aqueous, MA- Mustard aqueous and NA- Nutmeg aqueous) extracts in water and methanolic (CM- Cinnamon methanol, MM- Mustard methanol and NM- nutmeg methanol) extracts in 1:4 distilled water: methanol were prepared. The suspensions were kept overnight in shaking condition. After 24 h, the suspension was filtered and was allowed to dry. The dried solid mass was collected and stored at 4 °C [20].

2.4 Total phenolic content

The total phenolic content of the extracts was determined by Folin-Ciocalteu method. For this, 1 mL of crude extract (5 mg/mL) was mixed thoroughly with 1 mL 1N Folin-Ciocalteu reagent and 1 mL of 5% (w/v) sodium carbonate. The volume was made upto 6 mL using distilled water. The mixture was allowed to stand for a further 60 min in dark, and absorbance was measured at 640 nm. The total phenolic content was calculated from the calibration curve. The amount of phenolic content was expressed as mg per g of gallic acid [21].

2.5 Antioxidant assay

The antioxidant activity of the extract was determined by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH). For this, 200 µL of each extract (20 µg/mL) were mixed with 3.8 mL DPPH solution and incubated in the dark at room temperature for 1 h. The absorbance of the mixture was then measured at 517 nm. Ascorbic acid was used as a positive control and reaction volume with phytoextracts replaced with distilled water was used as control. The ability of the sample to scavenge DPPH radical was determined from [21]:

$$\text{DPPH scavenging effect} = \frac{\text{Absorbance Control} - \text{Absorbance Sample}}{\text{Absorbance Control}}$$

2.6 Effect on biofilm growth

Autoclaved microscope slides were kept in sterile Petri plates. To this, 6 mL of 1:10 diluted culture of *Pseudomonas aeruginosa* PAO1 and *lasR* mutant was added and 200 µL (20 µg/mL) of the phytoextract was added. The plates were incubated for 48 h at 37 °C. After the incubation, the broth was extracted using sterile micropipette and the slide was flooded with 2 mL of 0.02% acridine orange dye. The plates were incubated in dark for 10 min at room temperature. After incubation, the slides were washed gently using sterile distilled water, dried in dark for 24 h and then observed using fluorescent microscope. The fluorescent microscopy images were analysed using ImageJ software (NIH, USA) for the surface architecture of biofilm. Biofilm growth was quantified in terms of raw integrated density calculated using ImageJ and structural architecture was determined using 3D plot constructed using ImageJ [12].

3. Results and Discussion

3.1 Phenolic content

The total phenolic content of the phytoextracts was estimated from the FC reagent. Phenolic content was found to be highest in the cinnamon aqueous extract. Among the aqueous and methanolic extracts, cinnamon with 305.86 mg/g of gallic acid and mustard with 145.28 mg/ g of gallic acid showed the highest phenolic content respectively (Table 1). Overall, the highest phenolic content was observed in the aqueous extract of cinnamon. The total phenolic content in the (*Myristica fragrans*) nutmeg fruit was found to be 130.81 mg/g of gallic acid (aqueous extract) and 138 mg/g of gallic acid (methanolic extract). This content was higher than the previously reported values where the aqueous and methanolic extracts have 57.49 mg/g of gallic acid and 61.26 mg/g of gallic acid respectively [22]. The phenolic content in the cinnamon extracts was found to be 305.86 mg/g of gallic acid which is higher than the content reported [23].

3.2 Antioxidant assay

The DPPH radical is widely used in assessing free radical scavenging activity because if the ease of the reaction. The methanolic extract of mustard had the strongest antioxidant activity. DPPH scavenging activity at concentration of 20µg/µL of the positive control, ascorbic acid was 98.84%. The antioxidant activity of methanolic extract of mustard is highest with 93.2% (Table 1). Amongst all the extracts, the antioxidant activity of the methanolic extracts of mustard and nutmeg are closer to that of ascorbic acid.

