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Promising Phytochemicals that Show Antibiofilm Activity at Sub-Minimum Inhibitory Concentrations: Trans-Cinnamaldehyde, Limonene, Eugenol, and Curcumin

Alt-Minimum İnhibisyon Konsantrasyonlarında Antibiyofilm Aktivitesi Gösteren Umut Verici Fitokimyasallar: Trans-sinnamaldehit, Limonen, Eugenol ve Kurkumin

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ABSTRACT

As the challenges in the treatment of infections caused by multi-drug resistant microorganisms with well-known antimicrobial agents become a serious treat for the human health in worldwide, development of novel antimicrobials with potent antimicrobial activity has garnered significant attention. Therefore, this study aimed to investigate the antimicrobial and antibiofilm effects of four phytochemicals (trans-cinnamaldehyde, limonene, eugenol, and curcumin) against Gram-positive and Gram-negative bacteria and a yeast. Prior to antibiofilm assays, minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), and minimum fungicidal concentrations (MFC) were determined, with significant bactericidal and fungicidal effects being observed at low phytochemical concentrations. Also, biofilm inhibition efficiency of these phytochemicals was assessed at sub-MIC values (0.5x, 0.25x, and 0.125x MIC). At least 60% biofilm inhibition was observed for most of the microorganisms at the lowest tested concentrations (0.125x MIC) of the phytochemicals. Their biofilm inhibition capacity generally increased up to 80-90% depending on the concentration. Six data-driven models and their joint optimization adopted in this study yielded validation-based high predictive accuracy and identified optimal conditions. These data analysis models were applied in the antibiofilm activity investigation of natural compounds for the first time in this study to evaluate the accuracy of the results.

Key Words
Biofilm inhibition, phytochemical, bactericidal, fungicidal.

ÖZ

Dünya çapında, çoklu ilaç direncine sahip mikroorganizmaların neden olduğu enfeksiyonların bilinen antimikrobiyal ajanlarla tedavisinde karşılaşılan zorluklar insan sağlığı için ciddi bir tehdit haline geldikçe, güçlü antimikrobiyal aktiviteye sahip yeni antimikrobiyallerin geliştirilmesi önemli derecede ilgi görmektedir. Bu nedenle, bu çalışmada da dört fitokimyasalın (trans-sinnamaldehit, limonen, eugenol ve kurkumin) Gram-pozitif ve Gram-negatif bakteriler ve bir maya üzerindeki antimikrobiyal ve antibiyofilm etkisinin araştırılması amaçlanmıştır. Antibiyofilm analizleri öncesinde minimum inhibitör konsantrasyonları (MİK), minimum bakterisidal konsantrasyonları ve minimum fungisidal konsantrasyonları saptanmış olup ve düşük fitokimyasal konsantrasyonlarda bile önemli bakterisidal ve fungisidal etkiler gözlenmiştir. Ayrıca, bu fitokimyasalların biyofilm inhibisyon etkinliği alt-MIC değerlerinde (0,5x, 0,25x ve 0,125x MIC) değerlendirildi. Fitokimyasalların en düşük test edilen konsantrasyonlarında (0,125xMİK) mikroorganizmaların çoğunda en az %60 biyofilm inhibisyonu gözlenmiştir. Biyofilm inhibisyon kapasiteleri konsantrasyona bağlı olarak genellikle %80-90'a kadar arttı. Bu çalışmada kullanılan altı farklı veri güdümlü model ve bu modellerin ortak optimizasyon sürecine göre validasyon temelli yüksek tahmin doğrulukları elde edilmiş ve optimum koşullar belirlenmiştir. Bu çalışmada, sonuçların doğruluğunu değerlendirmek amacıyla, ilk kez doğal bileşiklerin antibiyofilm aktivitesinin araştırılmasında bu veri analiz modelleri uygulanmıştır.

Anahtar Kelimeler
Biyofilm inhibisyonu, fitokimyasal, bakterisidal, fungisidal.

