Research article

Chitosan nanoencapsulation of flavonoids enhances their quorum sensing and biofilm formation inhibitory activities against an E.coli Top 10 biosensor

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Abstract

Phytochemicals have been found to be promising alternatives to conventional antibiotic therapies for the control of bacterial infections, as they may entail less selective pressure and hence reduce the development of resistance. This study involved examining the inhibition of biofilm formation and of quorum sensing (QS), and the cytotoxicity on mammalian cells of two flavonoids, quercetin and baicalein, in free form and associated into chitosan-based nanocapsules. This was done by use of a transformed E. coli Top 10 biosensor strain, while the cytotoxicity was evaluated on MDCK-C7 cells. In free form, application both phytochemicals exhibited slight inhibitory activity on the QS response and biofilm formation, a scenario that was improved positively upon encapsulation with chitosan (Mw ~115 kg/mol and DA ~42%). The association efficiency of 99% (quercetin) and 87% (baicalein) was obtained, and both had an average diameter of 190 ± 4nm & 187 ± 2nm and zeta potential (ζ) of +48.1 ± 2.03mV & + 48.4 ± 3.46mV respectively. Both types of systems were stable against aggregation in M9 and MEM media. The in vitro release kinetics data of both baicalein and quercetin seemed to be similar with only ~20 % over the first 5 h, and only ~10% was released during the first 4h respectively with subsequent sudden release increase up to ~40 % afterwards. The free phytochemicals seemed to be cytotoxic to MDCK-C7 cells at higher doses, however, upon nanoencapsulation, a cytoprotective effect was evidenced. We have gained proof-of-principle of the advantages of encapsulation of two bioactive flavonoids.

Key words: Antiquorum sensing, quorum sensing, E. coli Top 10 biosensor, autoinducer, baicalein, quercetin.

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1. Introduction

Bacterial quorum sensing (QS) is an area that currently has drawn increasing interest especially in bacterial pathogenicity as its responsibility to their phenotypic traits. QS is a cell-to-cell communication process that enables bacteria to assume a collective synchronous behaviour under the influence of chemical signalling molecules called autoinducers (AIs) which mediates interspecies or intraspecies behaviours[1]. As the cell density increases, AIs accumulate in the environment and they are sensed by protein receptors, which in turn act as transcriptional activators for the expression of specific genes; the products of these genes direct activities that are beneficial when performed by bacterial communities acting in synchrony [1]. Homoserine lactones (HSLs) produced by Gram-negative bacteria are the best studied AIs and possibly the most common group of bacterial QS AIs while Gram-positive bacteria use small peptides called AI2 [2, 3]. Frequently, pairs of genes encoding the HSL synthase and the HSL-sensing transcriptional regulator are found close to each other in bacterial genomes [4]. This phenomenon was first discovered in the marine bacterium Vibrio fischeri [5] and the term quorum sensing was later coined by Fuqua et al., [6] referring to the acylated homoserine lactone (AHL)-mediated luxR/luxI regulated system.

Biofilm formation is one of the virulence traits associated to QS [7]. Bacteria in biofilms are not easily reached neither by antibiotic agents nor by the host immune system as they act as diffusion barriers hence they continue multiplying inside the extracellular matrix up to a point at which they disperse to start forming new biofilm communities [8]. Microbial biofilms readily form on indwelling medical devices and cause serious diseases that are hard to control since effective therapy is lacking. According with CDC and NIH, between 65%–80% of infections could be caused by biofilms, demonstrating the need to develop improved treatment options [9].

Therefore, targeting and blocking QS circuits in bacteria may represent a strategy to disarm their virulence and hence, making them more susceptible to elimination by the host immune system or low doses of antibiotics [10]. Anti-virulent agents possessing QS inhibitory activity might not pose selective pressure on bacterial pathogens and hence, may contribute to reduce the rapid emergence of so-called “superbugs”, bacteria resistant to several antibiotics [11, 12]. Disruption of QS-regulated processes has been accepted to reduce accumulation of virulence factors at the infection site, and dismantles the collective virulent power of pathogens. These strategies are collectively called quorum quenching (QQ) [13].

Plant phytochemicals may be possessing anti-quorum sensing activities based on the various studies. Among them include, but are not limited to, zingerone [12], curcumin [14], cinnamon oil [15], pure trans-cinnamaldehyde [16, 17], proanthocyanidins [18], and flavonoids like quercetin [19]. On the other hand it is now known that the activity of anti-virulent agents or antimicrobials may improve upon encapsulating them in biopolymer-based materials [20, 21]. This may be attributed to the overall increase in their bioavailability, lower dosage release for longer periods; to the overcoming physiological barriers resistances e.t.c as compared to free agents [20-22].

Different types of nanocarrier formulations have extensively been used for drug delivery [23, 24]. Despite this, minimal information is available for their use in delivering QS inhibitory

agents. However, biologically-derived materials such as polysaccharides and proteins can be used as components of nanoformulations as they are fully biodegradable, biocompatible and non-immunogenic. Hence, they are attractive for the development of innovative drug delivery vehicles [25]. Among the most interesting biopolymers in this area, is chitosan that has gained enormous traction. Chitosan are a family of amino linear heteropolysaccharides comprised by β (1-4) 2-acetamido-2-deoxy-β-D-glucopyranose (N-acetyl glucosamine) and 2-amino-2-deoxy-β-D-glucopyranose (D-glucosamine) units, randomly or distributed as blocks throughout the biopolymer [26]. Chitosan and its chemical derivatives are particularly attractive as a building blocks for drug delivery nanoformulations in light of its biocompatibility, biodegradability, bio– and mucoadhesivity, and hydrophilic character that facilitate the administration of poorly absorbable drugs across various epithelial barriers [27-31]. It can also be easily be manipulated chemically and hence forming a good vehicle for delivering both hydrophobic and hydrophilic drugs/agents to target sites [26].

Quercetin and baicalein (Supplementary data - S1) are both bioflavonoids that are lipophilic. They are the most common polyphenolic compounds in nature and are found ubiquitously in plants, including food products like onions, many fruits, or in herbs [32-35]. Previous studies have shown that quercetin and baicalein can act as competitive inhibitors for the signalling compound towards LasR/Rh1R and orphan regulator QscR receptors pathway commonly found with P. aeruginosa and can serve as novel QS-based antivirulence agents to manage such groups of pathogens [19, 36 - 38]. In these studies, it was deduced that both flavonoids have both antivirulence and antibiofilm formation activity against P. aeruginosa PA01. However, only few of the available studies have reported on the role of plant extracts and phytochemicals as QS inhibitors, deal with the potential mechanisms of action [39] and even their activity upon nanoencapsulation.

In this study, we report the AQS & antibiofilm activities of free and chitosan nanoencapsulated quercetin and baicalein against a bioengineered E. coli Top 10 biosensor that has a Lux R receptor pathway. Also their viability towards MDCK–C7 mammalian cell line was determined.

2. Materials and Methods

2.1: Materials

The parent chitosan (Mw 288 kDa and DA-16%) was provided by Mahtani Chitosan Pvt. Ltd., India (Sample code 132; batch no. SCCF 20140609). The chitosan used for this study was derived from this parent sample as described in previous studies [40, 41]; the Mw was 115 kg/mol as determined by HPSEC-MALLS and the DA of 42 % as measured by ^{1}H-NMR spectroscopy according with the method by Lavertu et al. [42]. Lecithin was a kind gift from Cargill (Epikuron 145 V, Cargill Deutschland GmbH & Co. KG, Hamburg, Germany); Miglyol 812 N was from Sasol GmbH (Witten, Germany). 3OC_6HSL and all other chemicals were of analytical grade and unless otherwise stated were from Sigma (Sigma-Aldrich, Hamburg, Germany).

2.2: Baicalein and quercetin nanocapsules formulation

CS-based nanocapsules of both baicalein (Mw- 270.24) and quercetin (Mw- 302.24) were prepared according with the protocol originally developed in our laboratory with some slight modifications [25, 43]. Briefly, due to the hydrophobic nature of the two phytochemicals 1mg of each was dissolved in 1mL of absolute ethanol. Then 256µL of the each of the phytochemical was taken, 250µL of lecithin (100mg/mL of lecithin in ethanol) and 62.5 µL of Miglyol 812® were added to a beaker with 4.75 mL of absolute ethanol. Immediately afterwards, this organic phase was poured into of 10 mL of CS solution (0.5 mg/mL) which turned milky. Ethanol and a portion of the volume of water were evaporated in a rotavapor at 40 °C for ~8-15 min to a final volume corresponding with one third of the original one. The blank NCs were also made according with the same protocol but no phytochemicals were added in the organic phase. All preparations were made in triplicates.

