Research Article

Antimicrobial Activity of Flavonoids against Extended-Spectrum β-Lactamase (ESβL)-Producing Klebsiella pneumoniae

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Abstract

Purpose: In the present study, six flavonoids (5,7-dimethoxyflavanone-4′-O-β-D-glucopyranoside, 5,7-dimethoxyflavanone-4′-O-[2′′-O-(5''′-O-trans-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside, narigenin-7-O-β-D-glucopyranoside, 5,7,3′-trihydroxy-flavanone-4′-O-β-D-glucopyranoside, rutin, and nicotiflorin) isolated from Galium fissurense, Viscum album ssp. album and Cirsium hypoleucum were screened against extended-spectrum β-lactamase producing multidrug-resistant (trimetoprime-sulphametoxazole, sulbactam-ampicillin, clavulonate-amoxicillin, ceftriaxon, cefepime, imipenem, ceftazidime, tobramicin, gentamicin, ofloxacin, ciprofloxacin) bacteria Klebsiella pneumoniae (ESβLs).

Methods: We performed susceptibility testing according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) and used an inhibition endpoint for determination of the minimum inhibition concentrations (MICs).

Results: All the flavonoids showed in vitro antimicrobial activity against all the isolated strains of K. pneumoniae similar to the control antibacterial (ofloxacin) at the concentrations of 32 - 64 µg ml⁻¹; another control, ampicillin, had no activity. Since, ESβL-producing strains are known to be resistant to all β-lactam antibiotics, our results fall notably within the concentration range for antimicrobial activity.

Conclusion: To the best of our knowledge, this is the first report of the study of the activity of these flavonoids against (ESβL)-producing K. pneumoniae and may throw light to the low-toxicity of flavonoids, and their potentials for developing therapies for infections caused by ESβL-producing bacteria in the future. Further work is under investigation to identify their precise antibacterial mechanism.

Keywords: Antimicrobial activity, ESβLs, Flavonoids, Klebsiella pneumoniae, Ofloxacin, Ampicillin.
INTRODUCTION

Multiple drug resistance has significantly increased in recent years. The existence of enzymes of extended-spectrum β-lactamases (ESβLs) producing organisms that are resistant to virtually all β-lactam antibiotics have been reported. ESβLs are plasmid-mediated class A enzymes commonly found in the family Enterobacteriaceae, mainly K. pneumoniae. These microorganisms are Gram-negative rods that cause bacterial pneumonia and hospital-acquired infections. The increase in ESβL-producing organisms is sure to create significant therapeutic problems in the future. The available treatment regimens for infections caused by ESβL-producing bacteria are not always effective. Therefore, it is necessary to discover new antimicrobial compounds against ESβL-producing K. pneumoniae strains.

Flavonoids occur as aglycones, glycosides and methylated derivatives and are widely distributed in the plant kingdom. They have been reported to possess a variety of biological activities including antiallergic, antidiabetic, antiinflammatory, antiviral, antiproliferative and anticarcinogenic, hepatoprotective, and antioxidant activities. Since these secondary metabolites are synthesized by plants in response to microbial infections caused by ESβLs, producing bacteria and hospital-acquired infections. The increase in ESβL-producing organisms is sure to create significant therapeutic problems in the future. The available treatment regimens for infections caused by ESβL-producing bacteria are not always effective. Therefore, it is necessary to discover new antimicrobial compounds against ESβL-producing K. pneumoniae strains.

In this study, six flavonoids isolated from three plants used in traditional Turkish medicine against ten extended-spectrum β-lactamase producing multidrug-resistant (trimetoprim-sulphamethoxazole, sulfactam-ampicillin, clavulanate-amoxicilin, ceftriaxon, cefepime, imipenem, ceftazidine, tobramicin, gentamycin, ofloxacin, ciprofloxacin) bacteria K. pneumoniae (ESβLs) were presented. In vitro broth microdilution testing was performed in accordance with the guidelines of CLSI. Ampicillin and ofloxacin were used as control agents while K. pneumoniae RSKK was used as the test microorganism.