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INTRODUCTION

In the history of the medicine, the discovery of antibiotics marked a medical milestone, dramatically reducing mortality and morbidity from infectious diseases [1]. Unfortunately, the development of antibiotic-resistant microorganisms due to the inappropriate use of antibiotics is posing a significant threat to human health by contributing to treatment failures and increased recurrent infections. Infections caused by multidrug-resistant bacteria lead to the death of approximately 700.000 people each year, and if considerable precautions are not taken, this number is expected to reach 10 million by 2050 [2]. Biofilm-associated multidrug-resistant microorganisms are a primary cause of recurrent infections. Biofilms, microbial communities embedded in an exopolysaccharide matrix adhering to biotic/abiotic surfaces, enhance microbial resistance to antimicrobial agents. [3, 4]. 70% of infections is caused by microorganisms that can form biofilm and this structure is the major source of hospital associated infections. Microorganisms living as part of biofilms exhibit characteristic features such as collective cooperation and increased survival against antimicrobial agents [5]. Once biofilm has formed on invasive medical equipment, long-term use of antimicrobials is required as it is not possible to effectively eliminate the biofilm using conventional treatments [6]. The high cost of current antimicrobial therapy and the risk for pathogens to develop drug resistance increase the need for the development of new and cost-effective antimicrobial agents [7]. Additionally, a surface containing nutrients and moisture is an eligible environment for the formation of biofilm [5]. Hence, biofilm-forming microorganisms not only cause infections via hospital associated but also significant challenges in the food industry by forming biofilm on food processing surfaces, thus leading to contamination and foodborne illnesses [8-11]. The escalating threat of multidrug-resistant and biofilm-forming microorganisms has spurred research into safer, effective natural antimicrobial alternatives. Among these antimicrobial compounds, phytochemicals have gained attention due to their versatile biologic activities and usability in different fields, such as treatment of various diseases in medicine and a non-toxic preservative in the food and cosmetic industry [12, 13]. Consequently, this study evaluated four phytochemicals with different chemical nature—(i) trans-cinnamaldehyde (phenyl aldehyde), (ii) limonene (terpene), (iii) eugenol (phenol), and (iv) curcumin (polyphenol)—for their bacteriostatic, bactericidal, fungicidal, and antibiofilm activities against widespread Gram-positive and Gram-negative bacteria and a yeast. Moreover, this study focused on the modeling and optimization of the five individual responses through the joint use of six algorithms and Monte Carlo-based sensitivity analysis to predict the relationships between predictors and biofilm inhibition.

MATERIALS and METHODS

Materials

Phytochemicals: trans-cinnamaldehyde, limonene, eugenol, and curcumin were obtained from Sigma-Aldrich, USA. Microorganisms used for determining antibacterial/antifungal and antibiofilm activities of the phytochemicals (Gram-positive *Staphylococcus aureus* (*S. aureus*) ATCC 25923 and *Enterococcus faecalis* (*E. faecalis*) ATCC 29212, Gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853 and *Escherichia coli* (*E. coli*) ATCC 25922 and a yeast *Candida albicans* (*C. albicans*) ATCC 10231) were purchased from American Type Culture Collection (ATCC), USA. Mediums used for microbial cultivation and antimicrobial/antibiofilm assays, such as Brain hearth infusion (BHI) broth, Nutrient agar (NA), Potato dextrose agar (PDA), and Potato dextrose broth (PDB), were obtained from Biokar, France. Crystal violet, ethanol, and dimethyl sulphoxide (DMSO) were purchased from Merck, Germany. All the media and the buffers were prepared with double distilled water purified via a milli pore Simplicity 185 Ultrapure Water System.

Cultivation of microorganisms

S. aureus, *E. faecalis*, *P. aeruginosa*, *E. coli*, and *C. albicans* strains stored at -80 °C were pre-cultured onto NA and PDA and grown overnight at 37 °C. Then single colony of each microorganism was inoculated into BHI broth for the bacteria and PDB for the yeast and incubated for 24 h at 37 °C under agitation (MCI 120; Mipro, Ankara, Turkey). After incubation, cells were harvested using the centrifugation of cultures at 3000 rpm for 15 min (Eppendorf Centrifuge 5810 R, Hamburg, Germany). Supernatants were removed, and collected cells were washed with phosphate buffer saline (PBS) (pH 7.4) for three times. While bacteria cells were diluted in PBS to adjust bacteria concentrations to $1.5x10^8$ CFU/mL according to McFarland standards by using UV-visible spectrophotometer (Shimadzu UV-1700, Kyoto, Japan), collected yeast cells were counted on a Thoma chamber to adjust cell concentrations as $1x10^5$ CFU/mL.

Detecting minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of phytochemical.