2.3: Physicochemical properties of the nanocapsules

The size distribution of the nanoformulations was determined by dynamic light scattering with non-invasive back scattering (DLS-NIBS) with a measurement angle of 173°. The zeta potential was measured by mixed laser Doppler velocimetry and phase analysis light scattering (M3–PALS). A Malvern Zetasizer NanoZS (Malvern Instruments Ltd., Worcestershire, UK) fitted with a red laser light ($\lambda = 632.8$ nm) was used for both methods. The samples were diluted 1:100 in acidified water before measurement.

2.4: Encapsulation/association efficiency

It was determined as per the method used by Kaiser et al. [25]. Briefly, aliquots of 500 µL of the flavonoid-loaded nanoformulations were pipetted into Vivaspin® 500 ultrafiltration spin columns and then were centrifuged by centrifuge (Mikro 220 R, Hettich GmbH & Co. KG, Tuttlingen, Germany) at 16,000 rpm for 1 h at 15 °C. Free baicalein and quercetin content of the subnatant were determined by UV spectroscopy at $\lambda = 324$ and 374 nm, respectively using the corresponding calibration curves (Supplementary data-S2). The association efficiency was calculated as the difference between the total amount of the flavonoid incorporated in the formulation and the amount present in the subnatant. The association of quercetin was also determined by resuspending the creamy layer with distilled water up to 1 mL and then 25 µL were taken and then mixed with 475 µL of absolute ethanol and the concentration was determined as described above, as a confirmation of the previous method results.

2.5: Stability test with M9 and MEM medium

The colloidal stability of both baicalein and quercetin NCs was investigated by using minimal essential medium Eagle (MEM M4655, Sigma-Aldrich) (pH 7.45) cell culture medium without supplements and M9 bacterial growth medium. Briefly 50 µLs of either baicalein or quercetin NCs were added to 950 µLs in a sterile cuvette of either MEM or M9 medium and incubated at right conditions (M9 at 37 °C for 48h and for MEM at 37 °C, 5% CO_2 environment & 24h). The stability was evaluated in terms of the evolution of the particle size distribution and size (nm) of the NCs over time during incubation at 37 °C for up to ~24 h for MEM or ~48 h for M9 medium by use of the Malvern Zetasizer NanoZS as described above.

2.6: Release assay

The release assay of the developed NCs was investigated in the M9 bacterial growth medium. Briefly an aliquot of 800 µL of either baicalein or quercetin NCs was transferred to a dialysis tube (Pure-a-lyzer Maxi 0.1–3.0 ml, Mw cut-off = 6 kDa, Sigma-Aldrich GmbH, Steinheim, Germany) and placed in a sterile glass beaker with 79.2 mL of M9 medium previously equilibrated at 37 °C in an incubator. This set-up ensured that "sink" conditions were achieved. Every hour an aliquot of 500 µl was drawn from the dialysate and the same amount of fresh M9 medium replenished. Baicalein or quercetin content of the aliquots was determined by UV spectrophotometry as described above. The release experiment lasted 6 h.

2.7: Bioassays

2.7.1: Bacterial strain

E. coli Top10 was transformed with the plasmid pSB1A3-BBa_T9002, carrying the BBa_T9002 genetic device (Registry of Standard Biological Parts: http://parts.igem.org/Part:BBa_T9002), kindly donated by Prof. Anderson Lab (UC Berkeley, USA). The sequence BBa_T9002 was introduced by chemical transformation (Invitrogen, Life Technologies Co., UK) and stored as a 30 % glycerol stock at –80 ºC. The transformed strain is a biosensor that can respond to the N-(3-oxohexanoyl)-L-homoserine lactone ($3OC_6HSL$) and is the same strain used in accompanying studies [44]. The sequence BBa_T9002, comprised the transcription factor (LuxR), under the control of the lux pR promoter from Vibrio fischeri, which is constitutively expressed with GFP production only in the presence of the exogenous cell-cell signaling molecule $3OC_6HSL$.

2.7.2: Growth media and conditions

Bacterial strains were cultivated using on Luria-Bertani (LB) and M9 minimal medium (Becton, Dickinson and company, Germany). We inoculated 10 mL of LB broth supplemented with 10µL (200 µg/mL) ampicillin (AppliChem GmbH, Germany) with a single colony from a freshly streaked plate of E. coli Top10 containing BBa_T9002 and incubated the culture for 18 h at 37ºC, shaking at 100 r.p.m. Each culture was then diluted 1:1000 into 20 mL M9 minimal medium supplemented with 0.2% casamino acids and 1 mM thiamine hydrochloride plus 20µL (200 µg/mL) ampicillin. The culture was maintained under the same conditions until the OD_{600} reached 0.15 (~5 h). Then 500 µL of the overnight culture were mixed with 500µl 30% sterile glycerol together in the white plastic vials and stored at -80˚C for future use. Before the biosensor assay, the bacteria cultivation was prepared by cultivating 40µL of thawed bacteria from the white plastic vial kept at -80˚ C unto 20 mL M9 medium plus 20µl of ampicillin (200 g/mL). This is incubated at 37 ˚C, 100 r.p.m until the OD_{600} reached 0.04~ 0.07 (~4 h).

2.7.3: E. coli Top10 biosensor QS assay

The N-(β-ketocaproyl)-L-homoserine lactone (Sigma- Aldrich) ($3OC_6HSL$) was dissolved in acetonitrile to a stock concentration of 100 mM and stored at –20ºC. Prior to the biosensor assay, a 5 µL aliquot of this stock solution was diluted with sterile milli-Q water to a final concentration of 10 nM. Then 10 µL of 10 nM $3OC_6HSL$ aqueous solution was placed with 10

µL of the treatment samples in the wells of a flat-bottomed 96-well plate (Greiner Bio-One, cat. # M3061) and each well was then filled with 180 µL aliquots of the bacterial culture of OD_{600} 0.04~ 0.07 to test for QS inhibition activity. Several controls were also set up. Blank 1 contained 180 µL of M9 medium and 20 µL of milli-Q water to measure the absorbance background. Blank 2 wells contained 180 µL of bacterial culture and 20 µL of milli-Q water to measure the absorbance background corrected for the cells. Finally, positive control wells contained 10 µL of water plus 10 µL $3OC_6HSL$ solution and 180 µL of the bacterial culture to measure the fluorescence background. In order to remove the effect of samples themselves with OD_{600} and fluorescence, 10 µL $3OC_6HSL$ solution was added with 10 µL of the treatment samples in the wells and each well was then filled with 180 µL M9 medium to test the samples control in biosensor assay.

The plates were incubated in a Safire Tecan-F129013 Microplate Reader (Tecan, Crailsheim, Germany) at 37 ºC and fluorescence measurements were taken automatically using a repeating procedure (λ_{ex}=480 nm and λ_{em}=510nm, 40 µs, 10 flashes, gain 100, top fluorescence), absorbance measurements (OD_{600}) (λ=600 nm absorbance filter, 10 flashes) and shaking (5 s, orbital shaking, high speed). The interval between measurements was 6 min. For each experiment, the fluorescence intensity (FI) and OD_{600} data were corrected by subtracting the values of absorbance and fluorescence backgrounds and expressed as the average for each treatment. All measurements were taken in triplicate.2.8: Antibiofilm assay

Microtiter plate assay as used before [45] with some modifications was performed to quantify the effect of both free and nanoencapsulated baicalein and quercetin on the biofilm formation of E. coli Top 10 biosensor strain. The test bacteria stored at -80˚C in vials was first thawed and inoculated on LB agar and incubated at right conditions at 37˚C overnight. Then a colony was identified and was picked and inoculated unto 10mLs of LB broth and was incubated 100 r.p.m, 37˚C overnight. 190 µls of M9 broth with and without the free or nanoencapsulated baicalein or quercetin (various concentrations) were then inoculated with 10µls of bacterial cultures and incubated at 37˚C for 48 h without shaking. Unloaded kanamycin (1mM) was also added as a positive control and M9 broth as negative control. The polystyrene tissue culture microplate was sealed with a para film to prevent medium evaporation prior to incubation. After incubation, plates were carefully rinsed 2-3 times with double-distilled water to remove loosely attached cells. The microplate was air dried for one hour before adding 200 µl per well of 0.4% crystal violet solution to the adhered cells in the wells and it was left at room temperature for 15min. Excess stain was then removed by rinsing the wells with 200 µl per well of distilled water three times. The microtiter plate was then air-dried again for 1 h after which 200 µl per well of absolute ethanol was added to solubilize the dye. Intensity was measured at OD_{590}nm by using a Safire Tecan-F129013 Microplate Reader (Tecan, Crailsheim, Germany). For each experiment background staining was corrected by subtracting the crystal violet bound to un-inoculated controls from those of the samples. The experiments were done in triplicates and average values were calculated. To estimate the antibiofilm activity (%) of a given treatment the following equation was used.

