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N-Acyl Homoserine Lactone-Mediated Quorum Sensing System Inhibition of Phenolic Strawberry Extract

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ABSTRACT

Phenolic compounds which have many bioactivities such as antioxidant, antimicrobial and also inhibition of quorum sensing-regulated violacein pigment production properties are one of the plant secondary metabolites. The objective of this study was to determine the antioxidant, antimicrobial activity and the inhibition of quorum sensing (QSI) activity by the phenolic extract obtained from strawberry fruits (*Fragaria×ananassa* cv. 'Rubygem'). The phenolic content of extract was determined as 3089 µg GAE g fw^{-1}. The antioxidant activity determined through the $ABTS^{+}$ and FRAP method was 28.4 and 26.2 mmol TE L^{-1} respectively. The phenolic extract was able to inhibit all the evaluated bacteria by the disc diffusion assay in the range of 3.12–18.36 mm and could inhibit the quorum sensing phenomena in bacteria. The strawberry phenolic extract exhibited high antioxidant capacity, antimicrobial and anti-quorum sensing properties. To our knowledge, this is the first report on the quorum sensing inhibition activity of strawberry fruit extract. The discovery of non-toxic novel compounds within antibacterial and QSI activity could lead to the development of antimicrobial therapeutic agents that can be alternative and complementary treatments against antibiotic resistant bacterial pathogens.

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Introduction

Bacteria have gained resistant to conventional antibiotics day by day which leads to deliver promising 'discovery of new drug' (Andrade et al., 2014; Borges et al., 2013; Andersson and Hughes, 2010; Projan and Youngman, 2002). The use of compounds which inhibit bacterial communication in biofilms provides a direct approach to this problem (O'Loughlin et al., 2013). The bacterial communication is regulated by a cell density-dependent chemical process called as "quorum sensing" (QS). In QS process, bacteria (gram-negative) release signal molecules by recognition of receptor proteins that control the expression of a particular set of genes and regulate varied properties which increase to a certain threshold concentration in the environment (Singh et al., 2012). QS targeting could be a promising strategy to control pathogenic bacteria while this mechanism is responsible for bacterial virulence induction (Jorge et al., 2012). The prevention of bacterial infection based on inhibition of bacterial communication system by natural products has emerged in recent years. Therefore, the current research for new antimicrobials is aimed to determine natural and non-toxic QS inhibitors that can be applied for bacterial infection treatments in humans (Hentzer and Givskov, 2003). Several plants and their fruits which have bioactive compounds could be developed as functional nutrition due to their ability to inhibit bacterial communication and prevent infection. The inhibition of QS mechanisms by natural products have been not identified completely yet but recent researches have been clarified some strategies included that the synthesis inhibition and/or the enzymatic degradation of auto inducer signal molecules, prevention of signal molecules secretion and receptor antagonism (McCarthy and O'Gara, 2015).

Phenolic compounds are secondary metabolites affect against phytopathogens in fruits and vegetables and are also capable of inhibiting the production of signal molecules and growth of microorganisms according to their concentration (Tiveron et al., 2012). The dietary phytochemicals have great attention for managing QS and infectious diseases recently (Martins et al., 2015). These phytochemicals are rarely associated with any side-effects as seen in many antibiotic regimens and generally recognized as safe (GRAS), because they are derived from dietary natural sources (Cowan, 1999). From recent several studies it was recognized that these phytochemicals could be used to a new antimicrobial therapeutics that overcome antibiotic resistance (Pinho et al., 2014). The fruits from the family *Rosaceae* have a great capacity of secondary metabolites such as

flavonoids, phenolics, alkaloids, quinones, polyacetylenes and terpenoids (Puupponen-Pimia et al., 2005). Although extensive works have been done on the antimicrobial properties of phenolic compounds of strawberry fruits, it is interesting that the literature about the dietary polyphenolics of strawberry as potential of QSI is very limited (Martins et al., 2015). 'Rubygem' is an early spring cultivar grown in most strawberry-growing areas (Herrington et al., 2007). The fruit is rich in anthocyanins content that presents its biological and therapeutic activity (Santoni et al., 2015; Campbell et al., 2015). Results obtained from strawberry extract provided high phenolic content, antioxidant, antimicrobial and QSI activities. Thus, the present study was performed to evaluate some bioactivities of phenolic compounds such as their antioxidant, antibacterial and QSI properties of strawberry cultivar 'Rubygem' (Figure 1).