MICs of trans-cinnamaldehyde, limonene, eugenol, and curcumin were determined in 96 well microtiter plate by the microdilution method against the above-mentioned microorganisms according to Balouri et al. [14]. Briefly, 20 μL BHI broth was added to each well in the plate. The same concentrations of each phytochemical compounds (500 μg/mL) which were prepared by solving eugenol and curcumin in ethanol and trans-cinnamaldehyde and limonene in DMSO, were added into the first wells of each column of the well plate, and then serial two-fold dilutions were realized to obtain concentration ranges of 0.98-500 μg/mL in each well except for the positive sample well. Then, bacteria solutions adjusted to $1.5x10^8$ CFU/mL were added to each well as 10 μL except for the negative sample well. The same process was applied for the yeast solution adjusted as the concentration of $1x10^5$ CFU/mL. After the incubation at 37 °C for the bacteria 24 h and for the yeast 48 h in a static incubator, the MIC of each compound was determined as the lowest concentration preventing visible growth of microorganisms. After incubation, MICs of each compound for the tested microorganisms were determined according to visually observed turbidity. Furthermore, a 100 μL sample was taken from the wells with no growth and inoculated onto NA for the bacteria and PDA for the yeast to determine MBC and MFC of the tested phytochemicals. MBC and MFC can be defined as the minimum concentration of a given antimicrobial agent that kills bacteria or fungi. Following the incubation period at 37 °C for 24 h, the concentration with no growth was determined as MBC or MFC. All the experiments were replicated triplicate.

Antibiofilm activity of phytochemicals

To evaluate the antibiofilm activity of the phytochemicals, crystal violet biofilm-forming assay was applied [15]. Briefly, 100 μL BHI broth, 100 μL from microorganism at the concentration of $1.5x10^8$ CFU/mL for the bacteria and $1x10^5$ CFU/mL for the yeast were added to each well. Sub-MIC concentrations (0.5x, 0.25x, and 0.125x MIC) of each phytochemical were tested to assess their concentration-dependent antibiofilm activity. Control biofilm formation of the used strains was performed in the wells prepared without phytochemical compounds. After incubation process at 37 °C for the bacteria for 24 h and for the yeast for 48 h in the static incubator, cultures were poured, and formed biofilms were fixed to the well surface by treating with methanol. Then, biofilms were rinsed with DI water for three times and stained with crystal violet for 45 min at ambient temperature. After removing crystal violet solution, stained biofilm was solubilized with 95% (v/v) ethanol and absorbance of the wells depending on turbidity difference, measured at 540 nm via a UV-visible spectrophotometry. Biofilm inhibition capacity (%) of each phytochemical against the strains was calculated as follows:

Eq 1:

$$\%\ \text{Biofilm inhibition} = \frac{(\text{ODcontrol} - \text{ODsample})}{\text{ODcontrol}} \text{x}100$$

Data Analysis

The data-driven prediction and joint optimization process adopted in this study consisted of the modeling of the five individual responses through six algorithms, followed by single-response joint optimizations of the six models and Monte Carlo-based sensitivity analysis. The (1) Lasso (least absolute shrinkage and selection operator)-based generalized regression (LGR), (2) least squares regression (LSR), (3) support vector machine (SVM), (4) boosted artificial neural network (BANN), (5) XGBoost, and (6) random forest (RF) were best fit to the five responses. The five responses comprised the inhibition (%) of biofilm formation by (1) *E. coli*, (2) *S. aureus*, (3) *P. aeruginosa*, (4) *E. faecalis*, and (5) *C. albicans*. The inputs of the best-fit models were a nominal phytochemical compound predictor with four levels (trans-cinnamaldehyde, limonene, eugenol and curcumin) and an ordinal concentration predictor with three levels (0.5x, 0.25x, and 0.125x MIC). The predictive performance of the models was tested against 5-fold cross-validation for the individual responses. Based on composite desirability function (*D* value ranging from 0 to 1) [16], the six best-fit models (LGR, LSR, SVM, BANN, XGBoost, and RF) were simultaneously optimized to maximize the biofilm inhibition (%) of each microorganism as a function of the predictors. Monte Carlo simulations were independently performed on the two predictors to pinpoint how sensitive the individual and simultaneous responses were to the total (main plus interaction) effects of the inputs [17]. All the modeling and optimization were conducted using JMP Pro 17.2.

RESULTS and DISCUSSIONS

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of phytochemicals

MIC, MBC, or MFC of the selected phytochemicals (trans-cinnamaldehyde, limonene, eugenol, and curcumin) were determined against Gram-positive *S. aureus* and *E. faecalis*, Gram-negative *P. aeruginosa* and *E. coli* bacterial strains and the yeast *C. albicans* (Tab. 1).

According to the MIC, MBC, and MFC assay results, all the tested plant-derived compounds showed significant bactericidal and fungicidal activities against the selected microorganisms at the lowest concentrations ≤ 125 μg/mL (Tab.1).