Antibiofilm activity (%) = $[1- (OD_{\text{Test sample}}-OD_{\text{Blank}})/ (OD_{\text{Untreated control}} - OD_{\text{Blank}})] \times 100$

2.9: Cell viability assay

2.9.1: Cell culture

Mandin Darby Canine Kidney (MDCK) cells clone 7 (C7) were cultured in 75 cm^2 flasks using MEM supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM) and 1% penicillin-streptomycin (10000 units penicillin, 10000 units streptomycin in 0.9% NaCl). The cell cultures were maintained in a humid atmosphere at 37 °C with 5% CO_2 (Sanyo MCO-19AIC, Panasonic Biomedical Sales Europe BV, AZ Etten Leur, Netherlands). Cells from passages 17 - 25 were used for all experiments, which were carried out as independent triplicates on different days. After reaching microscopic confluence, the cells were washed with 10 ml phosphate buffered saline (PBS) and trypsinized with 10 ml 0.05% trypsin in EDTA (1x) buffer. After detachment, 10 ml of MEM was added to the trypsin buffer. The cell suspension was centrifuged at 1000 r.p.m for 5 min (Rotina 420 R, Hettich GmbH, and Tuttlingen, Germany). The excess of medium was removed by sucking and the cell pellet was resuspended in 1 ml MEM. A 10-µl aliquot of the cell suspension was diluted with 90 µl trypan blue and the number of cells was counted with an improved Neubauer chamber before seeding. The cells were subcultured by splitting at a ratio of 1:10.

2.9.2: Mammalian cell (MDCK-C7) line cytotoxicity tests: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The cytotoxicity of both free and nanocapsulated baicalein and quercetin were evaluated using an MTT assay protocol as used before [46- 48]. Briefly, 100 µl of cell suspension was transferred to each well of a 96-well polystyrene tissue culture plate (~10^4 cells per well) and was incubated at 37 °C with 5% CO_2 (Sanyo MCO-19AIC, Panasonic Biomedical Sales Europe BV, AZ Etten Leur, Netherlands) for 24h to allow attachment of the cells. Then the attached cells were washed twice with supplement-free MEM before the various concentrations of either free or nanoencapsulated sample were added unto the wells and the cells were incubated for 24h at same conditions as above. The samples were then removed and replaced with 100 µl supplement-free MEM and 25 µl of MTT reagent (5 mg/ml of thiazolyl blue tetrazolium bromide) per well. After 4 h incubation at 37 °C with 5% CO_2, the medium and MTT reagent were removed and the dye was dissolved in DMSO (100µls per well). After orbital shaking at 300 r.p.m for 15 min, the absorbance was measured at $\lambda = 570$ nm in a Safire Tecan-F129013 Microplate Reader (Tecan, Crailsheim, Germany). We used 4% Triton X-100 in PBS as a positive control. The following formula was used to calculate the percentage relative viability of the treatments against the MDCK-C7 cell line.

$$\text{Pa\% Cell Survival} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

3. Results

3.1: Physicochemical properties of baicalein and quercetin nanocapsules

The MDP chitosan (Mw- ~115kDa and DA- ~42%) was used in nanocapsules formation. Table 1 presents the various physicochemical parameters and encapsulation efficiency of both baicalein and quercetin. The Z-average diameter of each formulation ranged within a narrow

range from ~185 to ~194 nm, with a low polydispersity index (PDI) fluctuating from ~ 0.12 to ~ 0.15. The nanocapsules were highly positively charged with ζ–potential values ranging between ~ + 47.6 and + 48.4 mV and a very high capacity of association with baicalein (87%) and quercetin (99%). For the resuspended quercetin NCs pellet, association efficiency of 99.9% was deduced.

3.2: Stability test for baicalein and quercetin nanocapsules

The colloidal stability of the both flavonoids-loaded and of unloaded (blank) nanocapsules was determined and figure 1 shows in both mediums (M9 and MEM) that the average size and PDI remained constant for all the systems, in agreement with previous studies on chitosan nanocapsules [49, 50].

3.3: Release assays of baicalein and quercetin nanocapsules

The release of both flavonoids-loaded nanocapsule formulations was examined in M9 bacterial growth medium as illustrated in Figure S4 (Supplementary Information) and the calibration curves (Supplementary Information S3). Both flavonoids-loaded NCs had a slow but steady release of the quercetin up to 5 h after which the release rate increased tremendously. But generally, baicalein-loaded nanocapsules had a more pronounced release as compared to quercetin.

3.4: Antiquorum sensing activity of chitosan nanoencapsulated and free baicalein and quercetin

We investigated the influence of applying different concentrations of both free and chitosan-nanoencapsulated baicalein and quercetin on the relative growth and the relative quorum sensing of the transformed E. coli Top 10 biosensor strain that expresses GFP production only under the influence external $3OC_6HSL$ [51-53]. A close inspection of Figure 2 reveals that free baicalein (Figure 2i-a) did not have any substantive inhibitory effects on the QS response of the bacteria. At high doses (> 2.53×10^{-3} mM) it seemed to be toxic as the bacteria OD was reduced. However, upon encapsulating baicalein and applied at similar doses as for the free form, the toxicity at high doses was abrogated. Baicalein encapsulation also seemed to enhance QS response especially at 2.53×10^{-4} mM and 6.25×10^{-5} mM concentrations.

Figure 3 (i) illustrates individual plots for fluorescence (a), OD_{600} (b), and FL/OD_{600} (c) for representative treatments of baicalein-loaded nanocapsules at the two selected flavonoid concentrations where inhibition of QS was observed (Figure 2i-b), namely 2.53×10^{-4} and 6.25×10^{-5} mM. Notice that the data confirm that there was a selective inhibition of the expression of fluorescence and that this effect was not at the expense of a reduction in cell viability, apart from a very slight reduction in OD_{600} at 2.53×10^{-4} mM. Comparable results as those obtained for baicalein were observed upon treatment of the E. coli Top 10 biosensor with free quercetin (Figure 2 ii). It's clear that at doses greater than 6.25×10^{-3} mM, quercetin exerted significant toxicity ($p<0.05$) in a dose dependent manner (Figure 2 ii-a). Also, at concentrations < 3.25×10^{-3} mM there seems to occur a consistent reduction in the relative QS response, though this effect does not reach statistical significance.

With quercetin-loaded nanocapsules treatments this scenario changed (Figure 2 ii-b). They exhibited a protective effect on the toxicity of quercetin on E. coli Top 10 bacteria even at the highest applied doses (8.4×10^{-3} mM). About the QS inhibition activity, notice that there was a "Goldilocks"-type of effect, and the minimum values were attained at intermediate concentrations ~3.91×10^{-4} mM with less pronounced effects either at greater or lower concentrations. Figure 3 (ii) depicts individual plots for fluorescence (a), OD_{600} (b), and FL/OD_{600} (c) for representative treatments of quercetin-loaded nanocapsules at three flavonoid concentrations around that where the maximum inhibition of QS was observed (Figure 2 ii-b). Notice that in this case, the results confirm that there was a selective inhibition of the expression of fluorescence and that this effect was not at the expense of a reduction in cell viability, apart from a very slight reduction in OD_{600} at 2.53×10^{-4} mM. It is evident that the unloaded nanocapsules had no effect neither on the fluorescence nor on OD_{600} of E. coli Top 10 biosensor. The null effect of unloaded nanocapsules on both the growth and the QS response was confirmed at various concentrations in the range 6.25×10^{-6} to 8.80×10^{-3} mM (See supplementary data-S5).