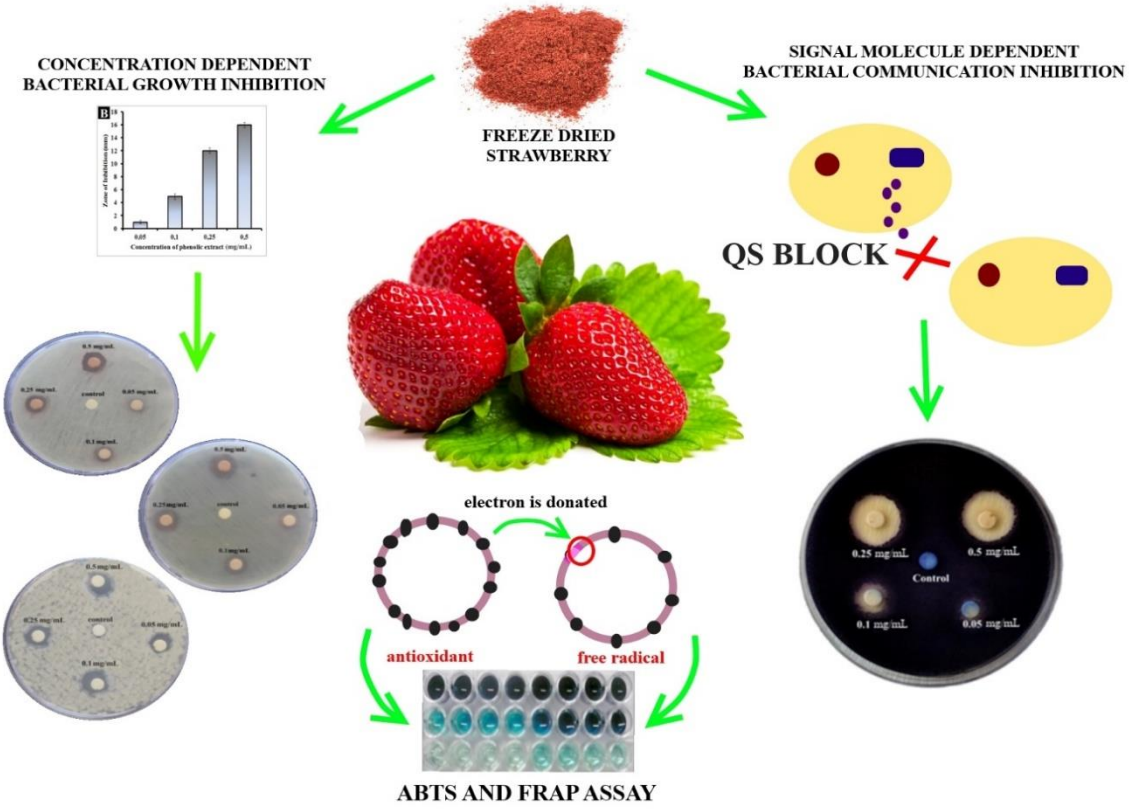

Figure 1 Schematic illustration of the objective of the study: determination of total phenolic content, antioxidant capacity, antimicrobial and quorum sensing inhibition properties of strawberry fruits (*Fragaria×ananassa* cv. 'Rubygem') phenolic extract.

Materials and Methods

Phenolic Extract Preparation

Strawberry fruits cv. 'Rubygem' were harvested from Silifke district of Mersin province, Turkey, frozen immediately and stored at -20°C until analyse. Samples were defrosted at room temperature and homogenized in a blender (WARING, 8010EG). The slurry was homogenized and dried into powder by using lyophilization. The seeds in the dry samples were removed. The total phenolic content of the extracts was determined by the Folin–Ciocalteu assay (Singleton and Rossi, 1965) and expressed as µg of gallic acid equivalent per g fresh weight basis (µg GAE fw^{-1}). The phenolic extract term used in this study has no relation to the solvent system used to obtain the extract.

