Tropical Journal of Pharmaceutical Research, December 2008; 7 (4): 1151-1157

Available online at http://www.tjpr.org

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, naringenin-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-amoxicilin, 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.*

Received: 10 July 2008 *Revised accepted: 22 August 2008*

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INTRODUCTION

Multiple drug resistance has significantly increased in recent years. The existence of enzymes of extended-spectrum β-lactamases (ESβLs) producing organism that are resistant to virtually all β-lactam antibiotics have been reported[1]. ESβLs are plasmid-mediated class A enzymes commonly found in the family Enterobacteriaceae, mainly *K. pneumoniae*[2]. These microorganisms are Gram-negative rods that cause bacterial pneumonia and hospital-acquired infections[3]. 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 infection, it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms[4-6]. Also, their antimicrobial activities against some Gram-negative and Gram-positive bacteria have been reported in many papers[2]. Therefore, the flavonoids may be promising new class compounds in antimicrobial therapy.

In this study, six flavonoids isolated from three medicinal plants, which are used in Turkish folk medicine, were screened against *K. pneumoniae. Galium fissurense* Ehrend. & Schönb.-Tem. (Rubiaceae), which is an endemic plant in Turkey, has long been used in folk medicine for a variety of purposes, especially as a diuretic, astringent, choleretic and in the treatment of some stomach, gout and epilepsy[7]. *Viscum album* L. (Loranthaceae) is reported to have various biological activities such as hypoglycemic, anti-inflammatory, anti-viral, anti-atherogenic, and analgesic activities. On the other hand, we have previously shown that the extracts of *V. album* ssp. *album* have inhibitory effects on IL-1α[8]. In folk medicine, the decoction of *Cirsium* sp. (Asteraceae) seeds and roots is used for healing hemorrhoids and the flowers are a good remedy for peptic ulcer. In addition, the stem is also used for treating ailments such as cough and bronchitis in Anatolia[9]**.** In a previous work of our group, the antimicrobial activities of various *C. hypoleucum* DC. extracts (methanol, n-hexane, chloroform, ethylacetate, n-butanol and remaining water extracts) were tested against various microorganisms including *Herpes simplex* Type-1 (*HSV*-1) and *Parainfluenza viruses* (*PIV*)[10].

In this study, antimicrobial activity results of six flavonoids isolated from three plants used in traditional Turkish medicine against ten extended-spectrum β-lactamase producing multidrug - resistant (trimetoprime - sulphametoxazole, sulbactam-ampicillin, clavulonate-amoxicilin, ceftriaxon, cefepime, imipenem, ceftazidime, tobramicin, gentamicin, 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[11].

A novel flavanone glucoside, 5,7,3'-trihydroxy-flavanone-4'-*O*-β-D-glucopyranoside **(3)**, was

isolated from the ethanol extract of the herb of *G. fissurense*, in addition to naringenin-7-*O*-β-D-glucopyranoside **(4)**[7]. 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[9]. 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)[12].

Microorganisms and Inoculum preparation

Isolated strains of ten *K. pneumoniae* that are resistant to trimetoprime-sulphametoxazole (SXT; Oxoid; 25 µg; ≤10 mm), sulbactam-ampicillin (SAM; Oxoid; 20 µg; ≤11 mm), clavulonate-amoxicilin (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), tobramicin (TOB; Oxoid; 10 µg; ≤12 mm), gentamicin (GM; Oxoid; 10 µg; ≤12 mm), ofloxacin (OFX; Oxoid 5 µg; ≤12 mm), ciprofloxacin (CIP; 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[12].

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 10^7 cfu (colony forming units)/ml by diluting fresh cultures at McFarland 0.5 density (10^8 cfu ml^{-1}). Suspensions of all bacteria were added in each well of the diluted test compounds density of 10^5 cfu ml^{-1} [12, 13].

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 cefotaxime (30 µg) and ceftazidime (30 µg) placed on Mueller-Hinton agar adjacent to a co-amoxiclav disc (20µg amoxicillin plus 10µg clavulanate). The procedures and interpretation of the double-disc synergy test were described previously[14]**.** The agar diffusion test was performed according to NCCLS guidelines[12]. 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[15].

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 remaining wells. 10 µl culture suspensions were inoculated into all the wells. The sealed microplates were incubated at 35ºC for 18h. The lowest concentration of the flavonoids that completely inhibit macroscopic growth was determined and the MICs were recorded[16].

