Antioxidant and Antibacterial Study of 10 Flavonoids Revealed Rutin as a Potential Anti-Biofilm Agent in *Klebsiella Pneumoniae* Strains Isolated From Hospitalized Patients

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Abstract

Background: The emergence of multidrug resistance (MDR) and extensively drug-resistance (XDR) of *Klebsiella pneumoniae* strain has brought great threaten to traditional antibiotics. Previous studies showed that plant-derived flavonoids have inhibitory functions on pathogens. However, In *K. pneumoniae*, the antibacterial activities of different flavonoids on the growth and biofilm formation remains a mystery. The aim of the present study is to evaluate the antioxidant abilities of different flavonoids and to identify their inhibitory effects on *K. pneumoniae* growth and biofilm formation.

Results: Totally 10 flavonoids representing 4 major categories were screened and used in this study. The antioxidant capacity of each flavonoid was evaluated through a DPPH assay. Rutin showed highest free radical scavenging capacity, followed by kaempferol, luteolin, quercetin, apigenin, hesperidin, sinensetin, naringenin, naringin and 3,5,6,7,8,3',4'-heptamethoxyflavone. The inhibitory effect of rutin and naringin on bacteria growth were compared. The antibacterial activity of flavonoid is highly correlated with the antioxidant capacity. Among these flavonoids, rutin showed the best inhibitory capacity against both the growth curve and biofilm production. The lowest value of MIC was found in rutin against *K. pneumoniae* ATCC700603 (1024μg/mL) and *E.coli* ATCC25922 (512μg/mL). The MBIC were not found. The expression profiles of 15 genes were analyzed in biofilm cells both with and without rutin treatment. The correlation analysis showed the *mrkA* gene expression was positively correlated with the biofilm biomass accumulation.

Conclusions: Our study indicated that flavonols showed stronger antibacterial activities. The biofilm production is correlated with several gene expression rather than one. Rutin is a potential agent to inhibit the *K. pneumoniae* biofilm formation.

Background

Klebsiella pneumoniae (*K. pneumoniae*), an ubiquitous gram-negative encapsulated bacterium, has been reported and recognized as a strong risk to public health due to its high frequency of drug-resistance[1]. This opportunistic pathogen is widespread in the mucosal surfaces of different environments, such as soil, water, plants, animals and humans, causing a wide range of infections including pneumonia, urinary tract infection and liver abscesses among people with low immunity, even healthy ones[2]. *K. pneumoniae* is always considered as a most important pathogen bacteria as it poses not only community infections but also nosocomial infections[3]. In recent years, it was newly reported that drug-resistant *K. pneumoniae* can acquire virulence gene and as well, virulent *K. pneumoniae* can also obtain drug-resistance genes, thus making this strain resistant to the antibiotic class of 'last resort'[4]. In addition, the biofilm forming ability of *K. pneumoniae* are further contribute to their drug resistance, resulting in higher mortality[5]. Thus, it is urgent to develop new strategies or novel antibacterial agents to fight this pathogen.

Development of new antimicrobial agents to *K. pneumoniae* is particularly challenging due to its complicated mechanisms of drug resistance[6]. Among these mechanisms, biofilm forming is a highly correlated one[7]. Biofilms are such complex assemblage of microbial cells covered with self-produced matrix[8]. Many *K. pneumoniae* are able to form biofilms[9], where bacteria are surrounded by extracellular polymeric substances (EPS) such as polysaccharides, nucleic acids and proteins[10]. Compared with none biofilm producer, pathogens inside the biofilms are notoriously known for higher resistance to both inflammatory defense system of the host and antibiotics, which can lead to persistent infections or even, treatment failure[11].

Associated with high cost and low efficiency, the pace of new antibiotics emergence is increasingly limited[12, 13]. However, plant-derived flavonoids have been increasingly attracting researcher's attention due to their potential to be substitutes for antibiotics[14]. Composed of more than 8000 known compounds, plant-derived flavonoids are rich resource for new antibacterials discovery[15]. Flavonoids are naturally-occurring polyphenols and have been reported to contain a wide range of significant pharmacological functions including anticancer[16], anti-inflammation[17] and antiviral[18]. In addition, flavonoids are also well known as antibacterial agents against a wide range of pathogenic microorganisms[14], such as *Staphylococcus aureus*[19], *Vibrio harveyi*[20], *Pseudomonas aeruginosa*[21] and *Enterococcus faecalis*[22]. Several flavonoids, namely, fisetin, phloretin and curcumin, showed inhibitory effect on biofilm of multidrug-resistant *Acinetobacter baumannii* isolates[23]. However, in *K. pneumoniae* strains, the antioxidant and antibacterial effects of most flavonoids are still unclear.

In the past few years, we have studied citrus flavonoids and find over 80 citrus components in citrus plants (unpublished data). These compounds can be classified into four major types, namely flavones, flavonols, flavanones, and flavanonols according to their basic structures[24]. In present study, 10 flavonoids representing 4 major classes were studied. The capacity of antioxidant were evaluated through DPPH assay. The functions of different flavonoids on strain growth and biofilm formation were identified and compared. The MIC and MBIC were screened. The relationship between the gene expression and the biofilm formation was discussed. The aim of this study is to screen active flavonoid and to reveal the inhibitory mechanism of active flavonoid in *K. pneumoniae*.