3.5: E. coli Top 10 biosensor antibiofilm formation

We examined the effect of free solution and flavonoid-loaded nanocapsules on the biofilm formation activity of the E. coli Top 10 QS biosensor strain (Figure 4). Notice that for both type of systems, biofilm formation was inhibited mostly at low concentrations upon treatment with both nanoencapsulated phytochemicals. Indeed, below 6.25×10^{-4} and 3.91×10^{-4} mM concentrations of baicalein- and quercetin-loaded nanocapsules, respectively, no significant differences were observed with respect to the positive control (kanamycin-1mM). By contrast, free solutions of both flavonoids showed invariably significantly lower inhibition of biofilm formation than kanamycin and the encapsulated phytochemicals at low concentrations. Supplementary data-S6 shows the image of representative appearance of microtiter plates during the biofilm formation assay by E. coli Top 10 biosensor upon treatment with varying concentrations of: (a) Baicalein- and (b) Quercetin-loaded nanocapsules after treatment with 0.4% CV and before dissolving the stain with absolute ethanol. Unloaded NCs seemed to also have a slight effect on biofilm formation inhibition especially at low dosages (supplementary data-S7).

3.6: Mammalian cell cytotoxicity tests – MDCK-C7 cell line

The influence of various concentrations of both free and nanoencapsulated baicalein and quercetin on the metabolic competence (mitochondrial activity) of MDCK-C7 cell line was assessed using MTT assay. The results (Figure 5) show that with free baicalein and quercetin in most of the concentrations the relative viability was below 50%, a scenario that improved with introduction of nanoencapsulated baicalein and quercetin. It is also quite clear that at higher concentration especially with quercetin there is more relative viability. This may be a false positive result as the MTT reagents used have been found to be influenced by certain doses of quercetin. But generally in both phytochemicals it can be concluded that nanoencapsulation improved their relative viability with the MDCK cell line.

4. Discussion

A combination of both physicochemical and biological analysis was used to investigate the effects of free and chitosan-based nanocapsules loaded with baicalein and quercetin against a bioengineered E.coli Top 10 biosensor and MDCK-C7 mammalian cells. The chitosan (DA 42%) used in this study, does not have antibacterial activity and, therefore, it is best suited for producing nanocapsules for bacterial quorum sensing studies [52]. The physicochemical characteristics of the nanoformulations in terms of size, zeta potential, encapsulation efficiency (Table 1) and stability in biological media (Figure 1) concurred closely with those of previous reports [25, 54]. The presence of the flavonoids did not have a dramatic effect on the Z-average size. Both flavonoids associated with very high efficiencies, namely quercetin (99%) and baicalein (87 %), in agreement with findings of other groups [50]. Their zeta potential was strongly positive, diagnostic that chitosan adsorbed at the outer shell and of the exposure its protonated amine groups. The colloidal stability of the nanocapsules in biological media (M9 and MEM), as assessed from the evolution of the particle size revealed that all the systems were stable as documented in previous studies [49, 50].

The in vitro release kinetics data of the payloads from both flavonoid-loaded NCs revealed a somewhat distinct pattern for each. Baicalein released only ~20 % (Figure S4, Supplementary Information) over the first 5 h, while quercetin released ~10% (Figure S4, Supplementary Information) over the first 4 h and thereafter the release suddenly increased up to ~40 % in both nanocapsules. The sui generis release pattern observed in both cases, particularly at long times, could not be attributed to disintegration of the nanocapsules, as no change in the size was noticed during the evaluation of the colloidal stability (Figure 1). Alternatively, erosion of the nanocapsule shell could explain this anomalous sudden increase in the release of both payloads at times exceeding ~4 h of incubation. The overall lower release of quercetin, as compared to baicalein, could be associated to slight differences in solubility (i.e., log P) that could result in differences in the partitioning of the compound between the oil-core of the droplet and the continuous aqueous phase. QS bioassays did indicate that free baicalein did not have much impact at most of the tested concentrations. In fact, at higher concentration it was observed that both free flavonoids seem to be toxic towards the E. coli Top 10 biosensor. Both phytochemicals had been reported to inhibit quorum sensing-dependent processes in a P. aeruginosa PA01 strain, but this is only achieved at higher concentrations too [19, 37]. However, in their encapsulated forms, our results demonstrate that their QS inhibition ability improves when tested in our E. coli Top 10 biosensor. PA01 strain has a specialized QS circuitry that includes LasR, Rh1R and QscR than the LuxR circuit of the transformed E. coli Top 10 strain used in this study. Each of these circuits use different type of autoinducers and therefore, it is likely that these two flavonoids do not compete for the binding site of the involved receptors with the cognate autoinducers, but they may act by different mechanisms. One possibility is that they inhibit specific enzymes like autoinducer synthases, this cannot be ruled out from our studies, as E. coli Top 10 QS biosensor strain does not synthesize the AHL AI. Also, it could be that both flavonoids bind at the LuxR receptor not at the binding site, but at a different domain that would affect the binding of the dimerized LuxR to the DNA promoter. Yet an alternative explanation is that flavonoids accumulate rapidly at the lipidic membrane of bacteria [55], and hence, block the diffusion of AHL to the cytosol. This last effect may explain why the free flavonoids exert a toxic response in a dose-dependent manner at high

concentrations, while when loaded into nanocapsules, it is likely that the flavonoids are released under a slow kinetics, and hence, bacteria can keep up with the pace to metabolise them. About the QS response, the available evidence shows that free baicalein does not have any significant effect on FL/OD_{600} when dosed at sub-lethal concentrations. In the encapsulated form, it seems that at intermediate concentrations (2.53×10^{-4} and 6.25×10^{-5} mM), there is an effect, although this does not seem to be consistent, as it was not evident at 1.24×10^{-4} mM. Even when we could not draw too many conclusions on baicalein-loaded nanocapsules in this regards, we observed a comparable, though much more defined trend in quercetin-loaded nanocapsules under the same experimental setup.

In the case of the effect of free quercetin, the toxic response was very much like that of baicalein, and a dose-dependent response was appreciated at concentrations below 6.25×10^{-3} mM and it also exerted no effect on the relative QS response at any of the tested doses. On the other, the quercetin-loaded nanocapsules exhibited no toxicity, in line with baicalein-loaded ones. What draws attention most in these systems though, was the effect of the concentration on the relative QS response and the clear so-called "Goldilocks" effect, which shows a clear maximum inhibition at an intermediate concentration of 3.91×10^{-4} mM. An interpretation to this effect can be offered in line with previous studies carried out in our laboratory showing that unloaded chitosan-based nanocapsules obtained from the same chitosan sample as in the present study and under an identical preparation protocol, at specific number ratios of nanocapsules/bacterium inhibit the relative QS response in a very similar way as we report here. This has been attributed to the aggregation exerted by the capsules on the E. coli bacteria, the result of electrostatic interactions [52]. Since in our experiments the dose of flavonoids was controlled not by the actual concentration loaded in the capsules, but by the total amount of added formulations, our results therefore, concur with the mentioned previous findings. Also, along with the aggregation effect, the possibility that the nanocapsules are able to deliver their payloads locally at the cell wall cannot be ruled out.

In turn, results of the quantitative biofilm inhibition studies clearly revealed that both flavonoids do have an incipient ability to inhibit biofilm formation of E. coli Top 10 when dosed in free form. However, this capacity was much clearly enhanced upon their nanoencapsulation when the doses were lower than ~$2.0\text{-}2.5 \times 10^{-4}$ mM, and in both cases, almost similar results were observed between the two types of systems. A possible explanation to the loss in activity observed at greater doses could be associated to the aggregation effects discussed above. It is likely that aggregation may favour biofilm formation, even when the QS response seems to be inhibited (cf. Figure 2 ii-b) [7, 56].