Total Phenolic Content Determination

The Folin-Ciocalteu assay was used for the determination of the total phenolic content of the extracts. Briefly; Folin–Ciocalteu reagent (1 mL) and obtained extract solution (1 mL) were mixed thoroughly (Singleton and Rossi, 1965). After 8 min., 7% Na_2CO_3 (10 mL) was added and then the mixture was kept at room temperature. The absorbance at 750 nm was measured by UV/Vis spectrophotometer (PG Instruments, T60U UV-Visible, UK). The total phenolic concentration was calculated as µg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The total phenolic compound assay was carried out in triplicate.

ABTS Radical Scavenging Assay

For the ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) assay, firstly ABTS was dissolved in buffer of acetate and mixed with potassium persulfate according to method with some modifications (Özgen et al., 2006). Then, the obtained solution was diluted in sodium acetate buffer (20 mM, pH 4.5). Finally, the $ABTS^{\cdot+}$ solution (2.97 mL) and fruit extract (30 µL) were mixed and kept for 10 min and the absorbance was determined at 734 nm.

FRAP Assay

The FRAP assay was carried out by three aqueous stock solutions containing 0.1 mol/L acetate buffer (pH 3.6), 10 mmol L^{-1} TPTZ [2,4,6-tris(2-pyridyl)-1,3,5-triazine] acidified with hydrochloric acid and ferric chloride (20 mmol L^{-1}) (Benzie and Strain, 1996). These solutions were prepared (10:1:1, *v/v/v*) as the FRAP reagent to analysis. The FRAP reagent (2.97 mL) and phenolic extract (30 µL) were mixed. After the incubation (10 min.), the reaction mixture's absorbance was determined at 593 nm using UV/Vis spectrophotometer. Each assay was done in triplicate.

Antimicrobial Activity

The antimicrobial activity of the strawberry phenolic extract within different concentrations was determined by disc diffusion method. The tests were performed against human pathogens gram negative bacteria *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6380, *Salmonella* spp. (Laboratory stock) and the gram positives *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 25175 and *Bacillus subtilis* ATCC 21332. All the strains were sub-cultured before use. For the disc diffusion assay, the procedure described by the CLSI disc-diffusion method was followed (Yağız et al., 2017). To adjust the turbidity of microorganisms, 0.5 McFarland standard was used as a reference. The fruit extract powder was dissolved in ethanol:water (5:95, v/v) solvent. Microorganism suspensions (100 µL) were inoculated (1×10^8 CFU mL^{-1}) onto Müller-Hinton medium. Filter discs (diameter:6 mm) were placed on inoculated petri plate and 20 µL of fruit extract solution was added onto each filter disc. Plates were incubated at 37°C for 24 h. Only ethanol:water (5:95,*v/v*) solvent containing discs were used as a negative control. After the incubation, the inhibition zone's diameter was calculated. All tests were carried out triplicate and mean values were calculated.

Quorum Sensing Inhibition Activity

The *Chromobacterium violaceum (C. violaceum)* ATCC 12472 biomonitor strain was employed to evaluate QSI properties of strawberry phenolic extract. The *C. violaceum* suspension was recovered by overnight incubation (30°C) in Luria-Bertani (LB) broth.

Quorum Sensing Inhibition in C. violaceum

Initially, the suspension of *C. violaceum* ATCC 12472 was standardized to an $OD_{620\ nm}$ of 1 and was seeded on the LB agar plates. Sterile discs (diameter: 6 mm) impregnated with the phenolic extract solution (20 µL) in different concentrations (0.5, 0.25, 0.1, 0.05 mg mL^{-1}) were placed over the plates and incubated (30°C, 24 h). Discs with distilled sterilized ethanol:water (5:95, *v/v*) were used as negative control. The quorum sensing inhibitory activity in *C. violaceum* was determined by a turbid halo formation that presented no pigment production by bacterial growth around the well on a purple background on the plate. The QSI, measured by the inhibition of violacein pigment, was calculated by subtracting the diameter of bacterial growth inhibition (d_1) from the total diameter (d_2) (QSI = d_2–d_1). Diameter 1 (d_1): the inhibition of bacterial growth in mm, total diameter 2 (d_2): both bacterial growth and pigment inhibition in mm (Ilk et al., 2017). The assay was performed in three independent experiments.