Materials And Methods

Clinical isolates and growth conditions

A total of 177 no duplicate *K. pneumoniae* isolates were collected from blood samples in the Affiliated Hospital of Southwest Medical University (Luzhou, China) over the period from July 2018 to June 2020. All strains were cultured in Luria-Bertani broth (LB, Haibo, Qingdao, China) media at 37℃ with shaking or as static cultures, as required.

Chemicals

Flavonoids including naringenin, naringin, hesperidin, kaempferol, quercetin, rutin, apigenin, luteolin, sinensetin and 3,5,6,7,8,3',4'-heptamethoxyflavone were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). The chemical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich Co. (Shanghai, China). The flavonoid stocks were prepared in dimethyl sulfoxide (DMSO). All chemicals used in this study were of analytical grade.

Antioxidant capacity of flavonoids

As a stable free radical，the DPPH was used to evaluate the efficiency of antioxidant and antiradical of 10 flavonoids. The method was adapted to previous study[25, 26] and slightly modified. The DPPH assay was conducted as follows. **Fabrication of standard curve-** The 2.5 mg DPPH powder was weighted and dissolved in methanol, the standard stock solution of 2.5×10^{-2} mg/mL was prepared. Then a series of solutions of 0, 5, 10, 15, 20 and 25 µg/mL were separately made. The absorbance of each solution at 515 nm was measured with a Beckman Model DU650 spectrophotometer. The standard curve was drawn according to the absorbance value. **DPPH residual rate detection-** The 12, 6, 3 and 1.5 mg/mL solutions were prepared separately of 10 flavonoids. Ascorbic acid was used as control. Reaction tubes of 10 mL were prepared and each was added in 100 µL flavonoids solution and 3.9 mL DPPH solution (2.5×10^{-2} mg/mL). Methanol was used as empty control. The absorbance was measured at different time points. The DPPH residual rate was calculated out according to the standard curve.

Determination of minimum inhibitory concentration

The *K. pneumoniae* ATCC700603 and *E.coli* ATCC25922 strain were cultured in LB agar medium for 24 h at 37°C. The antibacterial ability of 10 flavonoids were determined by minimum inhibitory concentration (MIC). The MIC was evaluated through micro-broth dilution method performed in 96-well micro-plates according to the methods previously reported[27]. Briefly, *K. pneumoniae* ATCC700603 and *E.coli* ATCC25922 were cultured in LB solid medium and grown overnight at 37°C without shaking. Then, 100µL bacterial suspension (10^6cfu/mL) was transferred to each well. Then, 100 µL of each dissolved flavonoid stock was twofold serially diluted (ranging from 1µg/mL to 1024µg/mL) and added to each well, making the final volume of 200 µL in each well. The empty control was performed by 3 wells filled with LB only and the positive control was conducted in 3 wells filled with LB and bacteria. Ciprofloxacin was used as positive drug control. The 96-well plates were covered and incubated for16 h at 37°C with shaking (150 rpm). The MIC was determined when no visible bacterial growth can be seen (absence of turbidity) in the well.

Bacterial strains and growth conditions

Bacterial species were identified by standard methods with a MicroScan WalkAway 96 Plus System (Siemens, Germany). *K. pneumoniae* ATCC700603 and *E.coli* ATCC25922 were purchased from China National Health Inspection Center.

All procedures performed were approved by the Ethical Committee of the Affiliated Hospital of Southwest Medical University and were in accordance with the tenets of the 1964 Helsinki declaration and its later amendments. All strains were cultured in LB medium at 37°C. Before testing, the microorganisms were transferred on Mueller-Hinton agar (MHA, Liofilchem, Italy) and cultured over night at 37 ˚C. Subsequently, 10 mL of Mueller Hinton broth (MHB, Scharlau, Spain) were inoculated with one representative colony of each organism taken from MHA, then cultured overnight (37˚C, 150 rpm) and used as source of inoculum for each assay.

Growth curve measurement

Stationary *K. pneumoniae* cultures were diluted 1:1000 in LB and incubated at 37°C with shaking (220 rpm). Each well of a 96-well polystyrene microtiter culture plate (Costar #3524, Corning, NY, USA) were filled with 10µL of the overnight culture diluted to a final concentration of 1.5×10^8 cfu/ml and 190 µL of LB broth mixed with rutin to a final concentration of 512 µg/mL. Sterile LB was added to the last three wells and used as a negative control. The 96-well plates were covered and incubated for 24 h at 37°C with shaking (150 rpm). The growth was measured every 2h at OD590 using an microplate reader. All experiments were performed in triplicate.

Biofilm formation assay

Crystal violet (CV) staining was used in this study to quantify the biofilm formation. The assay was performed as previously described[28, 29]. The procedures was slightly modified. Briefly, for each strain, three wells of the 96-well plate were filled with 180 µL LB broth and 20 µL of the overnight culture. The overnight culture was grown at 37 °C and diluted to a final optical density of 0.1 at 600nm before use. Wells filled with nothing and sterile LB broth were used as empty control and negative control, respectively. After incubation for 24h at 37°C, each well was washed three times with phosphate-buffered saline (PBS). The 96-well plate need to be dried at 60 °C for 30 minutes and stained for 15 minutes with 200 µL 0.1% (w/v) CV for 30min at room temperature. After that, each well was washed three times with distilled water and air-dried. The dye bound of the adherent cells was dissolved in 200 µL of 95% ethanol (Merck, Germany). The 590 nm absorbance was quantified using a microplate reader. All assays were performed in triplicate.