Overall, our findings concur with previous studies that have documented that these phytochemicals can inhibit biofilm formation [19, 37]. For instance, baicalein has been reported to inhibit P. aeruginosa PA01 biofilm formation at concentrations of 32-128 µg/mL [37], while quercetin at concentrations of 16 µg/mL produced the highest inhibitory antibiofilm formation effect against PA01 among the concentrations used - 8, 16, 32 and 64 µg/mL [19]. It is therefore, a clear indication that the inhibition of biofilm formation could be dependent on the pathogen of interest and that the observed effects are not necessarily dose-dependent as in both studies lower concentrations had better results than higher ones. Alternatively, since it is most likely that these two phytochemicals could not be competitors with autoinducers at the receptor level as they have shown inhibitory effects to bacteria of different QS circuits, it could

be argued that their biofilm inhibiting activity stems on other mechanisms. One such pathway could be inhibition of diguanylate cyclases (DCGs) that is responsible for the production bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) in bacteria cell; a molecule that controls the biosynthesis of adhesins and exopolysaccharides associated with biofilm formation [57]. A few phytochemicals have been reported to have such an activity. For instance, N-[4-(phenylamino)phenyl]-benzamide was reported to possesses a broad-spectrum activity, reducing biofilm formation of both V. cholerae and P. aeruginosa by inhibiting the DGCs and it did not possess any effect on the bacterial growth [58, 59]. Yet another possible target could be the phytochemicals compounds able to enhance dispersal of biofilms mainly by targeting the breakdown of the extracellular polysaccharide that makes up the extracellular matrix. Some compounds like norspermidine have been reported to have an enhanced dispersal of multispecies waste water biofilms when it was combined with silver nitrate [60, 61]. To account for the enhanced inhibition observed when the flavonoids were loaded in the nanocapsules, we can argue that there is a local delivery effect, that it is enhanced at low concentrations. In our previous study, we showed that 100% of the chitosan nanocapsules bind to E. coli Top 10, when they are dosed at low concentrations, below the optimal "stoichiometric" nanocapsule/bacterium binding point [52]. It is also conceivable, that under such scenario, the nanocapsules release their payloads in a sustained and controlled manner.

Cell viability studies were conducted using the MTT assay and they revealed that both baicalein and quercetin are cytotoxic to MDCK- C7 cells when dosed in solution, and produced a reduction on relative viability to less than ~50%. However, upon their nanoencapsulation, there was a notorious increase in cell viability. An explanation can be that the confinement of the phytochemicals as previously argued, could result in a controlled releases of the payloads, and hence, in lower doses over the time span of the experiment. In studies conducted in our laboratory on capsaicin-loaded nanocapsules [25], similar cytoprotective effects as those reported here were observed. In the same studies, it has been shown that the nanocapsules are up taken avidly by MDCK-C7 cells. If the NCs are up taken in intact form, hence, the loaded flavonoids are likely to bypass the cell membrane, and hence, be delivered intracellularly (possible antioxidant effect in mitochondria). Ongoing studies in our laboratory are addressing whether the nanocapsules are taken up in intact, partially or fully degraded form.

5. Conclusion

In this study, we have gained proof-of-concept that baicalein and quercetin-loaded nanocapsules modify favourably the bioactivity of both flavonoids, namely, they prevent E. coli Top 10 QS bacteria to "listening" the signalling of AHL (added exogenously). These effects were much clearly observed for quercetin-loaded nanocapsules and were maximized at an intermediate concentration below and above which the magnitude of the response decreases. This "Goldilocks" response, we attribute it to the possible role of bacteria aggregation exerted by the capsules. The consequences of encapsulating the flavonoids were even more accentuated on the capacity to inhibit biofilm formation in the same bacterial strain, and only at concentrations below that at which the maximum QS inhibition was observed. Whether the inhibition of QS resulted also in antibiofilm formation, under conditions that 100% of the capsules bound to bacteria, is yet to be confirmed. Yet another favourable effect of the

nanoencapsulation of the flavonoids was the cytoprotective effect observed in MDCK-C7 cells. The potential use of these systems in food and health is yet to be fully realized.

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Figures Captions

Figure1: Evolution of the Z-average diameter size with time of flavonoid-loaded and unloaded (blank) nanocapsules and incubated at 37 °C in: (a) M9 and (b) MEM media (n=3).

Figure 2: Flavonoid in vitro release profiles during incubation in M9 medium at 37 °C of: (a) Baicalein -loaded nanocapsules, and (b) Quercetin -loaded nanocapsules.

Figure 3: Effect of varying doses of baicalein (i) and quercetin (ii) as (a) free solution, and (b) loaded nanocapsules on the relative growth (OD_{600}) and relative quorum sensing activity (FL/OD_{600}), evaluated in a E. coli Top 10 quorum sensing biosensor strain. Average values represent average ± S.D. (n=3).

Figure 4: Evolution during the culture of E. coli Top 10 quorum sensing biosensor strain (in M9 medium at 37 °C). Panels: (a) fluorescence expression, (b) growth (OD_{600}), and (c) quorum sensing (Fl/OD_{600}) upon treatment with (i) baicalein and (ii) quercetin-loaded nanocapsules at different doses (as indicated in legend).

Figure 5: Effect of varying doses of: (a) baicalein and (b) quercetin after treatment in free solution and in both flavonoids-loaded nanocapsules (as shown in legends), on the relative inhibition of biofilm formation of E. coli Top 10 biosensor strain (cultured in M9 liquid medium at 37 °C). Kanamycin (conc. 1mM) was used as positive control. Average values represent average ± S.D. (n=3).

Figure 6: Effect of varying doses of: (a) baicalein and (b) quercetin after treatment in free solution and in both flavonoids-loaded nanocapsules (as shown in legends), on the cell viability of MDCK-C7 cells (cultured in supplemented MEM at 37 °C). Average values represent average ± S.D. (n=3).

Table 1: Characteristics of chitosan-coated nanocapsules (mean averages ± SD, n=3)

Nanocapsules Formulation	Z-av. diameter (nm)	PDI[a]	ζ–potential (mV)	Flavonoid association efficiency(A.E., %)[b]
Quercetin-loaded	194 ± 4	0.12	+48.4 ± 3.46	99 ± 1.2
Baicalein-loaded	187 ± 2	0.12	+48.1 ± 2.03	87 ± 5.1
Unloaded	185 ± 2	0.15	+47.6 ± 2.38	------

[a] Polydispersity index

[b] Flavonoid A.E. (%) = $[(\text{Conc. flavonoid}_{total} - \text{Conc. flavonoid}_{unloaded})/(\text{Conc. flavonoid}_{total})] \times 100$

Figures Captions

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Figure 1

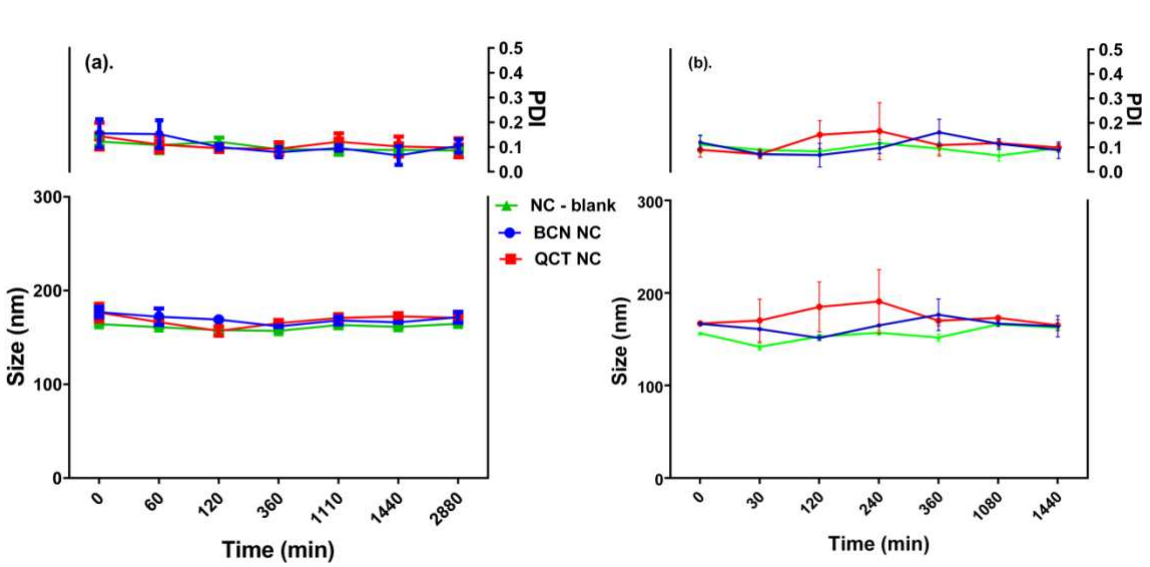

(a).
(b).
PDI
0.5
0.4
0.3
0.2
0.1
0.0
Size (nm)
300
200
100
0
NC - blank
BCN NC
QCT NC
0
60
120
360
1110
1440
2880
30
240
1080
Time (min)