Curcumin was the only compound that showed both bacteriostatic and bactericidal effects at the same concentration. A higher fungicidal effect was also detected at the low curcumin concentrations (31.25 μg/mL) than that at the other compound concentrations. In the study of Neelakantan et al. in which anti-biofilm effect of curcumin was evaluated against biofilm formation of *E. faecalis* on tooth substrate, they found the MIC of curcumin as 625 μg/mL [18]. In another study, antimicrobial and anti-biofilm efficacy of curcumin and piperine on *C. albicans* were investigated and 128 μg/mL MIC was recorded [19]. Previous studies [18, 19] indicated that bactericidal and fungicidal curcumin concentrations were 6-10 times higher than those observed in this study for both Gram-positive and Gram-negative bacteria, as well as yeast. The purity or extraction method used to obtain the compound was most likely to affect the bioactivity of the plant-derived compounds. Curcumin possesses antimicrobial behavior by affecting the different functions of bacteria, such as cell membrane or wall damage, inhibition of cell division, downregulation of gene expression, and inhibition of the quorum sensing (QS) mechanism [20]. Consistent with this study, it was previously shown that curcumin has broad spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria by targeting the different functions of bacteria [21, 22].

On the other hand, while eugenol showed similar antibacterial properties in the similar MBC of curcumin against *E. coli*, *P. aeruginosa* and *S. aureus*, MBC for *E. faecalis* and MFC for *C. albicans* were determined to be slightly high (125 μg/mL). Lou et al. [23] showed the inhibitory effect of eugenol and eugenol nano-emulsion at a sub-MIC of 0.2 mg/mL on QS activity and virulence factors production of *P. aeruginosa*. Eugenol and its nano-emulsion were able to show QS inhibitory effect by downregulating the genes in charge of the synthesis of QS signal molecules [23]. The given MBC concentrations of eugenol for the tested strains in this study were correlated with the findings of Lawrence and Jayekumar [24]. They also examined the inhibition mechanism of eugenol against *E. coli* by evaluating depolarization of cell membrane with the use of fluorescent dye. An increased fluorescent dye concentration inside the cell was observed depending on the exposure of eugenol.

Even though the antimicrobial activity of limonene was found to be effective against all the tested bacteria, its antimicrobial activity on Gram-positive bacteria was sharply observed at the lowest concentrations. In a study in which inhibitory effect of limonene and various natural compounds on the growth of *E. coli* and *S. aureus* were investigated, while no inhibitory

Table 1. MIC, MBC, and MFC of plant-derived compounds against a yeast and several bacteria strains.

	Plant-Derived Compounds							
	trans-Cinnamaldehyde (μg/mL)		Limonene (μg/mL)		Eugenol (μg/mL)		Curcumin (μg/mL)	
Strains	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	31.25	125	3.9	7.8	15.6	15.6	31.25	31.25
E. faecalis	31.25	125	7.8	7.8	31.25	125	31.25	31.25
P. aeruginosa	31.25	62.5	125	125	15.6	31.25	31.25	31.25
E. coli	62.5	62.5	125	125	7.8	31.25	31.25	31.25
		MFC		MFC		MFC		MFC
C. albicans	31.25	62.5	125	125	62.5	125	15.6	31.25

effect was determined for *E. coli* at the used concentration, MIC was found as 21 µg/mL for *S. aureus* [25]. Limonene shows an antimicrobial effect by causing cell membrane damage that leads to intracellular material leakage and finally death. Thus, the primary target of limonene is changed according to bacteria group as cell membrane in Gram-positives and outer membrane in Gram-negatives [25]. The lipopolysaccharide-rich outer membrane of Gram-negative bacteria forms a hydrophilic permeability barrier, increasing their resistance to hydrophobic compounds like limonene [27]. Additionally, lower MIC and MFC values (125 µg/mL for both) were observed for *C. albicans* in this study, compared to the study by Ahmedi et al. in which they observed MIC and MFC concentrations of 300 µg/mL and 400 µg/mL, respectively [28].

Trans-cinnamaldehyde demonstrated greater bactericidal efficacy against Gram-negative bacteria at lower concentrations than against Gram-positive bacteria as well as exhibiting fungicidal activity against *C. albicans*. The aldehydic group in trans-cinnamaldehyde easily penetrates the hydrophilic outer membrane of Gram-negative bacteria and passes through the cell wall, thus disrupting cell wall integrity and leading to loss of proteins, ions and nucleic acids [29, 30]. In this study, lower MIC values (31.25-62.5 µg/mL) were obtained with trans-cinnamaldehyde on both tested microorganisms when compared with the MICs such as 310 µg/mL [31], 125 µg/mL [32] and 250 µg/mL [33] in the literature. Trans-cinnamaldehyde demonstrates moderate fungicidal activity against *C. albicans*, inducing morphological changes and inhibiting adhesion and biofilm formation [34].