Biofilm-forming capacities of the isolates were evaluated by the value of cut-off OD, termed as ODC. The OD was defined as three standard deviations above the mean OD of the negative control, and the following categories were established: OD ≤ ODC, non-biofilm producers (-); ODC < OD ≤ 2×ODC, weak biofilm producers (+); 2×ODC < OD≤ 4×ODC, moderate biofilm producers (++) and OD > 4×ODC, strong biofilm producers (+++)[23].

Quantitative reverse transcription (qRT)-PCR

The expression levels of 15 genes were determined from biofilm-grown cells using qRT-PCR. Briefly, bacteria were grown in LB broth at 37 °C in a 6-well microtiter plates(LG18-301C-1H, Lige Science, Guangzhou, China) and, after incubation for 24 hours, each well was gently washed and biofilm cells were removed from the well surface using a cell scraper and resuspended in saline solution. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) and cDNA was synthesised using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturers' instructions. The qRT-PCR was performed with an Applied Biosystems 7500 RT-PCR system using a SYBR Green RT-PCR Kit (Qiagen) and the primer pairs listed in Table 1.

In *K. pneumoniae*, biofilms formation are mediated by a wide rage of biological processes and genetic factors, including type I fimbriae biosynthesis(*fimH*)[30], type III fimbriae biosynthesis(*mrkA* and *mrkD*), virulence genes(*entB* and *fimH-1*)[31], lipopolysaccharide (LPS) biosynthesis(*wzm* and *wbbM*)[32], poly-β-1,6-N-acetyl-d-glucosamine polysaccharide secretion(*pgaA*)[5], quorum-sensing(QS)(*luxS*)[33], capsular polysaccharide(CPS)(*treC*, *wabG*, *rmpA* and *magA*)[5], colonic acid (*wcaJ*)[34], allantoin (*allS*)[35], capsule-associated virulence

gene(*wcaG*)[36] and siderophore-associated gene(*entB*)[31]. Most of these genes are virulence gene.

Primers were synthesized by Shanghai Biotech (Shanghai, China). The relative expression levels of tested genes were normalised to the expression of the 16sRNA gene. Each sample was run in triplicate and the means of Ct values were obtained for analysis. The relative gene expression was represented as fold change between the (-rutin) and (+rutin) treatment. Data were analysed using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The Student's t-test was used to analyse the difference in biofilm formation ability and gene expression levels. Statistical analysis was performed with GraphPad Prism 7.01 software and IBM SPSS Statistics 23 software.

Correlation analysis

The correlation analysis was conducted through Pearson's correlation analysis. Three separated biological replicates of each assay were used for analysis.

Results

Antioxidant capacity of flavonoids

To identify the correlation between the antioxidant capacity and the related molecular structures of active flavonoids, and to develop new drugs that are less likely to induce resistance to treat drug-resistant bacteria, 10 components representing 4 major flavonoids categories were screened and evaluated through DPPH assay in present study (Table 2). The results showed that among the 4 major classes of flavonids, flavonols showed the highest free radical scavenging capacity, followed by flavones and flavanones. The polymethoxylatedflavones were supposed to have the lowest antioxidant activity (Figure 1). Furthermore, within the flavonols, rutin exhibited highest free radical scavenging ability, followed by kaempferol and quercetin, respectively.

Clinical characteristics and biofilm formation abilities of *K. pneumoniae* strains

The biofilm formation abilities of 177 clinical isolates of *K. pneumoniae* were evaluated by crystal violet staining. The results were shown in Table 3. Among the 177 isolates, the positive rate of biofilm producer was 88.14% (156/177), of which strong (+++), moderate (++), and weak (+) biofilm producing strains account for 10.73% (19/177), 16.95% (30/177) and 60.45% (107/177), respectively. The rate of strong biofilm producer was low among these clinically isolated *K. pneumoniae* strains, though the positive rate of biofilm producer was high.

Minimum inhibitory concentration of 10 flavonoids

The minimum inhibitory concentration, termed as MIC of flavonoids against *K. pneumoniae* ATCC700603 and *E.coli* ATCC25922 were evaluated. Flavonols showed higher level of inhibitory effect than other categories of flavonoids, maybe due to their strong antioxidant capacity. However, most of the flavonoids are not strong inhibitory agents against neither *K. pneumoniae*, nor *E.coli*, as the lowest MIC was 512 μg/mL of rutin against *E.coli* ATCC25922 and 1024 μg/mL of rutin against *K. pneumoniae* ATCC700603. Other flavonids were found no obvious inhibitory effects even when the concentration of these flavonoids increased to 1024 μg/mL (Table 4).

Inhibitory effect of rutin on *K. pneumoniae* growth

From the results above, we found rutin was the most potential antimicrobial agent. In order to confirm the inhibitory effect of rutin against *K. pneumoniae* strains, the growth of two drug-resistant *K. pneumoniae* isolates (Kpn495 and Kpn499) were evaluated by morphological observation and growth curve changes. Furthermore, naringin and the combination of rutin and naringin were also used to evaluate their inhibitory effect against *K. pneumoniae* (Figure 2). The concentration of both rutin and naringin used in this session was increased to 50 mg/mL as no obvious inhibitory effect was found at low concentration.

In solid media, both rutin and naringin showed inhibitory effect on *K. pneumoniae* growth. Rutin showed better inhibitory effect than naringin both in Kpn495 and Kpn499 strains. In addition, rutin showed significant inhibitory effect on the 24-h growth curve of both Kpn495 and Kpn499(Figure 2 c and d).

The combination of these two components were found no significant inhibitory effects. In contrast, the combination of rutin and naringin even promoted the growth of Kpn495 strain.