Table 1: Total phenolic content and antioxidant activity of phytoextracts

Phytoextract	**Total phenolic (mg/g) gallic acid**		**Antioxidant activity (%)**	
Plant	***Aqueous***	***Methanol***	***Aqueous***	***Methanol***
Cinnamon	305.86	86.44	79.28	88.96
Mustard	85.38	145.28	78.44	93.2
Nutmeg	130.81	138	83.28	91.6

The antioxidant activity of nutmeg was reported to be highest in acetone extract (63.04 %) [22], while those in the above studies were highest in the methanolic extract of mustard (93.2%). The radical scavenging in cinnamon is reported 92% [24], which is higher than the results obtained in this study. The *Cinnamomum zeylanicum* (cinnamon bark) showed 88.96 % and 79.28 % in methanolic and aqueous extracts respectively.

3.3 Biofilm growth of wild type and *lasR*

The biofilm of the wild type and *lasR* mutant of *Pseudomonas aeruginosa* PAO1 were grown in absence of the phytoextracts and used as control. The raw integrated density of the wild

type was higher than the *lasR* mutant (Fig. 1). Also, the 3D surface plot and plot profiles show more uniform growth in the wild type as compared to the *lasR* mutant biofilm (Fig. 2). The *lasR* regulates transcription of virulence associated elastase (*lasB*) and LasA protease (*lasA*) in *P. aeruginosa* two proteases associated with virulence. *lasR* in association with *lasI (*N-3-oxo-dodecanoyl-L-homoserine lactone) coordinate the expression of target genes, including many genes for biofilm development [12, 25].

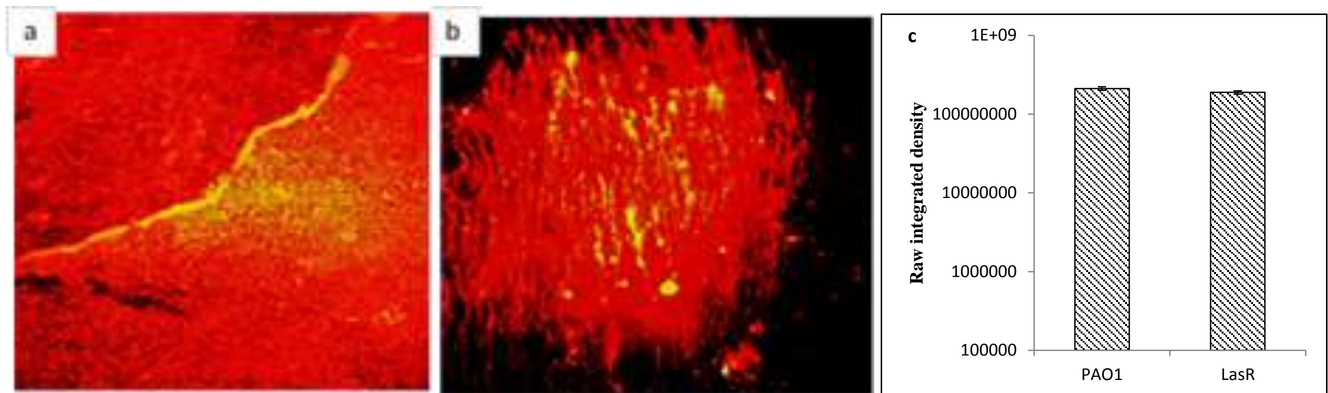

Fig 1: Biofilm formed by *P. aeruginosa* PAO1 a. wild type b. *lasR* mutant biofilm in absence of phytoextracts c. Biofilm growth in terms of raw integrated density

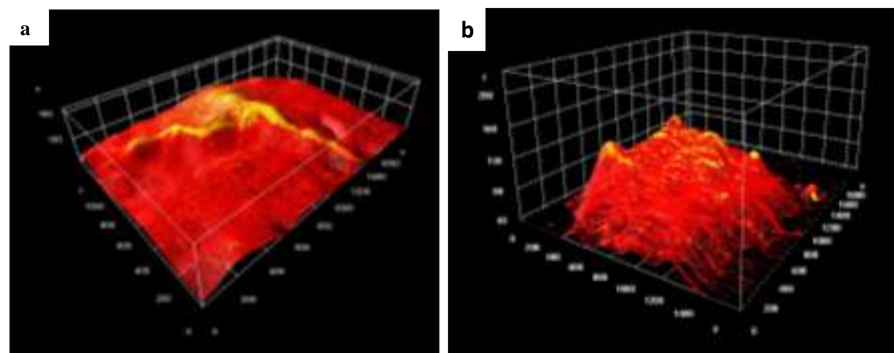

Fig 2a: 3D surface plot. **b.** Plot profile of biofilm formed by wild type and *lasR* mutant of *P. aeruginosa* PAO1 in absence of phytoextracts respectively.