EXPERIMENTAL

Test compounds

5,7-dimethoxyflavanone-4'-O-β-D-glucopyranoside (1) was isolated from the ethylacetate extract of the leaves and stems of V. album ssp. album (Loranthaceae) from Armeniaca vulgaris Lam. by medium pressure liquid chromatography (MPLC) and 5,7-dimethoxyflavanone-4'-O-[2''-O-(5''-O-trans-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside (2) was also isolated from the n-butanol extract of the same plant using several chromatographic methods. A novel flavanone glucoside, 5,7,3'-trihydroxyflavanone-4'-O-β-D-glucopyranoside (3), was
isolated from the ethanol extract of the herb of *G. fissurensen*, in addition to naringenin-7-O-β-D-glucopyranoside (4). From the n-butanol extract of *C. hypoleucum* aerial parts (Asteraceae), after multi-stage column chromatographies, two flavonoids, quercetin-3-O-rutinoside (rutin) (5) and kaempferol-3-O-rutinoside (nicotiflorin) (6) were previously obtained. The structures of isolated compounds were elucidated by conventional methods of analysis, as well as by different NMR and MS techniques.

**Microbiological studies**

**Preparation of the test materials**

The flavonoids were dissolved in dimethylsulphoxide to a concentration of 256 µg ml⁻¹ and sterilized by filtration using 0.22 µm Millipore filter (MA 01730, USA) and then used as the stock solutions. Reference antibacterial agents of ampicillin (AMP; Faco), ofloxacin (OFX; Hoechst Marion Roussel) were obtained from their respective manufacturers and dissolved in phosphate buffer solution (ampicillin; pH: 8.0, 0.1 mol/L), and in water (ofloxacin). The stock solutions of these agents were prepared in medium according to as CLSI (formerly National Committee for Clinical Laboratory Standards) recommendations (CLSI; formerly NCCLS).

**Microorganisms and Inoculum preparation**

Isolated strains of ten *K. pneumoniae* that are resistant to trimetoprime-sulphamethoxazole (SXT; Oxoid; 25 µg; ≤10 mm), sulbactam-ampicillin (SAM; Oxoid; 20 µg; ≤11 mm), clavulanate-amoxicillin (AMC; Oxoid; 20 µg; ≤13 mm), ceftriaxon (CRO; Oxoid; 30 µg; ≤25 mm), cefepime (CPM; Oxoid; 30 µg; ≤14 mm), imipenem (IMP; Oxoid; 10 µg; ≤13 mm), ceftazidime (CAZ; Oxoid; 30 µg; ≤14 mm), tobramycin (TOB; Oxoid; 10 µg; ≤12 mm), gentamicin (GM; Oxoid; 10 µg; ≤12 mm), ofloxacin (OFX; Oxoid 5 µg; ≤12 mm), ciprofloxacin (CLP; Oxoid 5 µg; ≤13 mm) in disc diffusion test were used for the determination of antibacterial activity (minimum inhibition concentration, MIC). *K. pneumoniae* RSKK 574 (Refik Saydam Central Hygiene Institute-Culture Collection, The Ministry of Health of Republic of Turkiye, Ankara) was used as the control strain.

Mueller Hinton Broth (MHB; Oxoid) and Mueller Hinton Agar (MHA; Oxoid) were applied for growing and diluting of the bacteria suspensions. The microorganism suspensions used for inoculation were prepared at 10⁷ cfu (colony forming units)/ml by diluting fresh cultures at McFarland 0.5 density (10⁵ cfu ml⁻¹). Suspensions of all bacteria were added in each well of the diluted test compounds density of 10⁵ cfu ml⁻¹.

**Confirmatory test for ESβL-producing *K. pneumoniae* isolates**

The double-disc synergy and agar diffusion tests were used as screening tools to detect ESβL-producing strains. In the double-disc synergy test, the antibiotic discs (Oxoid) used were ceftoxime (30 µg) and ceftazidime (30 µg) placed on Mueller-Hinton agar adjacent to a co-amoxiclav disc (20 µg amoxicillin plus 10 µg clavulanate). The procedures and interpretation of the double-disc synergy test were described previously. The agar diffusion test was performed according to NCCLS guidelines. A ≤5 mm increase in a zone diameter for either ceftazidime/clavulanic acid (30 µg/ 10 µg) or cefotaxime/clavulanic acid (30 µg/ 10 µg) versus its zone when tested alone was taken as being indicative of ESβL-production.