Inhibitory effect of rutin on biofilm formation

The minimum biofilm inhibitory (MBIC) effect of flavonoids against biofilm formation were measured in 96-well polystyrene plates using a crystal violet assay. The tested flavonoids exhibited varying levels of anti-biofilm activity against *K. pneumoniae* strains though the MBIC was not observed in this study (Figure 3). Of the 10 flavonoids, rutin, kaempferol and quercetin at 256 μg/ml exhibited biofilm inhibition, whereas naringin, sinensetin and 3,5,6,7,8,3',4'-heptamethoxyflavone increased biofilm formation in Kpn140. When the concentration of rutin increased to 512 μg/ml, more flavonoids, like naringin, kaempferol, quercetin, rutin, luteolin, sinensetin and 3,5,6,7,8,3',4'-heptamethoxyflavone showed inhibitory effect, but only kaempferol and rutin showed significant variations (Figure 3a). Regarding to Kpn 173, four flavonoids, that is kaempferol, quercetin, rutin and luteolin showed inhibitory effect on biofilm

formation when the concentration was at 256 μg/ml, others had little effect (Figure 3b). When the concentration increased to 512 μg/ml, another three flavonoids, that is, naringin, sinensetin and 3,5,6,7,8,3',4'-heptamethoxyflavone reduced biofilm formation. Among these 10 flavonoids, only kaempferol, quercetin, rutin and luteolin showed dose-dependent inhibitory effect and only rutin showed significant inhibition. The strongest inhibitors against biofilm formation were rutin, kaempferol and quercetin, they are all flavonols.

To identify the correlation between the concentration of rutin and its anti-biofilm capacity, five different concentrations were used to treat the biofilm and the inhibitory effects were compared (Figure 3c and d). Rutin significantly decreased the biofilm production when the concentration was at 512 μg/mL and 256 μg/mL. However, when the concentration lowered to 128 μg/mL, the inhibitory effect was not significant. When the concentration decreased to 64 μg/mL or 32 μg/mL, no inhibition was found.

Gene expression analysis

To identify key factors that involved in biofilm formation process, totally 15 genes related to type I and III fimbriae biosynthesis, virulence factors, lipopolysaccharide (LPS) biosynthesis, poly-β-1,6-N-acetyl-d-glucosamine polysaccharide secretion, quorum-sensing(QS), capsular polysaccharide(CPS), colonic acid (wcaJ), allantoin, capsule-associated virulence gene and siderophore-associated gene were analyzed in biofilm cells before and after the rutin treatment, respectively. In Kpn140, 7 genes (*mrkA*, *wzm*, *wbbM*, *luxS*, *treC*, *wabG*, *entB*) were detected to show significant expression differences with and without rutin treatment. Among these genes, *wzm*, *wbbM* and *entB* were up-regulated while, *mrkA*, *luxS*, *treC* and *wabG* were down-regulated after rutin treatment in biofilm cells.The expression level of *luxS*, *wabG*

and *mrkA* gene were down regulated 69-fold, 61-fold and 8.8-fold after rutin treatment, respectively. However, the up-regulated genes were found to have very low expression level though the changes were significant after rutin treatment(Figure 4a).

In Kpn173, 8 of the 15 genes were found to significantly changed their transcription levels after rutin treatment. Among the 8 genes, 3 (*wzm*, *wbbM* and *entB*) were up-regulated and the others were down-regulated (Figure 4b). The *wabG* and *luxS* gene decreased their expressions by approximately 34- and 7-fold, respectively.

Among the 15 genes analyzed in this study, most of them showed low level of expression alterations after rutin treatment. However, the *luxS* gene and *wabG* gene showed high level of expression in the biofilm cells and decreased their expression level sharply after rutin treatment in both Kpn140 and Kpn173.

Correlation between biofilm and gene expression

To identify the function of these genes in biofilm cells, in particular their response to rutin treatment and biofilm formation, the correlation between gene expression and biofilm biomass was studied (table 5). Of these genes, 7 of them were highly correlated with biofilm biomass. The expression of *mrkA* gene was positively correlated with the biofilm production(R=0.694). However, the results showed the genes, *wzm*, *wbbM*, *magA*, *wcaJ*, *allS* and *entB* were negatively correlated with biofilm biomass, as their coefficients were -0.846, -0.815, -0.903, -0.823,-0.669 and -0.737, respectively. Though the *luxS* gene showed highest level of expression, the correlation between its gene expression and the biofilm biomass was not significant. Furthermore, co-expression of genes were found in this study.

Discussion

As an opportunistic Gram-negative pathogen, *K. pneumoniae* can cause a wide range of severe infections like pneumonia, sepsis and urinary tract infections[37]. Furthermore, this strain is associated with invasive disease in community, while in hospital, it has a marked propensity to acquire drug resistance[38]. From previous studies we observed, associated with multi-drug resistance, *K. pneumoniae* is resistant to all, or almost all, available antibiotics[39]. As a result, it is urgent to develop new antibacterial agents or novel strategies to fight the strain.

Plant-derived flavonoids have potential properties in the discovery of new antibacterial agents[40]. In some cases, it was showed that flavonoids exhibited up to 6-fold stronger antibacterial activities than standard drugs in the market[41]. However, in this study, we evaluated 10 flavonoids and found the MICs of most tested flavonoids were higher than 1024 μg/mL, indicating that the antibacterial ability of flavonoids was not strong especially when the flavonoids were in low concentration. The lowest MIC values were observed in rutin of 1024 μg/mL and 512 μg/mL against *K. pneumoniae* ATCC700603 and *E.coli* ATCC25922, respectively. This result showed the inhibitory effect varied among different bacteria species. Regarding to biofilm, no MBIC was observed but the inhibitory effect of rutin against the biofilm formation was proved when the concentration of rutin was at 256 μg/mL or higher than that.