Antibiofilm capacity of phytochemicals

This study investigated the antibiofilm activities of the four phytochemicals at the sub-MIC concentrations. Results obtained with crystal violet biofilm-forming assay are presented in Fig. 1.

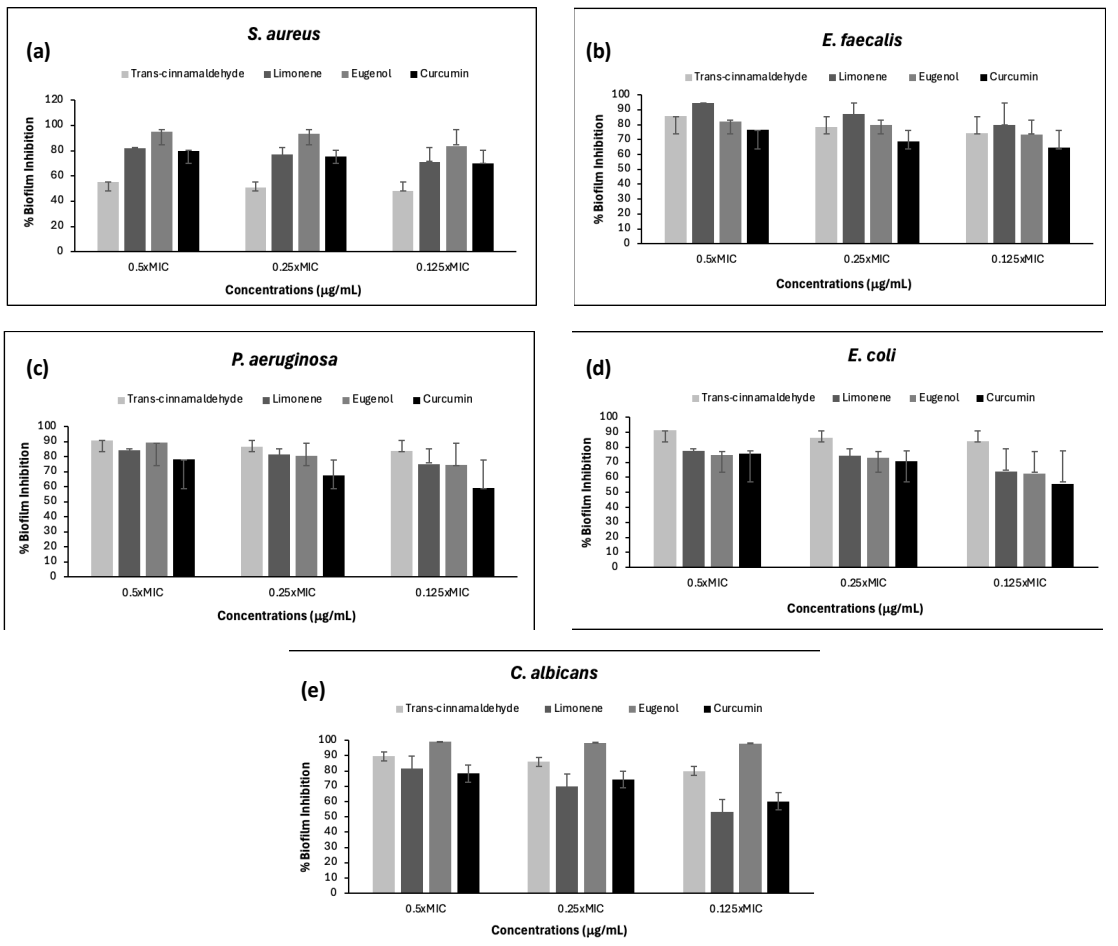


Figure 1. Biofilm inhibition (%) of the phytochemicals (trans-cinnamaldehyde, limonene, eugenol and curcumin) at the three sub-MIC concentrations against **(a)** *S. aureus*, **(b)** *E. faecalis*, **(c)** *P. aeruginosa*, **(d)** *E. coli*, and **(e)** *C. albicans*.