**Antimicrobial activity evaluation**

The microdilution method was employed for antibacterial tests. Media were placed into each 96 wells of the microplates. Sample solutions at 256 µg ml⁻¹ were added into first rows of microplates and two-fold dilutions of the compounds (128-0.0312 µg ml⁻¹) were made by dispensing the solutions into the microplates. Sample solutions at 256 µg ml⁻¹ were added into first rows of microplates and two-fold dilutions of the compounds (128-0.0312 µg ml⁻¹) were made by dispensing the solutions into the remaining wells. 10 µl culture suspensions were inoculated into all the wells. The sealed microplates were incubated at 35°C for 18h. The lowest concentration of the flavonoids that completely inhibit macroscopic growth was determined and the MICs were recorded.
RESULTS
Antimicrobial effects of six flavonoids isolated from three Turkish plants on ten isolated strains of ESβL-containing *K. pneumoniae* are presented in Table 1. In our previous studies, we reported that two new flavonoids, 5,7,3’-trihydroxy-flavanone-4’-O-β-D-glucopyranoside (1) and 5,7-dimethoxyflavanone-4’-O-[2’’-O-(5’’’-O-trans-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside (2), were isolated for the first time from *V. album* ssp. *album* and *G. fissurense*, respectively. When compared with control agents (ampicillin and ofloxacin), all of the tested flavonoids (1-6) showed remarkable activities against isolated strains of all *K. pneumoniae* at 32 and 64 µg ml⁻¹ concentrations, which are close to the effective concentrations exhibited by the control agents. Notably, these compounds possessed quite remarkable antimicrobial activities against isolates, *Kp₃*, *Kp₅*, *Kp₆* and *Kp₁₀*, similar ampicillin and ofloxacin (32 µg ml⁻¹). Isolates *Kp₁*, *Kp₂*, *Kp₄*, *Kp₇₋₉* were inhibited at a concentration of 64 µg ml⁻¹ by the tested flavonoids, the observed activities being twice the dose of ampicillin and ofloxacin (32 µg ml⁻¹). On the other hand, the activity at 8 µg ml⁻¹ concentration of the flavonoids (1-6) against *K. pneumoniae* RSKK 574 seems less active when compared with ampicillin (2 µg ml⁻¹) and ofloxacin (<0.12 µg ml⁻¹).

**Fig 1:** Structures of investigated compounds
Table 1: Antimicrobial activity as MICs (µg ml⁻¹) of flavonoids and the references against tested isolated strains of Klebsiella pneumoniae (Kpₙ₁-10)

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<td>R (SXT; AMC; SAM; CRO; CPM; TOB)</td>
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<td>7-O-β-D-glucopyranoside (4) Quercetin-3-O-rutinoside (rutin) (5) Kaempferol-3-O-rutinoside (nicotiflorin) (6) Ampicillin</td>
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DISCUSSION
Plasmid-mediated ESβLs pose a worldwide resistance problem. These isolates are usually resistant to all aminoglycosides, third- and fourth-generation cephalosporins and monobactams. Such isolates are involved frequently in outbreaks of infection, particularly in high-risk areas, such as...
active flavonoids
are in consonance with the findings of Xu and Lee. These results
showed no activity against
K. pneumoniae
ATCC 13883.
These inconsistencies may be
due to variations within each assay.
Furthermore, it was not stated whether the
test flavonoids were obtained from a
commercial or natural source.

To the best of our knowledge, this is the first report on the inhibitory activity of 5,7-
dimethoxyflavanone-4'-O-β-D-glucopyranosi-
de (1), 5,7-dimethoxyflavanone-4'-O-[2''-O-
(5''-O-trans-cinnamoyl)-β-D-apiofuranosyl]-β-
D-glucopyranoside (2), 5,7,3'-trihydroxy-
flavanone-4'-O-β-D-glucopyranoside (3),
naringenin-7-O-β-D-glycopyranoside (4), and
nicotiflorin (6) against ESβL-producing K.
Pneumoniae isolates.

CONCLUSION
All the flavonoids (1-6) showed in vitro
antimicrobial activity against all the isolated
strains of K. pneumoniae, similar to that
produced by the control antibacterial
(oxefloxacin) at the concentration of 32-64 µg
ml⁻¹; on the other hand, another control,
ampicillin, had no activity. To the best of our
knowledge, this is the first report of the
evaluation of the activity of these flavonoids
against (ESβL)-producing K. pneumoniae. On
the basis of these data presented, these
flavonoids may be considered potential
therapeutic compounds for infections that may
be caused by ESβL-producing bacteria in the
future. Therefore, further work is under way to
develop better drugs against clinical ESβL-producing K.
Pneumoniae.

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