Flavonoids are secondary metabolites with C6-C3-C6 skeleton and widely distributed in plant kingdom[42], including more than 8,000 compounds[43]. As a result, it is not possible to evaluate them one by one. For our first step, in present study, 10 flavonoids representing 4 major flavonoid categories were screened and studied.

Previous study showed free hydroxyl can donate hydrogen and electron and hence responsible for the radical scavenging ability[44]. In this study, we found the free radical scavenging ability was also correlated with the number of hydroxyl. Flavonols contain more (at least 4 hydroxyl) hydroxyl and they showed stronger free radical scavenging ability than others. In addition, luteolin showed higher free radical scavenging ability in comparison with apigenin, may be due to its 4 hydroxyl, though both of them are in flavones group.

Flavonoids were found to perform antibacterial actions through a series of mechanisms, such as bacterial toxin production, inhibition of nucleic acid synthesis and biofilm formation[45]. Biofilm formation ability is increasingly considered when develop new antibacterial drugs as bacteria inside biofilms can increase 10-1000 fold resistance[46]. Compared with conventional antibiotics, flavonoids are rich in nature and not easily to generate drug-resistance.

However, the inhibitory effects of flavonoids on *K. pneumoniae* are still unclear. In this study, 15 genes related to biofilm formation, involving in various biological processes were studied with and without rutin treatment. The *mrkA* gene was revealed to tightly correlated with the biofilm formation. This result indicated that the *mrkA* gene may play vital role in biofilm formation.

The *luxS* gene is a key factor involved in quorum sensing (QS) system in bacteria[47]. QS is a universal mechanism of cell density and interspecies communication through which the bacteria can regulate various gene and biological process, including biofilm formation[48]. In both Kpn140 and Kpn173, the *luxS* gene's expression was high in biofilm cells without rutin. However, *luxS* gene's expression decreased sharply when the biofilm was inhibited by rutin. Furthermore, we can conclude the importance of *luxS*, as several biofilm related genes were found to be co-expressed with *luxS*, though the correlation between *luxS* gene expression and biofilm formation was not significant. These results shed light on the inhibitory mechanism of rutin on the biofilm formation in *K. pneumoniae* strains, which could aid in developing new strategies for infection control.

Conclusion

Among these flavonoids, rutin showed stronger free radical scavenging capacity and antibacterial activity. Of the biofilm related genes, the expression of *luxS* gene was significantly induced by rutin treatment in biofilm cells. The biofilm biomass was positively correlated with the expression of a type III fimbriae biosynthesis gene, *mrkA*. Rutin could be a potential agent to inhibit the *K. pneumoniae* biofilm formation.

Abbreviations

MDR: multidrug resistance; XDR: extensively drug-resistance; DPPH: 2,2-diphenyl-1-picrylhydrazyl; MIC: minimum inhibitory concentration; MBIC: minimum biofilm inhibitory concentration; EPS: extracellular polymeric substances; LB: Luria-Bertani broth; DMSO: dimethyl sulfoxide; MHA: Mueller-Hinton agar; MHB:Mueller-Hinton broth.

Declarations

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Authors' contributions

Z-BW, F-J and J-BL designed the experiments; Z-BW and Z-XD conducted the experiment; Z-BW and Z-YQL isolated the strains; Z-BW and Y-HD identified the strains; Z-BW and J-BL analysed the data; Z-BW, F-J and J-BL prepared manuscript; F-J and J-BL supervised the research and revised the manuscript. All authors have read and approved the manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data set supporting the conclusions in this article is available from the corresponding author on reasonable request.

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Tables

Table 1 RT-qPCR primers used for studying expression profiles in response to rutin treatment in biofilm of *Klebsiella pneumoniae* isolates

Target gene	Function description	Primer sequence(5'→3')	Product length(bp)	Source or reference
fimH	type I fimbriae biosynthesis	CCATCACCGCAGGATCGTTA ACCACGTCGTTATTGGCGTA	109	This study
mrkA	type III fimbriae biosynthesis	ACGTCTCTAACTGCCAGGC TAGCCCTGTTGTTTGCTGGT	115	[9]
mrkD	type III fimbriae biosynthesis	CCACCAACTATTCCCTCGAA ATGGAACCCACATCGACATT	226	[30]
wzm	lipopolysaccharide (LPS) biosynthesis	TGCCAGTTCGGCCACTAAC GACAACAATAACCGGGATGG	148	[9]
wbbM	lipopolysaccharide (LPS) biosynthesis	ATGCGGGTGAGAACAAACCA AGCCGCTAACGACATCTGAC	122	[9]
pgaA	poly-β-1,6-N-acetyl-d-glucosamine polysaccharide secretion	GCAGACGCTCTCCTATGTC GCCGAGAGCAGGGGAATC	157	[9]
luxS	quorum-sensing	AGTGATGCCGGAACGCGG CGGTGTACCAATCAGGCTC	148	[9]
treC	capsular polysaccharide	CCGACAGCGGGCAGTATT CGCCGGATTCTCCCAGTT	71	[38]
wabG	capsular polysaccharide			
rmpA	capsular polysaccharide	TTCAGGGAAATGGGGAGGGTA AAACGTCAAGCCACATCCATTG	77	This study
magA	capsular polysaccharide	GTCAGGCAGCTGTTGTGAAC ACTTCTCGTATTTGCGGCGA	119	This study
wcaJ	colonic acid	AAATGGCGTACCGGTTGTTC CGGCCCTTTCGAGGTAGTTT	163	[35]
allS	allantoin	GTTTTCCGCTAACGGTTCGG ACCCAGCGCTACCCTTTTAC	129	This study
wcaG	capsule-associated virulence gene	TTCCACTACCCCAAACAACGA TGCGGCGCTTTCATGA	63	[39]
entB	siderophore-associated gene	CTGCTGGGAAAAGCGATTGTC ATGATCGACGGTTCGGCAAA	128	This study
16S rRNA	reference gene	ATGACCAGCCACACTGGAAC CTTCCTCCCCGCTGAAAGTA	157	[40]