The highest inhibitory effect against the biofilm formation of *S. aureus* was observed for eugenol in a concentration-dependent way. Approximately 70% biofilm inhibition was recorded for curcumin and limonene at the lowest sub-MIC concentrations. Trans-cinnamaldehyde was found to be less effective as a phytochemical against *S. aureus* than the others (Fig.1a). As for the results of *E. faecalis*, although limonene was found to exert a slightly higher effect than did the others, the other phytochemicals also offered a high potential for preventing the biofilm formation (Fig. 1b). All the tested phytochemicals were found to be quite effective against both *E. coli* and *P. aeruginosa* (Fig. 1c-d). However, trans-cinnamaldehyde, known to easily pass through the outer membrane of Gram-negatives with the aldehyde groups it carries, was found to be slightly more effective than the others [2]. All the phytochemicals showed a remarkable antibiofilm effect against *C. albicans*. Due to the biofilm inhibition up to 98%, eugenol was determined as the most effective one among the others against *C. albicans* (Fig. 1e). Thus, this study demonstrated the antibiofilm effects of the four phytochemicals against the Gram-positive and Gram-negative bacteria and yeast at the lowest concentrations (≤ 0.125x MIC). All the tested bacteria and yeast can cause serious infections, food spoilage, and water contamination with their planktonic cells as well as biofilm association [18, 35-37]. Biofilm is a complex structure formed by several microorganisms adhering to the biotic or abiotic surfaces and extracellular polymeric compounds secreted by them. Biofilm formation on foods is an ongoing concern for the food industry and still remains poorly controlled although extended studies have been established in the past decades [38]. Approximately 65% of microbial infections are associated with the pathogens that are able to form biofilm. In addition, bacteria presented in the biofilm become 10-100-fold more resistant to antibiotics due to the low biofilm permeability [39]. Preventing biofilm associated infections was previously reported to be only possible with the very high MIC and MBC values of antibiotics [40, 41]. Therefore, novel and effective strategies need to be developed rather than using high amounts of antibiotics. Unlike antibiotics with single mechanisms of action, phytochemicals often exhibit multiple mechanisms to inhibit the bacteria [42]. While high antibiotic doses can accelerate resistance development, phytochemicals with multiple targets may hinder this process [43]. The determination of natural components without significant side effects on humans that have ability to show antimicrobial and antibiofilm effects on various microorganisms even at low doses will guide the development of novel antimicrobial agents and the fabrication of biomaterials with antimicrobial features which can be used in different fields.

Data Analysis

As a function of the four phytochemical compounds and three sub-MIC concentrations, the inhibitory fractions for each of the five microorganisms were trained via the best-fit LGR, LSR, SVM, BANN, XG Boost, and RF models (n = 29). Based on the 5-fold cross-validation data (n = 7), the two strongest predictive models were presented for each response variable in Table 2. The predictive power of the 10 models varied between 96.52% for the *S. aureus* biofilm inhibition (%) by BANN and 79.23% for the *E. faecalis* biofilm inhibition (%) by LSR.

Tables 3-5 present key hyperparameters and their estimates for the best-fit LGR for the biofilm inhibition of *S. aureus* and LSR for the biofilm inhibition of *P. aeruginosa* and *E. faecalis*, respectively. Regardless of the response variable, the architecture of the best-fit BANN consisted of one hidden layer with three neurons, hyperbolic tangent (tanh) activation function, squared penalty method, an additive sequence of 30 models, and a learning rate of 0.1 (Fig. 2). The hyperparameters of the other best-fit models were identified as follows:

SVM for *E. coli* biofilm inhibition (%): radial basis function, cost value of 1, gamma value of 0.5, and 23 support vectors; XG Boost for *C. albicans* biofilm inhibition (%): the loss function = reg: squared error, maximum depth = 6, subsample = 1, the subsample ratio of columns when constructing each tree = 1, the minimum number of instances required in a child node = 1, alpha = 0, lambda = 1, a learning rate of 0.3, and 30 iterations; and RF for all the responses: number of trees in forest = 100, number of terms sampled per split = 1; training rows = 28; validation rows = 8; bootstrap samples = 28, minimum splits per tree = 10, and minimum size split = 5.

The joint optimization of the six best-fit models resulted in the different optimal combinations of the predictors for all the models for the maximum biofilm inhibition of a given microorganism and the different sensitivity levels of each model-based biofilm inhibition to each predictor (Fig. 3a-e). The red-to-white gradient depicts the most-to-least relative sensitivity level of the individual predictors based on Monte Carlo simulations, with

optimal values marked in red and with 95%-confidence intervals in blue.