Quantitative Evaluation of Violacein Inhibition

Quantitative evaluation of QSI activity of the strawberry phenolic extracts was determined to inhibit the purple pigment 'violacein' production by *C. violaceum* ATCC 12472. The inhibition of violacein production by *C. violaceum* ATCC 12472, when exposed to strawberry phenolic extract, was quantified according to described method with slight modifications (Ilk et al., 2017). The *C. violaceum* ATCC12472 suspension was adjusted to an OD_{620} nm of 0.1 and different concentrations of phenolic extract (0.5, 0.25, 0.1, 0.05 mg mL^{-1}) were added to the bacterial suspension. The ethanol (5%, v/v) was used as a control. After the incubation (24 h, 30°C, 150 rpm), the violacein extraction was carried out. Briefly, to precipitate the insoluble violacein and bacterial cells, each culture of samples was centrifuged (11000 rpm, 10 min). Then, the pellet was solubilized in ethanol/water (1 mL), vortexed for 1 min to solubilize the violacein and centrifuged again (10000 rpm, 10 min). Finally, the absorbance at 585 nm of the obtained supernatant within the violacein pigment was measured by UV/Vis spectrophotometer. These experiments were performed in triplicate. The results were presented as percentage of violacein inhibition (VI%) and calculated by using the following formula:

$$VI\% = ((Abs_{control} - Abs_{test})/Abs_{control}) \times 100$$

Results and Discussion

Phenolic content and antioxidant activity

Strawberry phenolic extract was exhibited at high amount of total phenolics and antioxidant capacity for both ABTS and FRAP methods tested (Table 1). Total phenolic content was average 3089 ± 112 µg GAE g fw^{-1}. Antioxidant capacities were 28.4 ± 0.3 and 26.2 ± 0.5 mmol TE L^{-1} for both ABTS and FRAP methods, respectively. Results obtained from this study were compatible with previous strawberry studies (Özgen et al., 2007) conducted by different commercial strawberry cultivars. The average values of phenolic content and antioxidant capacity of mature strawberry fruits obtained from four different cultivars were 2639 µg GAE g fw^{-1} and 22.0 mmol TE L^{-1}, respectively.

Table 1 Total phenolic content and antioxidant activity of strawberry fruits (Fragaria × ananassa cv. 'Rubygem') phenolic extracts

Total Phenolic Content	Antioxidant Capacity	
Folin-Ciocalteu Assay (µg GAE g fw^{-1})	ABTS Assay (mmol TE L^{-1})	FRAP Assay (mmol TE L^{-1})
3089 ± 112	28.4 ± 0.3	26.2 ± 0.5

Antimicrobial Activity

The fruit phenolic extracts and compounds have gained attention and have been investigated especially in *in vitro* systems in recent decades. The antibacterial activity of strawberry phenolic extracts was studied on six