Effect of phytoextract on biofilm growth of wild type and *lasR* mutant of *P. aeruginosa* PAO1

QS is a system that is widely used by pathogenic bacterial species to regulate the expression of virulence factors associated with different pathogenic phenotypes through adherence and biofilm formation [26]. Thus, QS inhibition has emerged as a promising target in a wide variety of bacterial infections following the emergence of antibiotic-resistant phenotypes, which has resulted in a search for new therapeutic alternatives.

For decades, plant products and their derivatives have predominated infection therapy. The spices used in this study showed inhibitory effect on *lasR* mutant biofilm of *Pseudomonas aeruginosa* PAO1. The raw integrated density of the biofilm with aqueous and methanolic extracts showed considerable difference among the wild type and the *lasR* mutant (Fig. 3, Fig. 4). Also, the raw integrated density of the wild type is higher than the *lasR* mutant. Among all the aqueous extracts, nutmeg showed highest inhibitory action while among the methanolic extracts, mustard showed the highest inhibitory action against the *lasR* mutant biofilm.

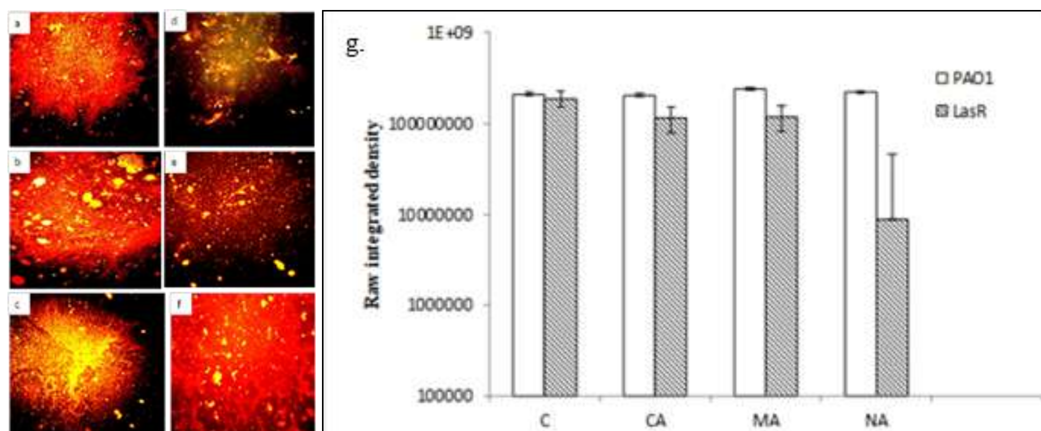

Fig 3: Raw integrated density of biofilm of **a-c:** PAO1 wild type **d-f:** *lasR* mutant in presence of aqueous phytoextracts CA, MA and NA respectively **g:** Comparison of raw integrated density of biofilms of wild type and *lasR* mutant in presence of aqueous extracts.