Table 2 Ten components of flavonoids and their structure variations

Code	Name	Substituent							Hydroxyl n (%)
		R1	R2	R3	R4	R5	R6	R7	
Flavanone									
1	Naringenin	OH	H	OH	H	H	OH	H	3(42.86)
2	Naringin	OH	H	O-Neo	H	H	OH	H	2(28.57)
3	Hesperidin	OH	H	O-Neo	H	OH	OCH3	H	2(28.57)
Flavonols									
4	Kaempferol	OH	OH	H	OH	H	OH	H	4(57.14)
5	Quercetin	OH	OH	H	OH	OH	OH	H	5(71.43)
6	Rutin	O-Rut	OH	H	OH	OH	OH	H	4(57.14)
Flavone									
7	Apigenin	H	OH	H	OH	H	H	OH	3(42.86)
8	Luteolin	H	OH	H	OH	H	OH	OH	4(57.14)
Polymethoxylatedflavones									
9	Sinensetin	H	OCH3	OCH3	OCH3	H	OCH3	OCH3	0(0)
10	3,5,6,7,8,3',4'-heptamethoxyflavone	OCH3	OCH3	OCH3	OCH3	OCH3	OCH3	OCH3	0(0)

Table 3 Characteristics of distribution and biofilm forming ability of 177 *K. pneumoniae* strains

Distribution n(%)			Biofilm model n(%)			
			None(-)	Weak(+)	Moderate(++)	Strong(+++)
Paediatric patients (≤ 16 years)			(n=21)	(n=107)	(n=30)	(n=19)
0−28 days	15	8.47%	5(2.82%)	2(1.12%)	2(1.13%)	6(3.39%)
29 days−1 year	5	2.82%	1(0.56%)	2(1.12%)	0	2(1.12%)
2−16 years	7	3.95%	2(1.12%)	1(0.56%)	4(2.26%)	0
Adult patients (> 16 years)						
17−49 years	51	28.81%	2(1.12%)	25(14.12%)	21(11.86%)	3(1.69%)
50−65 years	47	26.55%	1(0.56%)	45(25.42%)	0	1(0.56%)
> 65 year	52	29.38%	32(18.08%)	11(6.21%)	4(2.26%)	5(2.82%)
Gender						
Male	97	54.80%	12(6.78%)	64(36.16%)	12(6.78%)	9(5.08%)
Female	80	45.10%	9(5.08%)	43(24.29%)	18(10.17%)	10(5.65%)

Table 4 Minimum inhibitory concentration of 10 flavonoids against *K. pneumoniae* ATCC70060 and *E.coli* ATCC25922

Flavonoids		MIC (µg/mL)	
Code	Name	*K. pneumoniae*ATCC700603	*E.coli*ATCC25922
1	Naringenin	>1024	>1024
2	Naringin	>1024	>1024
3	Hesperidin	>1024	>1024
4	Kaempferol	>1024	1024
5	Quercetin	>1024	>1024
6	Rutin	1024	512
7	Apigenin	>1024	>1024
8	Luteolin	>1024	1024
9	Sinensetin	>1024	>1024
10	3,5,6,7,8,3',4'-heptamethoxyflavone	>1024	>1024

Table 5 Pearson's correlation coefficients between gene expression and biofilm biomass

Note: *P<0.05 vs control(-rutin); **P<0.01 vs control(-rutin).