The order of the predictors on the *x*-axis in Fig. 3 indicates the most sensitive predictor on the far left, revealing that the phytochemical compound type was the primary driver of the maximum biofilm inhibition regardless of the microorganism. The color gradient on the *y*-axis in Fig. 3 (with red representing the most sensitive and white the least) illustrates the individual sensitivity of each predictor. The composite desirability (*D*) curve in Fig. 3 indicates that the higher the D values were, the greater the simultaneously optimal biofilm inhibition level of the six models was for a given microorganism. As shown in Fig. 3, the optimal combinations that maximized the biofilm inhibition levels were achieved as follows:

Trans-cinnamaldehyde and concentration 0.5xMIC for *E. coli* (*D* = 0.849; Fig. 3a) and *P. aeruginosa* (*D* = 0.869; Fig. 3c)

Eugenol and concentration 0.5xMIC for *S. aureus* (*D* = 0.884; Fig. 3b) and *C. albicans* (*D* = 0.946; Fig. 3e)

Limonene and concentration 0.5xMIC for *E. faecalis* (*D* = 0.814; Fig. 3d)

The small difference in the *D* values between the concentrations in Fig. 3c and e suggested that the concentration levels did no exert a significant main and synergistic impact on the maximum biofilm inhibition percent of *P. aeruginosa* and *E. faecalis*.

Table 2. The two best-fit data-driven models for each response variable and their performance measures based on 5-fold cross-validation (*n* = 7).

Response (%)		R^2	RMSE	SD
E. coli	Boosted Artificial Neural Network	0.9165	2.4	1.3
	Support Vector Machine	0.8295	3.4	1.6
S. aureus	Boosted Artificial Neural Network	0.9652	2.5	0.3
	Generalized Regression Lasso	0.9222	3.6	0.5
P. aeruginosa	Boosted Artificial Neural Network	0.9059	2.6	1.7
	Least Squares Regression	0.7809	4.0	1.6
E. faecalis	Boosted Artificial Neural Network	0.8970	2.4	0.5
	Least Squares Regression	0.7923	3.3	1.0
C. albicans	Boosted Artificial Neural Network	0.9393	2.9	0.7
	XGBoost	0.8665	4.2	0.5

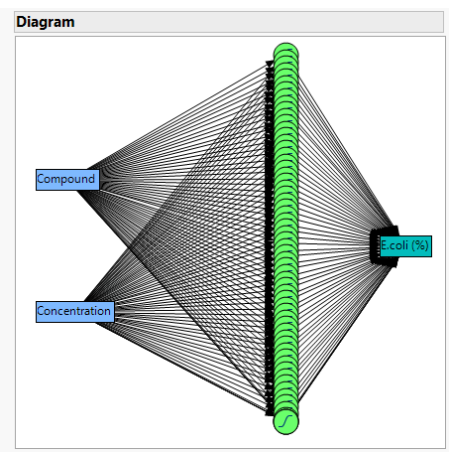


Figure 2. The architecture of the best-fit boosted artificial neural network for each response variable (E. coli is presented as an example) according to the following hyperparameters: one hidden layer with three neurons, hyperbolic tangent (tanh) activation function, squared penalty method, an additive sequence of 30 models, and a learning rate of 0.1.

Table 3. The hyperparameters of the best-fit generalized regression with the estimation method of Lasso for *S. aureus* (%) (150 grid points and square root grid scale; (with Curcumin and concentration 0.5xMIC as the baseline indicators).