strains of gram-positive and negative bacteria by using the disc diffusion method, because it is cheap, simple and has practical assay (Bachir and Benali, 2012). The results for the disc diffusion assay with the strawberry phenolic extracts at different concentrations against human pathogens gram negative bacteria *E. coli* ATCC 25922, *P. vulgaris* ATCC 6380, *Salmonella* spp. and the gram positive bacteria *S. aureus* ATCC 25923, *S. mutans* ATCC 25175 and *B. subtilis* ATCC 21332, are shown in Figure 2 and 3. As presented in Figure 2, the strawberry phenolic extract at least at three concentrations inhibited all the tested gram negative bacteria. The most sensitive bacterium in gram negative bacteria was *Salmonella* spp. that was inhibited in all evaluated concentrations and demonstrated as the highest zone of inhibition (16.56 ± 0.42 mm) (Figure 2 E-F). The antimicrobial activity of phenolic extracts against gram positive bacteria is shown in Figure 3. The phenolic strawberry extract exhibited antibacterial activity all the evaluated gram positive bacteria, at least at three concentrations. The most sensitive bacterium in gram positive bacteria was *S. mutans* ATCC 25175 which was inhibited in all evaluated concentrations of extract and showed the highest zone of inhibition (17.12 ± 0.45 mm). The results were presented in Figure 2 and 3 and it was possible to conclude that strawberry phenolic extract was more effective inhibitor against the gram positive microorganisms, while the inhibition zones' diameters were greater than the gram negatives. In addition, in all tested bacteria, it was observed that with increasing concentration of phenolic extract increased the zone of inhibition. Once again, no studies were found for the antimicrobial activity of strawberry fruit extracts. The studied phenolic extract can act as potential improving agents of antibiotics against gram positive and negative bacteria, considering the synergic effect obtained between these phenolic extract and conventional antibiotics.

Quorum sensing inhibition in C. violaceum (Plate diffusion assay)

In this study, the anti-QS activity of strawberry phenolic extracts was evaluated using the biomonitor strain *C. violaceum* ATCC12472. This biomonitor strain is a gram negative bacterium and produces the 'violacein' purple pigment for the bacterial communication system (QS) which is regulated by C6-AHL (Tan et al., 2012). Therefore, this may be excellent strain for screening of the QSI properties of compounds (Adonizio et al., 2006). When the results of the disc diffusion assay concerned to screen of the QSI activity of phenolic compounds (Figure 4), it was verified that strawberry phenolic extracts inhibited the violacein production by *C. violaceum* ATCC 12472. The inhibition of violacein production was seen by the turbid halo formation on a clear background around the disc. The strawberry phenolic extract inhibited the violacein production (Figure 4A) that demonstrates bacterial growth can be observed but no color production by the turbidity of the halo formation. The obtained results from the assay of disc diffusion were more promising than the results of other studies (Khan et al., 2009; Singh et al., 2009 and Del Monte et al., 2015). The QSI properties of the strawberry phenolic extracts at different concentrations were also evaluated (Figure 4B).

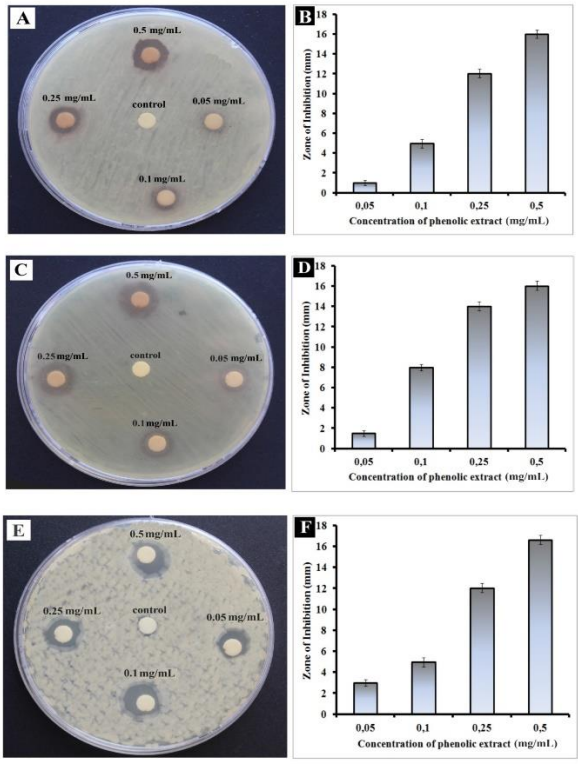

Figure 2 Growth inhibition against Gram negative bacteria *E. coli* (A and B), *P. vulgaris* (C and D), *Salmonella spp*. (E and F) by different concentrations of strawberry (*Fragaria×ananassa* cv. 'Rubygem') phenolic extract, measured as inhibition zone around the discs (mm), by the plate diffusion assay