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[7,11]. 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, *Kp$_3$, Kp$_5$, Kp$_6$* and *Kp$_{10}$,* similar ampicillin and ofloxacin (32 μg ml^{-1}). Isolates *Kp$_1$, Kp$_2$, Kp$_4$, Kp$_{7-9}$* 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}).

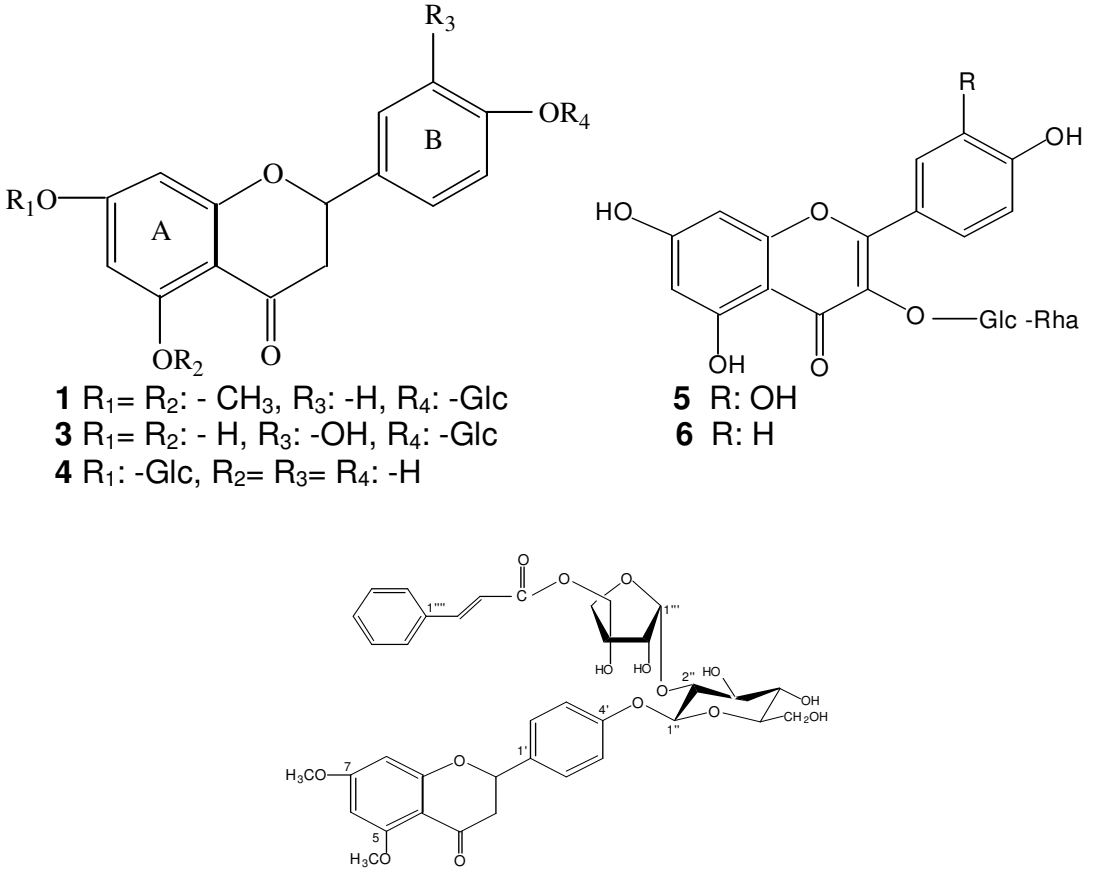

Fig 1: Structures of investigated compounds

Table 1: Antimicrobial activity as MICs (μg ml^{-1}) of flavonoids and the references against tested isolated strains of *Klebsiella pneumoniae* (*Kp*$_{n: 1-10}$)