Figure 2

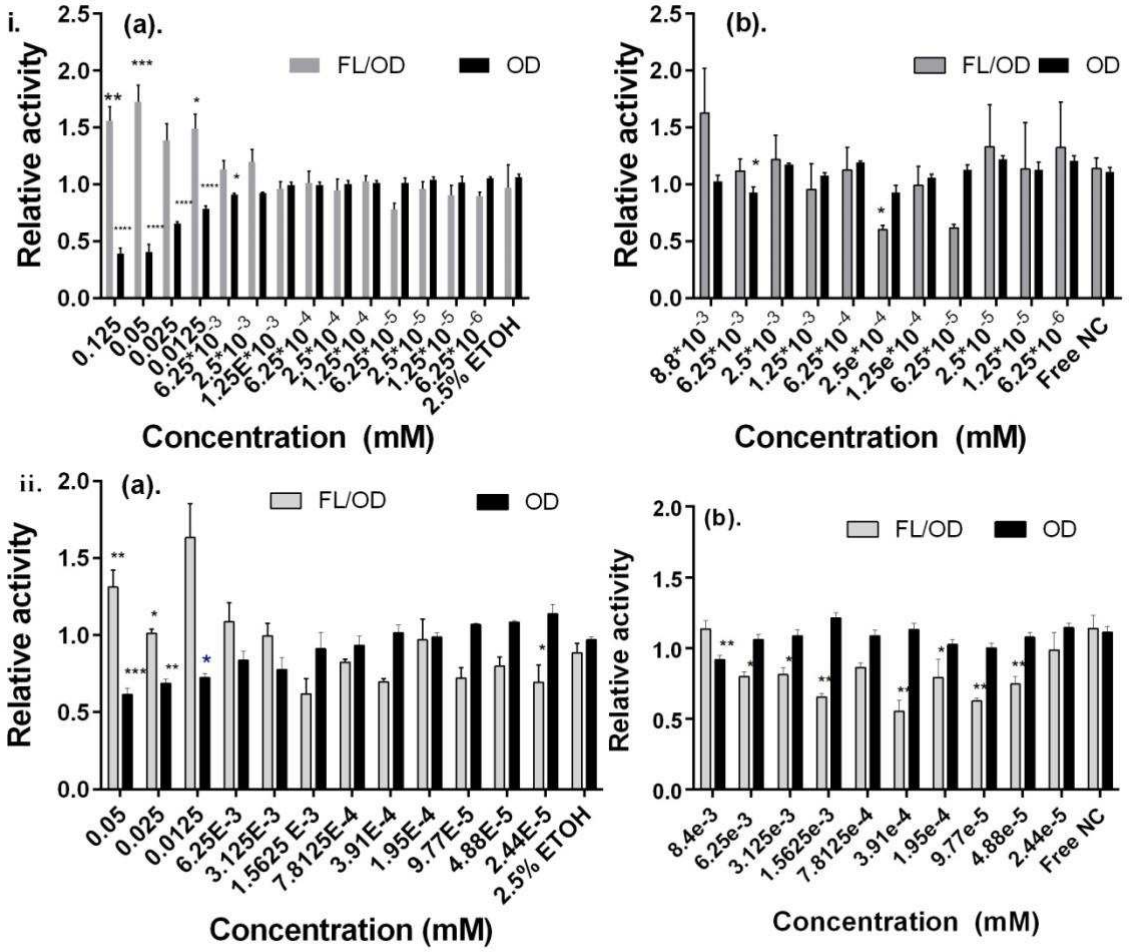

i.
(a).
FL/OD
OD
Relative activity
Concentration (mM)
0.125
0.05
0.025
0.0125
6.25*10-3
2.5*10-3
1.25E*10-3
6.25*10-4
2.5*10-4
1.25*10-4
6.25*10-5
2.5*10-5
1.25*10-5
6.25*10-6
2.5% ETOH
(b).
8.8*10-3
6.25*10-3
2.5*10-3
1.25*10-3
6.25*10-4
2.5e*10-4
1.25e*10-4
6.25*10-5
2.5*10-5
1.25*10-5
6.25*10-6
Free NC
ii.
(a).
0.05
0.025
0.0125
6.25E-3
3.125E-3
1.5625 E-3
7.8125E-4
3.91E-4
1.95E-4
9.77E-5
4.88E-5
2.44E-5
2.5% ETOH
(b).
8.4e-3
6.25e-3
3.125e-3
1.5625e-3
7.8125e-4
3.91e-4
1.95e-4
9.77e-5
4.88e-5
2.44e-5
Free NC

Figure 3

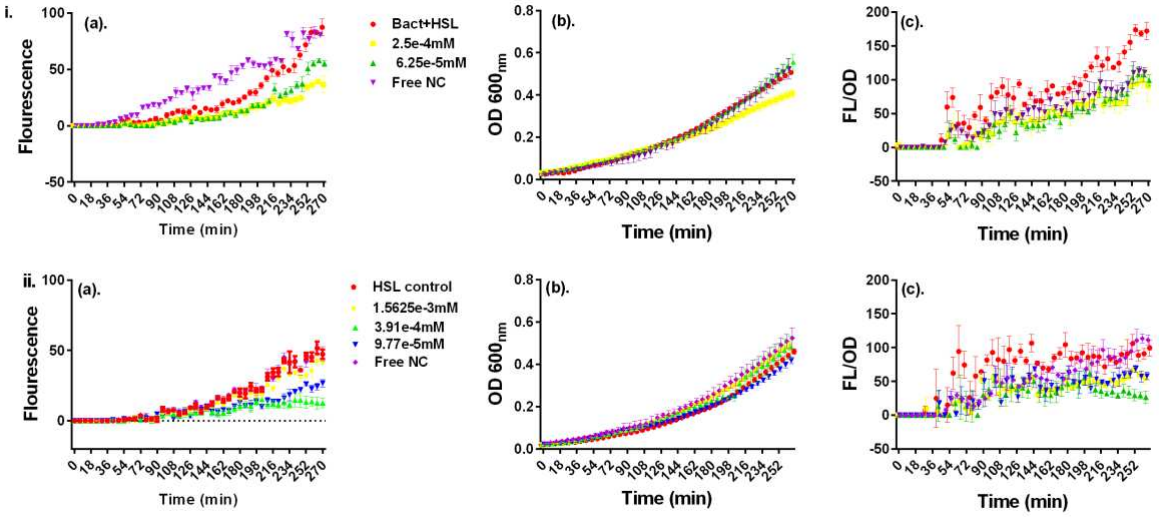

i.
(a).
Flourescence
Time (min)
Bact+HSL
2.5e-4mM
6.25e-5mM
Free NC
(b).
OD 600nm
Time (min)
(c).
FL/OD
Time (min)
ii.
(a).
Flourescence
Time (min)
HSL control
1.5625e-3mM
3.91e-4mM
9.77e-5mM
Free NC
(b).
OD 600nm
Time (min)
(c).
FL/OD
Time (min)