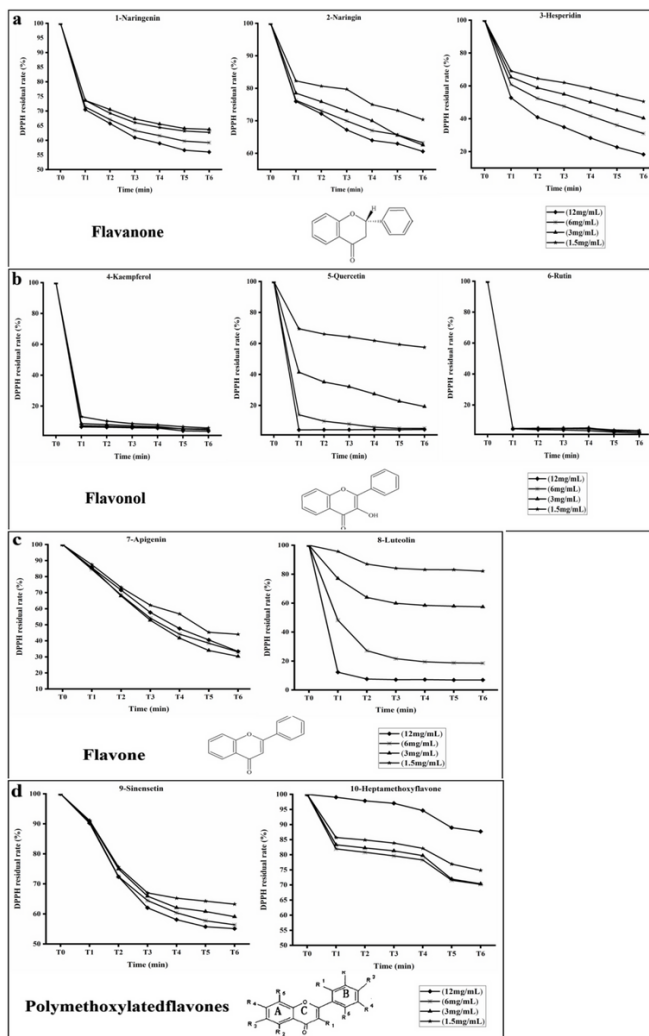| Coeffcient	mrkA	mrkD	wzm	wbbM	pgaA	luxS	treC	wabG	rmpA	magA	wcaJ	allS	wcaG	entB
fimH	0.678*	-0.216	-0.327	-0.414	0.005	0.007	0.034	0.104	0.362	-0.331	-0.341	-0.079	-0.081	-0.297
mrkA		0.036	-0.553	-0.638*	0.331	0.281	0.353	0.392	0.578*	-0.403	-0.526	-0.304	0.153	-0.391
mrkD			-0.053	-0.232	0.921**	0.941**	0.898**	0.872**	0.748**	0.465	-0.135	-0.177	0.032	-0.059
wzm				0.955**	-0.418	-0.361	-0.471	-0.489	-0.521	0.822**	0.933**	0.870**	-0.073	0.708**
wbbM					-0.584*	-0.531	-0.631*	-0.650*	-0.695*	0.718**	0.969**	0.867**	-0.006	0.801**
pgaA						0.994**	0.997**	0.984**	0.913**	0.116	-0.479	-0.465	0.046	-0.336
luxS		.					0.989**	0.974**	0.913**	0.187	-0.420	-0.411	0.011	-0.274
treC								0.988**	0.920**	0.057	-0.528	-0.509	0.035	-0.380
wabG									0.922**	0.036	-0.542	-0.513	0.141	-0.389
rmpA										-0.020	-0.561	-0.460	-0.061	-0.400
magA											0.790**	0.720**	0.010	0.731**
wcaJ												0.914**	0.021	0.888**
allS													-0.112	0.797**
wcaG														0.172
entB														

Figures

Figure 1

DPPH residual rate of 10 different flavonoids. Antioxidant ability of flavanones(a). Antioxidant ability of flavonols(b). Antioxidant ability of flavones(c). Antioxidant ability of polymethoxylatedflavones(d).
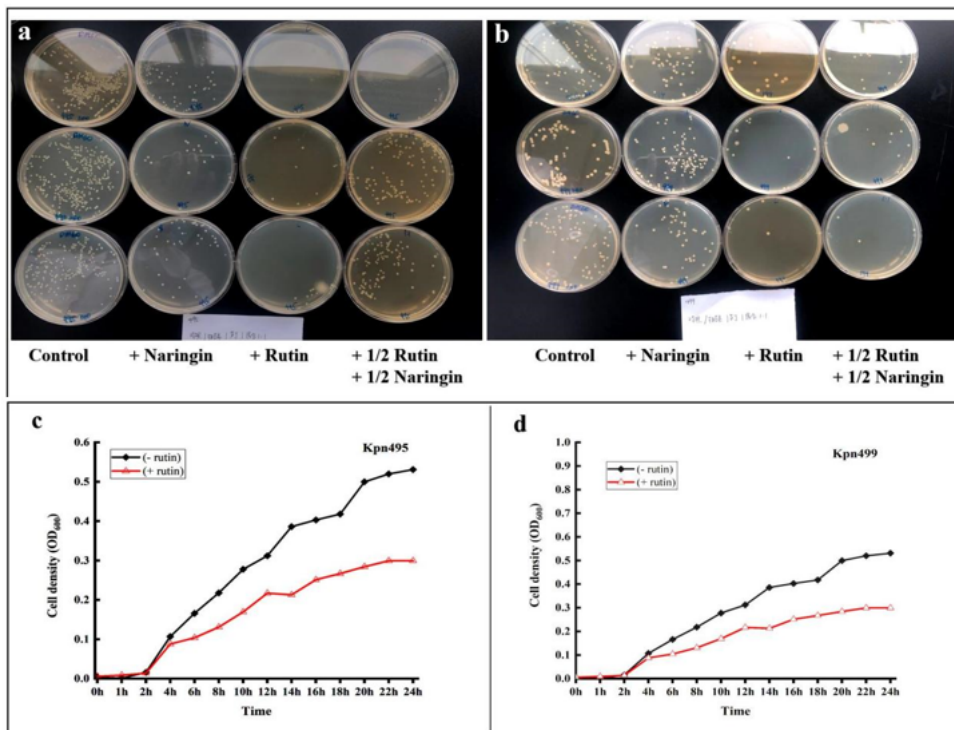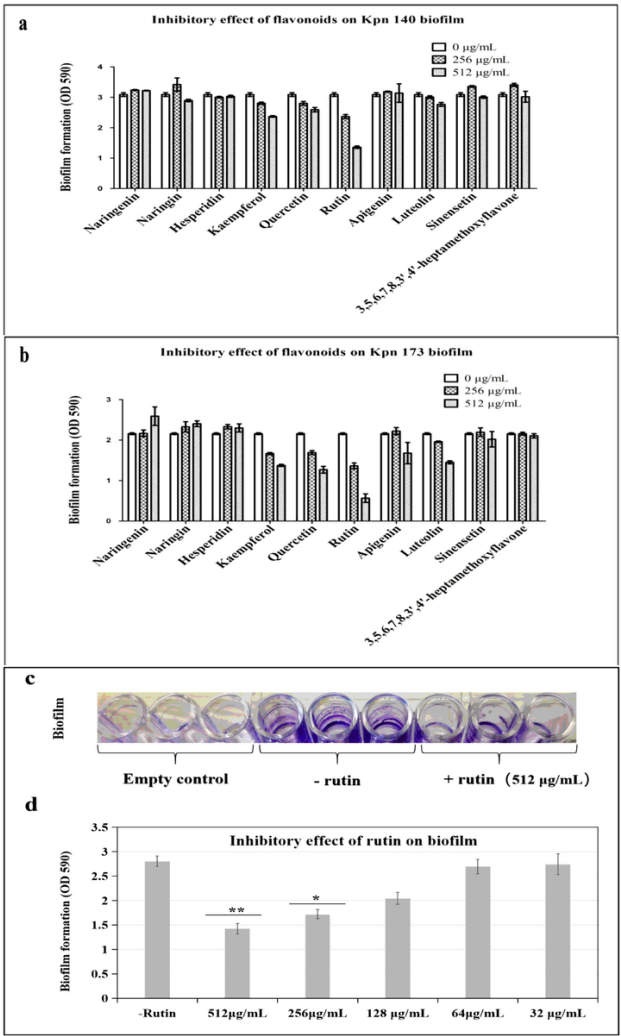
Figure 2