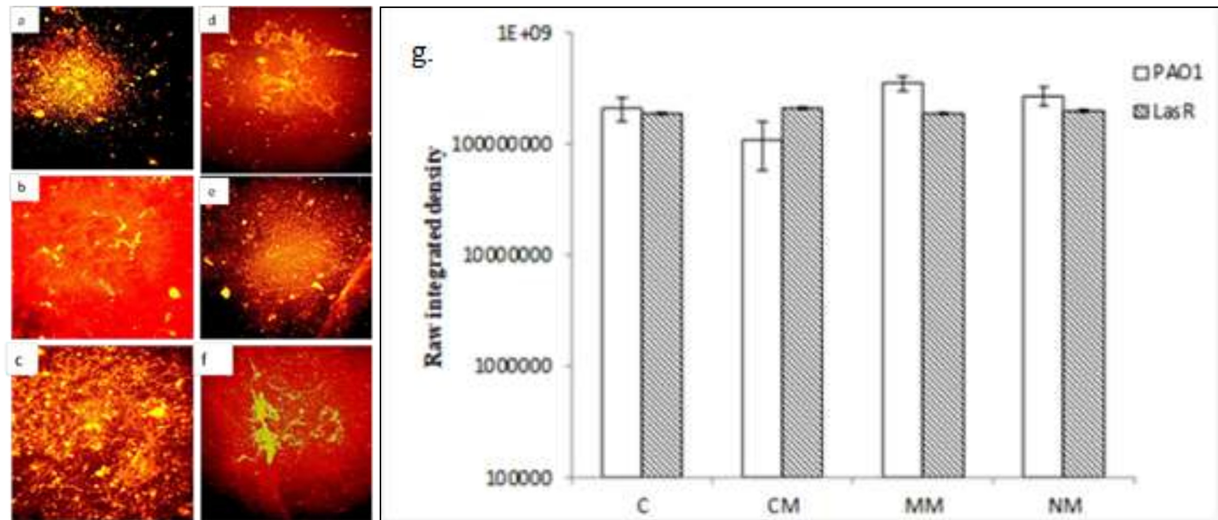

Fig 4: Raw integrated density of biofilm of. **a-c:** PAO1 wild type. **d-f:** *lasR* mutant in presence of methanolic phytoextracts CM, MM and NM respectively **g:** Comparison of raw integrated density of biofilms of wild type and *lasR* mutant in presence of methanolic extracts.

The 3D surface plot and plot profile of the biofilms were constructed to analyse the surface architecture of the biofilm. The plot profiles show more uniform growth of the wild type biofilm as compared to the *lasR* mutant biofilm (Fig. 5). This shows that the phytoextracts were more effective as biofilm inhibitors in *lasR* mutant as compared to the wild type. Furanone derivatives from plants in South Florida [27] and garlic [28] as well as other dietary phytochemicals, such as *Curcuma longa* [29], caffeine [30] and vanilla extract [31], are considered to be potent inhibitors of QS and its regulatory factors, one among which is the biofilm formation.