Figure 4

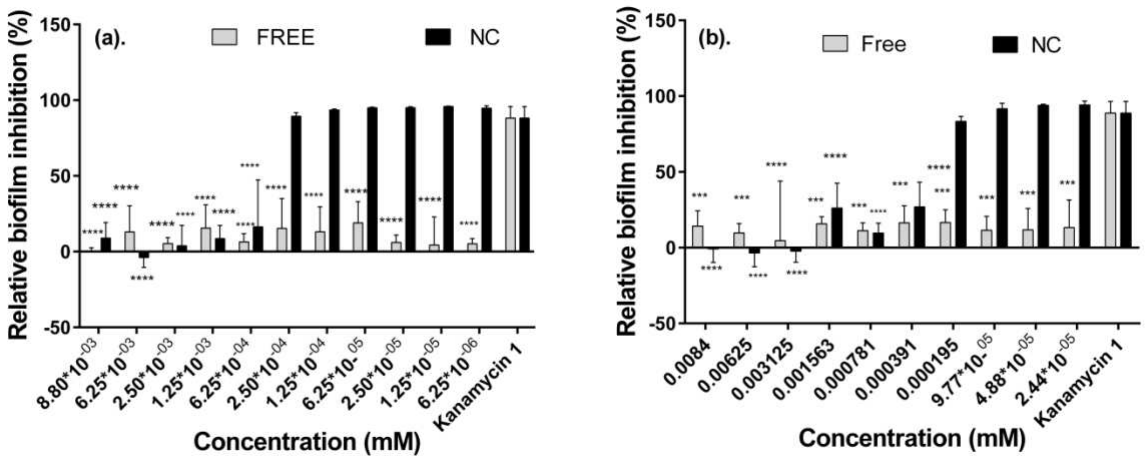
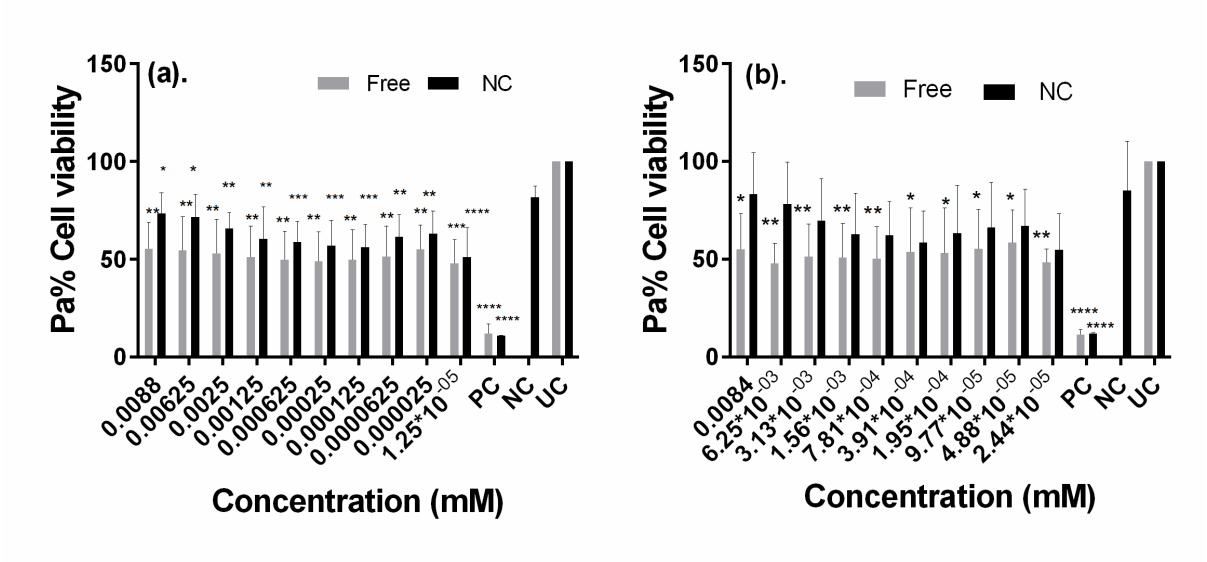

(a).
FREE
NC
Relative biofilm inhibition (%)
150
100
50
0
-50
8.80*10-03
6.25*10-03
2.50*10-03
1.25*10-03
6.25*10-04
2.50*10-04
1.25*10-04
6.25*10-05
2.50*10-05
1.25*10-05
6.25*10-06
Kanamycin 1
Concentration (mM)
(b).
Free
NC
Relative biofilm inhibition (%)
150
100
50
0
-50
0.0084
0.00625
0.003125
0.001563
0.000781
0.000391
0.000195
9.77*10-05
4.88*10-05
2.44*10-05
Kanamycin 1
Concentration (mM)

Figure 5


(a).
Free
NC
Pa% Cell viability
150
100
50
0
0.0088
0.00625
0.0025
0.00125
0.000625
0.00025
0.000125
0.0000625
0.000025
1.25*10-05
PC
NC
UC
Concentration (mM)
(b).
Free
NC
Pa% Cell viability
150
100
50
0
0.0084
6.25*10-03
3.13*10-03
1.56*10-03
7.81*10-04
3.91*10-04
1.95*10-04
9.77*10-05
4.88*10-05
2.44*10-05
PC
NC
UC
Concentration (mM)

Supplementary Information

Chitosan nanoencapsulation of flavonoids enhances their quorum sensing and biofilm formation inhibitory activities against an E.coli Top 10 biosensor

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Figure S1 (a): Quercetin hydrate
MW- 302.24
$C_{15}H_{10}O_7 \cdot xH_2O$
Log P, 2.16

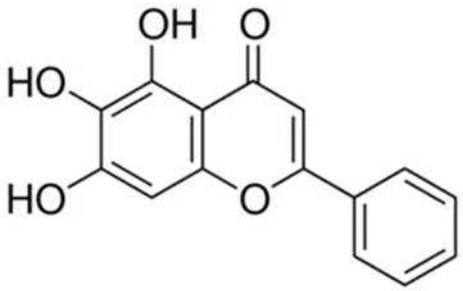
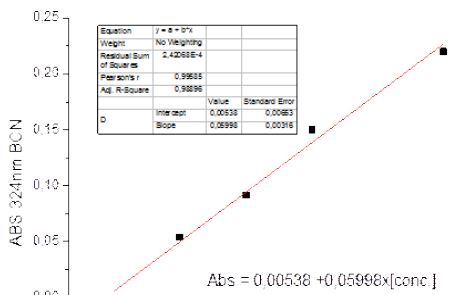


Figure S1 (b): Baicalein
MW- 270.24
$C_{15}H_{10}O_5$
Log P, 3.311

Figure S1: Chemical structures of baicalein and quercetin



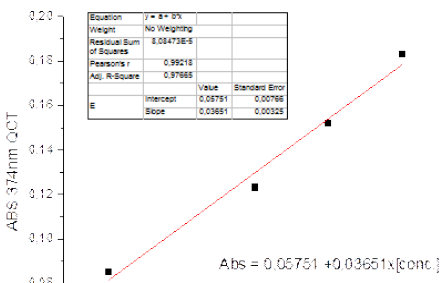


Figure S2: Calibration curves for baicalein (a) and quercetin (b) diluted in absolute ethanol

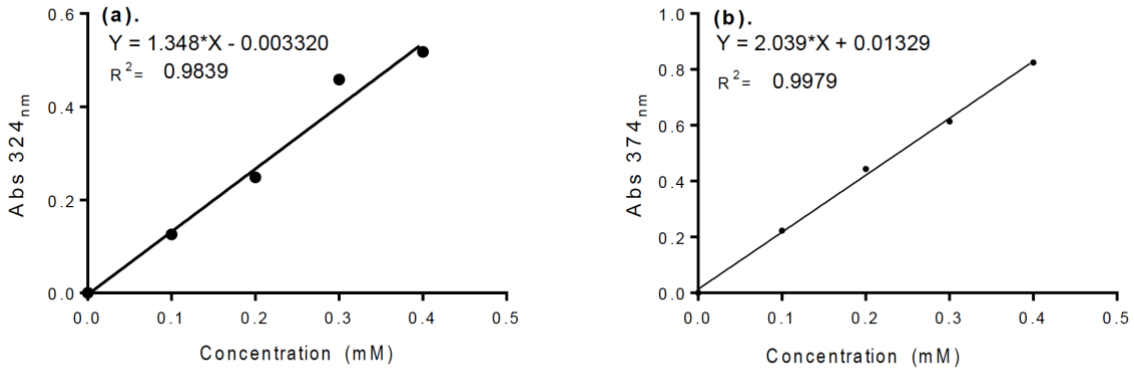


Figure S3: Calibration curves for baicalein (a) and quercetin (b) diluted in M9 medium.