	Microorganisms										
Compounds	*Kp*$_1$ R (SXT; AMC; SAM; CRO; CPM; IMP; TOB)	*Kp*$_2$ R (SXT; AMC; CRO; IMP; TOB)	*Kp*$_3$ R (AMC ; SAM; CRO; CPM)	*Kp*$_4$ R (AMC; CRO; CAZ; CPM; TOB; GM)	*Kp*$_5$ R (AMC ; CRO; CPM; GM)	*Kp*$_6$ R (AMC; SAM; CRO; CPM)	*Kp*$_7$ R (SXT; AMC; SAM; CRO; CPM; OFX)	*Kp*$_8$ R (SXT; AMC, SAM; CRO; CPM; OFX; CIP)	*Kp*$_9$ R (SXT; AMC, SAM; CRO; CPM; OFX; CIP)	*Kp*$_{10}$ R (AMC; SAM; CRO; OFX)	*K. pneumoniae* RSKK 574
5,7-dimethoxyflavanone-4'-*O*-β-D-glucopyranoside (**1**)	64	64	32	64	32	32	64	64	64	32	8
5,7-dimethoxyflavanone-4'-*O*-[2''-*O*-(5'''-*O*-*trans*-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside (**2**)	64	64	32	64	32	32	64	64	64	32	8
5,7,3'-trihydroxy-flavanone-4'-*O*-β-D-glucopyranoside (**3**)	64	64	32	64	32	32	64	64	64	32	8
naringenin-7-*O*-β-D-glucopyranoside (**4**)	64	64	32	64	32	32	64	64	64	32	8
Quercetin-3-*O*-rutinoside (rutin) (**5**)	64	64	32	64	32	32	64	64	64	32	8
Kaempferol-3-*O*-rutinoside (nicotiflorin) (**6**)	64	64	32	64	32	32	64	64	64	32	8
Ampicillin	-	-	-	-	-	-	-	-	-	-	2
Ofloxacin	32	32	32	32	32	32	32	32	32	32	<0.12

R: Resistant, SXT: Trimetoprime-Sulphametoxazole, SAM: Sulbactam-Ampicillin, AMC: Clavulonate-Amoxicillin, CRO: Ceftriaxon, CPM: Cefepime, IMP: Imipenem, TOB: Tobramicin, CAZ: Ceftazidime, GM: Gentamicin, CIP: Ciprofloxacin.

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

intensive care or neonatal units. Since the discovery of ESβL-producing coliforms in the mid-1980s, over 100 types of different enzymes have been described and have become a worldwide problem. Thus, the members of Enterobacteriaceae producing ESβL are a clinical threat and have been associated with increasing mortality in patients with severe infection. ESβLs are mutant enzymes, which derived from TEM or SHV (class A) enzymes. They confer variable levels of resistance to cefotaxime, ceftazidime and other broad-spectrum cephalosporins and to monobactams such as aztreonam. Thus, the choice of effective and safe drugs to be used against ESβL is shrinking rapidly. Therefore, in recent years, researches are increasingly turning their attention to folk medicine for new leads to develop better drugs against clinical ESβL-producing *K. pneumoniae* isolates[2,17-19]

In this study, structure-activity relationships do not appear to play a role here as all six flavonoids exhibited similar MICs (32–64 µg ml^{-1}) against all of the *K. pneumoniae* strains. We observed that active flavonoids **3–6** have an obligatory C–4 keto group and hydroxyl group substitutions at C–5 and have at least one hydroxyl group on ring B. These results are in consonance with the findings of Xu and Lee[19]. On the other hand, although the other active flavonoids **1** and **2** have methoxyl group substitutions at C-5 and C-7, these compounds also exhibited antimicrobial activity against *K. pneumoniae* at similar MICs values to those of ampicillin and ofloxacin. Alcaraz *et al* and Xu and Lee demonstrated that substitution with methoxyl groups drastically decreased the antibacterial of flavonoids[20,21]. Additionally, it is reported that rutin **(5)** from *Linum capitatum* Kit. flowers inhibited the growth of *P. aeruginosa*, but did not show any activity against *E. coli* different from other Gram-negative bacteria[21]. According to the other report, the same compound tested by the disc-diffusion method showed no activity against *K. pneumoniae* ATCC 13883[20]. 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-glucopyranoside **(1)**, 5,7-dimethoxyflavanone-4'-*O*-[2''-*O*-(5'''-*O*-*trans*-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside**(2)**, 5,7,3'-trihydroxyflavanone-4'-*O*-β-D-glucopyranoside **(3),** naringenin-7-*O*-β-D-glucopyranoside **(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 (ofloxacin) 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 identify their precise antibacterial mechanism. Additionally, the antimicrobial activity of some antibacterials in combination with these flavonoids against extended-spectrum β-Lactamase (ESβL)-producing *K. pneumoniae* may also need to be evaluated for the treatment of infections caused by ESβL-producing bacteria.

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