Inhibitory effect of flavonoids on the growth of drug-resistant K. pneumoniae. Effect of high concentration of naringin, rutin and the mixture(1/2 rutin+1/2 naringin) on Kpn495(a). Effect of high concentration of naringin, rutin and the mixture(1/2 rutin+1/2 naringin) on Kpn 499(b). Effect of rutin on the cell growth of K. pneumoniae isolates(c,d).

Figure 3

Inhibitory effect of flavonoids on the biofilm formation of K. pneumoniae. None dose-dependent effects of different flavonoids on biofilm formation by Kpn140(biofilm+++) (a). None dose-dependent effects of different flavonoids on biofilm formation by Kpn173(biofilm+++) (b). Effect of rutin on the biofilm formation of Kpn173(c). Effect of different concentrations of rutin on the biofilm formation of Kpn173(d). Biofilm was measured through crystal violet staining method. *P<0.01 vs control(-rutin). **P<0.05 vs control(-rutin).
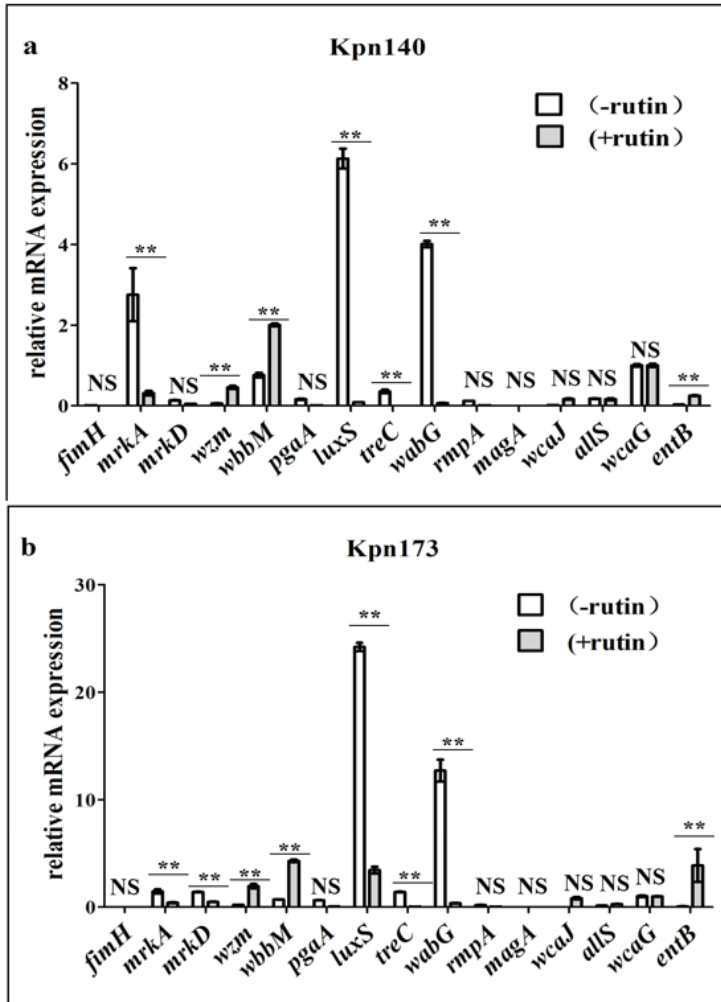
Figure 4

Gene expression profiles with and without rutin treatment. NS indicate not significant. *P<0.05 vs control(-rutin); **P<0.01 vs control(-rutin).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Detailinformationofthe177Kpstrains.xlsx
- StandardcurveforDPPHassay.xlsx
- Supplementaryfigure.docx