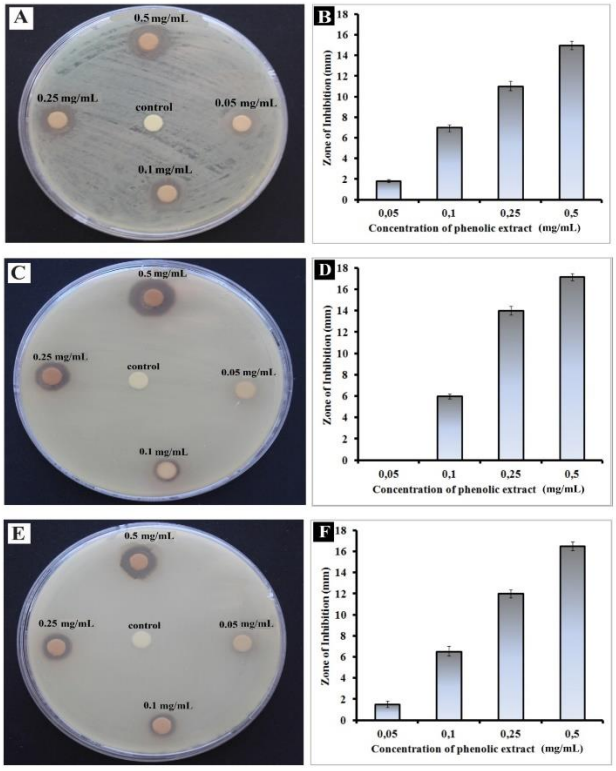

Figure 3 Growth inhibition against Gram positive bacteria *S. aureus* (A and B), *S. mutans* (C and D), *B. subtilis* (E and F) by different concentrations of strawberry (*Fragaria×ananassa* cv. 'Rubygem') phenolic extract measured as inhibition zone around the discs (mm), by the plate diffusion assay

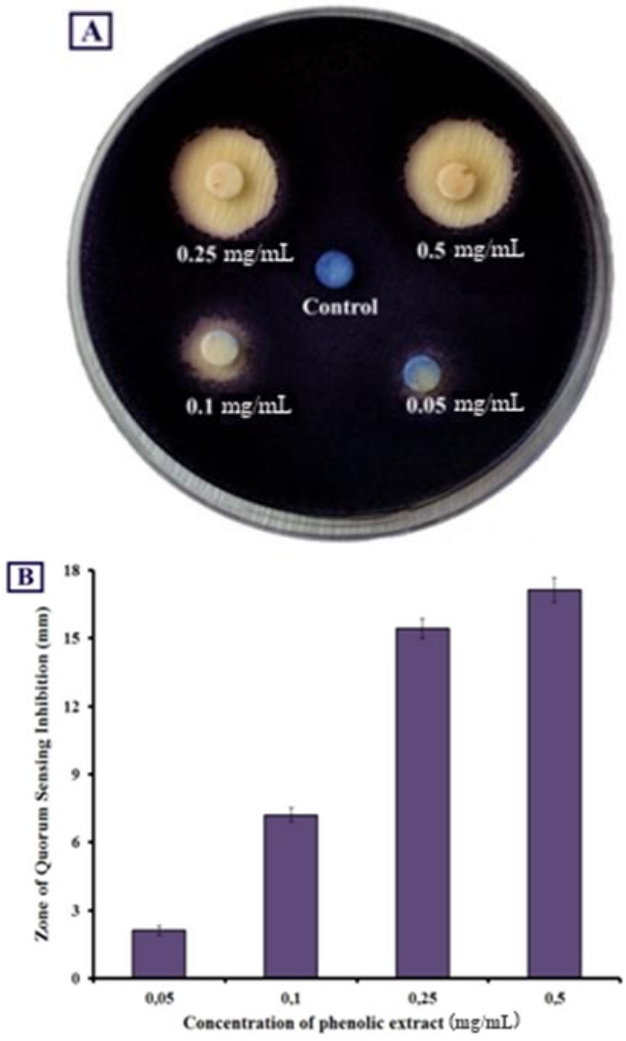

Figure 4 Plate diffusion assay for the quorum sensing inhibition in *C. violaceum* by different concentrations of strawberry (*Fragaria×ananassa* cv. 'Rubygem') phenolic extract (A) Dual plate assay (B) Quorum sensing inhibition zone (mm)