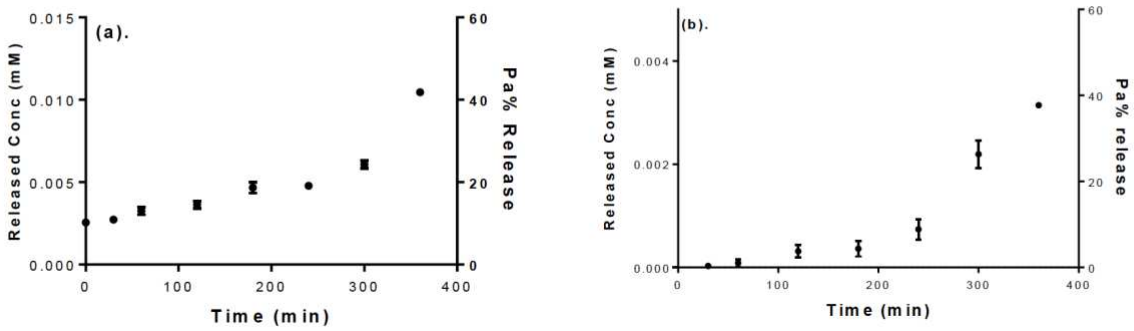


Figure S4: Flavonoid in vitro release profiles during incubation in M9 medium at 37 °C of: (a) Baicalein-loaded nanocapsules, and (b) Quercetin-loaded nanocapsules

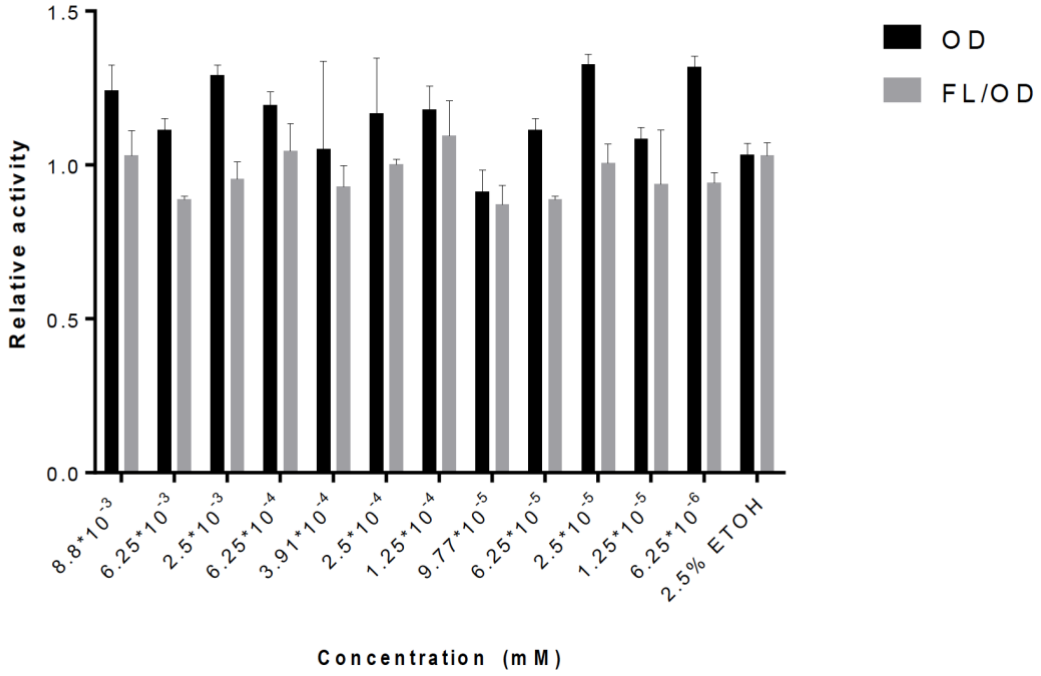


Figure S5: Effect of blank NCs on the quorum sensing of E. coli Top 10 biosensor.

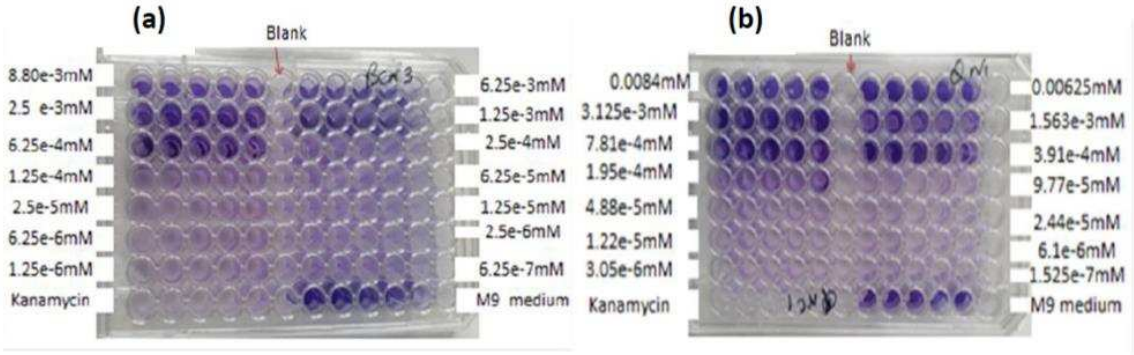


Figure S6: Appearance of microtiter plates during the biofilm formation assay by E. coli Top 10 biosensor upon treatment with varying concentrations (shown on legends on both plates) of: (a) Baicalein-loaded nanocapsules, and (b) Quercetin-loaded nanocapsules. Biofilms are stained with 0.4% crystal violet. Negative and positive controls were M9 medium and kanamycin (1mM) respectively.

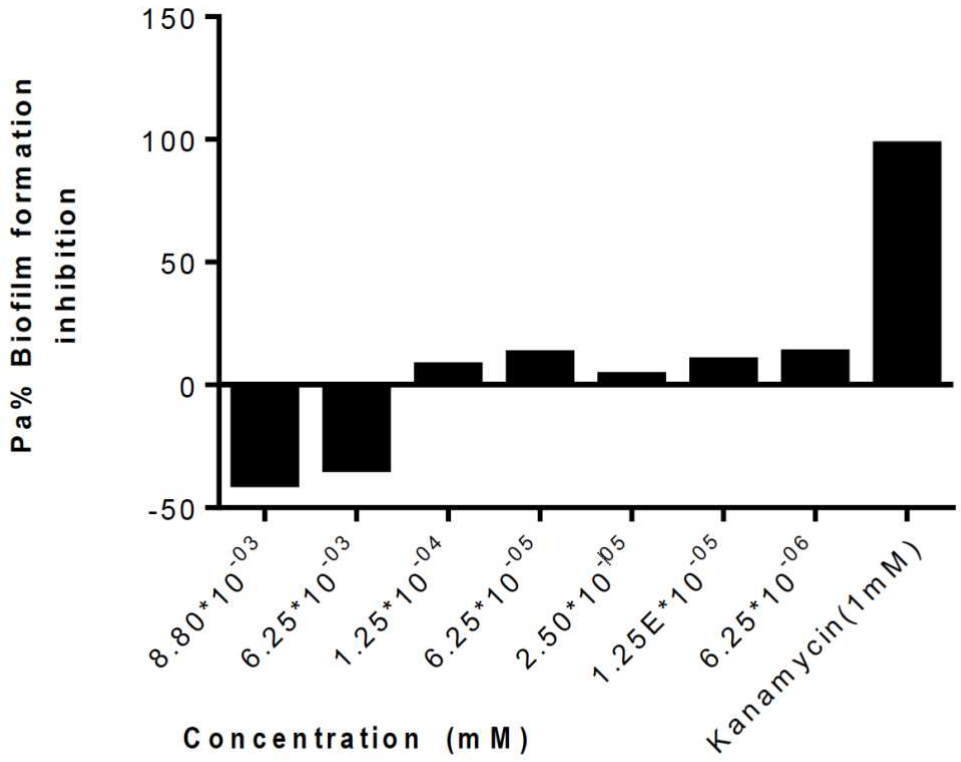


Figure S7: Effect of unloaded chitosan nanoparticles on biofilm formation of E. coli Top 10 biosensor.