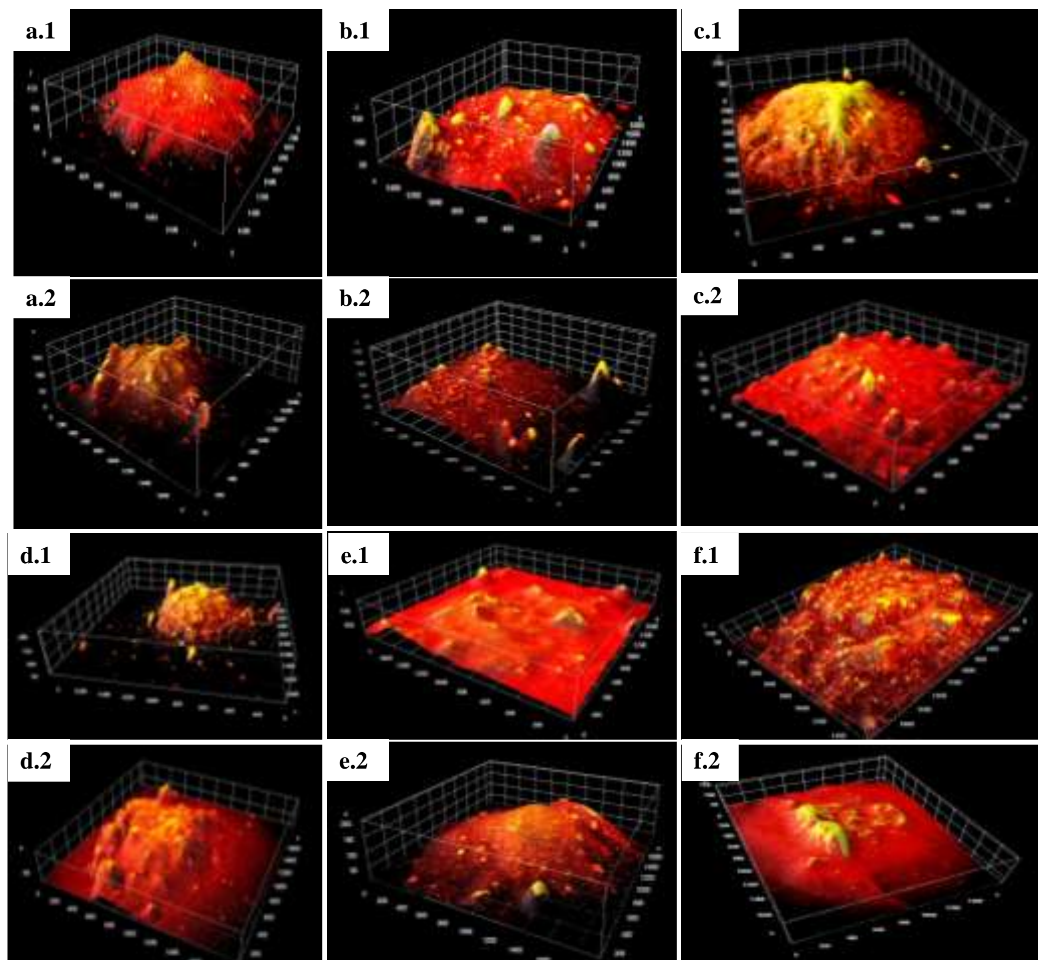

Fig 5: 3D surface plot of PAO1 and *lasR* in aqueous extracts CA: a.1, a.2; MA: b.1, b.2; NA: c.1, c.2 and methanolic extracts CM: d.1, d.2; MM: e.1, e.2; NM: f.1, f.2 respectively

Cinnamon oil at low concentrations (0.1 and 0.2 µl/ml) inhibits QS, while the remaining concentrations tested in the study affect both QS and the growth rate. Kim *et al*. (2015) reported decrease in biofilm formation and toxin production in the presence of different sub-lethal concentrations of cinnamaldehyde [32].

Complete cinnamon oil is active against QS-based virulence factors in *P. aeruginosa* PAO1, in which the QS molecules have been reported to be 3-oxo-C_{12}HSL and C_4HSL. The presence of many major and minor components in cinnamon oil, including cinnamaldehyde and eugenol, suggests that the QSI activity of cinnamon oil may lie in its constituents either individually or synergistically [32].

Naringenin and taxifolin also reduce the expression of several QS-controlled genes (*i.e.*, *las*I, *las*R, *rh*lI, *rh*lR, *las*A, *las*B, *phz*A1 and *rh*lA) in *P. aeruginosa* PAO1. Naringenin also dramatically reduce the production of the acylhomoserine lactones *N*-(3-oxododecanoyl)-l-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-l-homoserine lactone (C_4-HSL), driven by the *las*I and *rh*lI gene products, respectively [33]. The antibiofilm potential of several EOs, including eugenol, cinnamaldehyde, citral, and geraniol, had been elucidated [34].

Spices can be used as an effective method for food preservation. They possess phenolic content that allow them to elicit the antioxidant action over the microorganisms and thus prevent their growth [35, 36]. The spices are also capable of inhibiting the biofilm formation of the *lasR* mutant of *Pseudomonas aeruginosa* PAO1 by interfering with the quorum sensing, which tends to be the only means of communication among the microorganisms in a biofilm. The results imply that the spices could also be used as anti-biofilm agents to inhibit the biofilm growth in the nature and thus, prevent the damages caused by biofouling.

The overall findings showed the effective use of spices in the food biopreservation and control of biofilms formed by the *lasR* mutant of *Pseudomonas aeruginosa* PAO1.

4. Conclusion

The control of biofilms by phytoextracts is possible. Overuse of chemical preservatives results in the formation of multi-resistant strains of bacteria which makes the process of their control even more difficult. In the above study, the phytoextracts showed considerable inhibition of the biofilm formation of the *lasR* mutant of *Pseudomonas aeruginosa*PAO1. The extracts had phenolic content and antioxidant activity that attributed to their antimicrobial activity. The spices with the inhibitory activity make them eligible to be used in a wide range of products to control biofilms such that the use of chemical alternatives could be minimised.

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