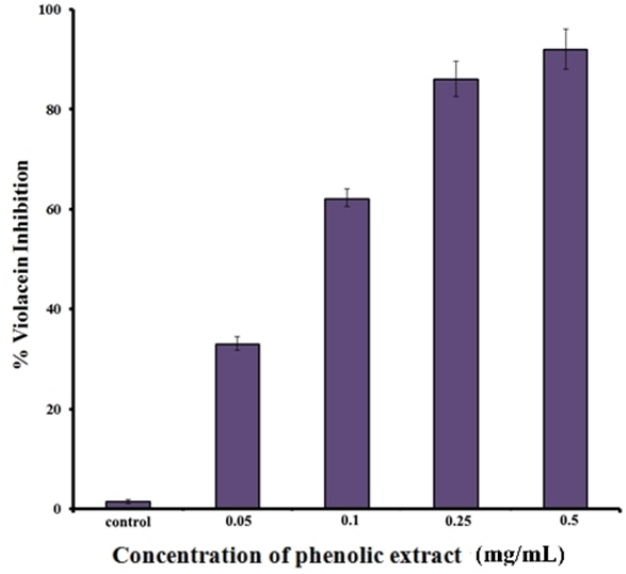

Figure 5 Percent inhibition of violacein production by the strawberry (*Fragaria×ananassa* cv. 'Rubygem') phenolic extract at different concentrations. The control consisted of LB medium added of 200 µL of ethanol:water (5:95)

Violacein production was inhibited at all the sample concentrations tested. There was not any inhibition on violacein production in the control. These results were confirmed with QSI capacity of the strawberry phenolic extract which was able to inhibit the violacein production in a concentration dependent manner. The inhibition of QS by other natural extracts was reported with the same findings in other studies (Ilk et al., 2017; Singh et al., 2009; Abraham et al., 2012). Overall, strawberry phenolic extract can inhibit the QS-regulated violacein pigment production in bacteria without interrupting on their growth and this finding was reported first time with this study.

Quantification of violacein inhibition of phenolic extract

The strawberry phenolic extract was highly effective for the inhibition of the violacein production at all evaluated concentrations (Figure 5). The absorbance of viable cells evaluated after 24 h of incubation was indicated that there was inhibition at presence of phenolic extract against violacein production (Figure 5). The violacein inhibition by the phenolic extracts, especially at 0.5 mg mL^{-1} was 92.6 ± 4.2%. No studies have been found on the effect of strawberry extract on the production of violacein. In general, the studies for finding new anti-pathogenic and QSI therapeutic compounds have been performed with plants, however studies showing the QSI effect of natural phenolic extracts are scarce (Adonizio et al., 2006; Abraham et al., 2011; Kalia, 2013).

Conclusions

In this study, we reported the bioactive potential (antioxidant, antimicrobial and anti-quorum sensing activities) of strawberry fruits cv. 'Rubygem' with the presence of phenolic compounds. The significant inhibition of the quorum-sensing regulated pigment production against the bacteria *C. violaceum* CV026 was observed in the first time using only the phenolic extract of strawberry without the addition of any toxic and synthetic chemical agents. Moreover, the bacterial growth was significantly interfered by the phenolic content of strawberry. Although the exact mechanism for observing strong anti-QS effect of the phenolic compounds is still to be unraveled, it is believed to be related to the flavonoids contents of the phenolics such as kaempferol and quercetin in the antioxidant rich strawberry. Since the autoinducer for the pigment production of *C. violaceum* CV026 also regulates many other important physiological processes of bacteria, it is expected that our natural strawberry freeze dried powder can be potentially used to inhibit those physiological processes including biofilm formation. Strawberry may be a good source for new bioactive agents with QSI activity which could be further discovered as a means to produce new additives and anti-virulence drugs for the several industrial applications.

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