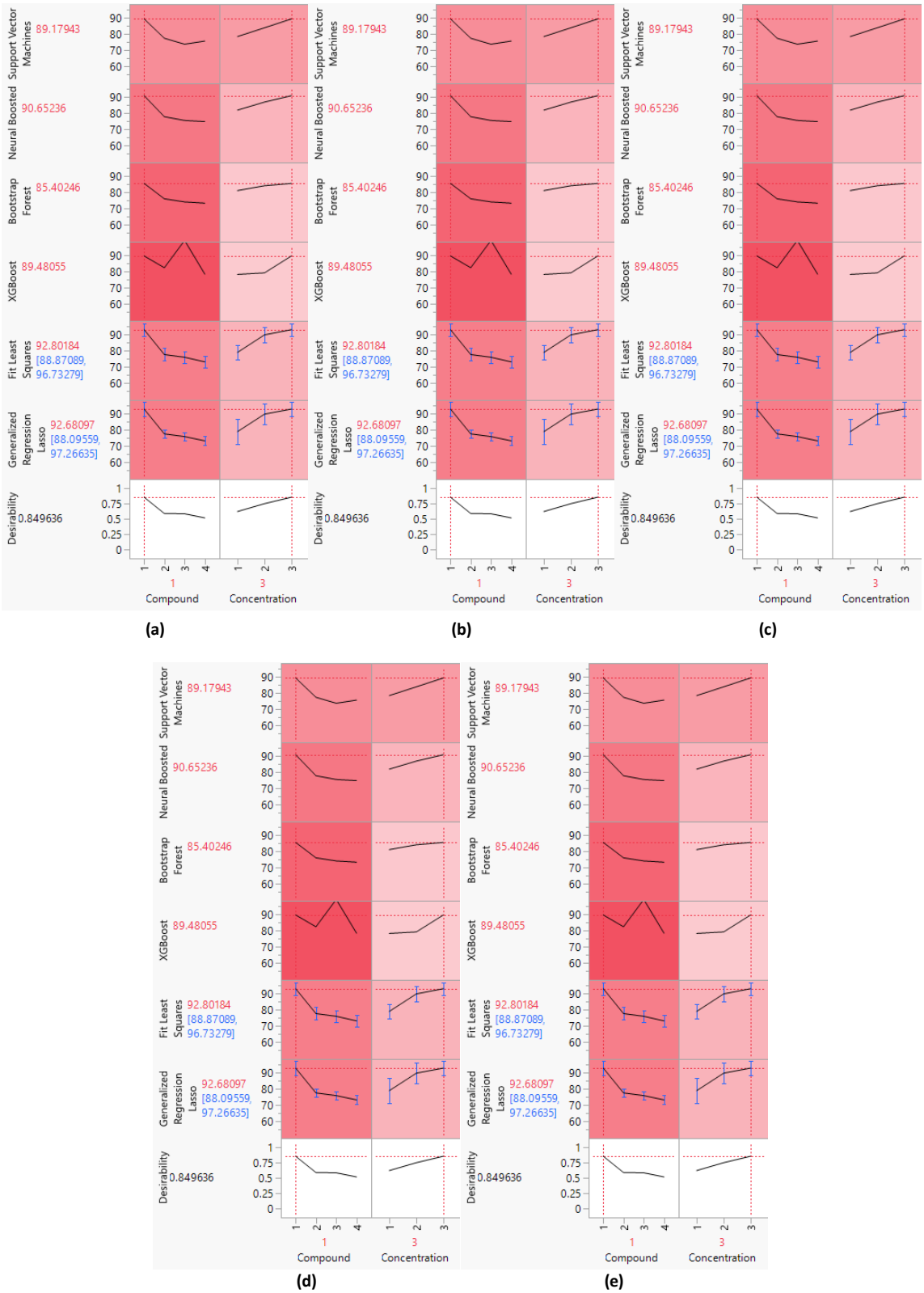
Term	Estimate	SE	P	VIF
Intercept	70.369377	1.5721055	<.0001	
Trans-cinnamaldehyde	-24.02409	2.0253256	<.0001	2.4
Limonene	1.5374062	2.0127024	0.445	1.9
Eugenol	15.116328	1.7536624	<.0001	1.8
Concentration 0.125xMIC	5.5892122	1.3295349	<.0001	1.2
Concentration 0.25xMIC	3.7341516	1.5597927	0.016	1.6

Table 4. The hyperparameters of the best-fit least squares regression for *P. aeruginosa* (%) (with Curcumin and concentration 0.5xMIC as the baseline indicators).

Term	Estimate	SE	P	VIF
Intercept	72.839545	1.0567	<.0001	.
Trans-cinnamaldehyde	6.9308238	0.987433	<.0001	1.4
Limonene	2.2118911	1.108662	0.058	1.6
Eugenol	2.0440967	0.992923	0.051	1.4
Concentration 0.125xMIC	6.3728334	1.463585	0.0002	1.3
Concentration 0.25xMIC	5.3333503	1.444107	0.0012	1.3

Table 5. The hyperparameters of the best-fit least squares regression for *E. faecalis* (%) (with Curcumin and concentration 0.5xMIC as the baseline indicators).

Term	Estimate	SE	P	VIF
Intercept	72.820726	1.172417	<.0001	.
Trans-cinnamaldehyde	1.2134686	1.173332	0.311	1.6
Limonene	7.7228431	1.144619	<.0001	1.6
Eugenol	-0.120014	1.144619	0.9174	1.6
Concentration 0.125xMIC	5.5695572	1.610092	0.0021	1.3
Concentration 0.25xMIC	6.5824108	1.598382	0.0004	1.3



Compound 1: Trans-cinnamaldeyde, Compound 2: Limonene, Compound 3: Eugenol, Concentration 3: 0.5xMIC

Figure 3. The joint optimization of the six best-fit models for the maximum inactivation responses (%) of **(a)** *E. coli*, **(b)** *S. aureus*, **(c)** *P. aeruginosa*, **(d)** *E. faecalis*, and **(e)** *C. albicans* as a function of nominal compound predictor with four levels and ordinal concentration predictor with three levels. The red-to-white gradient depicts the most-to-least relative importance of the individual predictors based on Monte Carlo simulations, with the optimal values marked in red and with 95%-confidence intervals in blue.

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