Antiviral and Antimicrobial Assessment of Some Selected Flavonoids

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In the current study, the results of antibacterial, antifungal, and antiviral activity tests of four flavonoid derivatives, scandenone (**1**), tiliroside (**2**), quercetin-3,7-*O*-α-L-dirhamnoside (**3**), and kaempferol-3,7-*O*-α-L-dirhamnoside (**4**), are presented. Antibacterial and antifungal activities of these compounds were tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Enterococcus faecalis*, as well as the fungus *Candida albicans* by a microdilution method. On the other hand, both DNA virus *Herpes simplex* (HSV) and RNA virus *Parainfluenza-3* (PI-3) were employed for antiviral assessment of the compounds using Madin-Darby bovine kidney and Vero cell lines. According to our data, all of the compounds tested were found to be quite active against *S. aureus* and *E. faecalis* with MIC values of 0.5 μg/ml, followed by *E. coli* (2 μg/ml), *K. pneumoniae* (4 μg/ml), *A. baumannii* (8 μg/ml), and *B. subtilis* (8 μg/ml), while they inhibited *C. albicans* at 1 μg/ml as potent as ketoconazole. However, only compound **3** displayed an antiviral effect towards PI-3 in the range of 8–32 μg/ml of inhibitory concentration for cytopathogenic effect (CPE).

Key words: Flavonoids, Antiviral Activity, Antimicrobial Activity

Introduction

Flavonoids are a group of polyphenolic compounds ubiquitous in many plants, in which they occur as the free forms, glycosides, as well as methylated derivatives. In general, flavonoids possess a chromane ring attached to an additional aromatic ring, which derives from malonyl-CoA and *p*-coumaroyl-CoA (Marten and Mithöfer, 2005). Up to date, over 5000 flavonoids have been isolated from fruits, vegetables, and beverages (*e.g.* wine and tea) derived from plants and a high portion of flavonoids occurs naturally as water-soluble glycosides (Kuhnau, 1976). Flavonoids have been in focus due to their nutraceutical and therapeutical significance, as they exhibit divergent biological activities such as antioxidant, anti-inflammatory, cardioprotective, antibacterial, antitumor, hepatoprotective, antiviral activities (Havsteen, 1983; Silver and Bostian, 1990; Ielpo *et al.*, 2000; Xu and Lee, 2001; Matsuda *et al.*, 2002; Toker *et al.*, 2004a; Haenen *et al.*, 2006; Moonfrom *et al.*, 2006; Alvesalo *et al.*, 2006).

On the other hand, microbial resistance has become a major concern all over the world and, therefore, ample investigation and research in this scope are dreadfully compulsory. Coming to the fore more recently is that there is an increasing interest in flavonoids due to their anti-infective properties (Cushni and Lamb, 2005). For instance, the flavonoids quercetin, kaempferol as well as the flavonoid glycosides rutin and isoquercitrin were reported to have antibacterial and antifungal activities (Beschia *et al.*, 1984). Therefore, in this study, we aimed to examine *in vitro* antibacterial, antifungal, and antiviral activities of four flavonoid derivatives; scandenone (**1**), tiliroside (**2**), quercetin-3,7-*O*-α-L-dirhamnoside (**3**), and kaempferol-3,7-*O*-α-L-dirhamnoside (**4**).

Material and Methods

Isolation of the compounds

Compounds **1**–**4** were previously isolated by conventional chromatographic methods. Compound **1** was isolated from the chloroform extract of the fruits of *Maclura pomifera* (Rafin.) Schnider (Moraceae) by preparative thin layer chromatography followed by crystallization in methanol as described formerly and identified as scandenone (**1**), a prenylated isoflavone derivative (Toker and Erdogan, 1998). Compounds **3** and **4** were obtained from the ethanolic extract, prepared with

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the leaves of *Tilia argentea* Desf. ex DC. (Tiliaceae), by silica gel column chromatography, as we reported previously, and elucidated as quercetin-3,7-*O*-α-L-dirhamnoside (**3**) and kaempferol-3,7-*O*-α-L-dirhamnoside (**4**), respectively, by one- and two-dimensional spectroscopic techniques (Toker *et al.*, 2004b). Compound **2** was also isolated from the same extract of *T. argentea* as well as compounds **3** and **4** and has not been reported before elsewhere. It was isolated from fractions 30–42 collected from the first column chromatography mentioned in Toker *et al.*'s study (2004b), and crystallized in a mixture of chloroform/methanol. Compound **2** was recognized as tiliroside [kaempferol 3-*O*-β-D-(6″-*O*-coumaroyl) glucopyranoside] using chromatographic and spectroscopic techniques by comparing with its authentic sample.

Microbiological studies

Compounds **1**–**4** were dissolved in ethanol/hexane (1:1, v/v) by using a 1% Tween 80 solution at a final concentration of 1024 μg/ml, sterilized by filtration using a 0.22 μm Millipore (MA, USA) filter, and used as the stock solutions. Standard antibacterial powders of ampicilline (AMP; Fako Drug Company, Istanbul, Turkey) and ofloxacine (OFX; Hoechst Marion Roussel, Istanbul Headquarters, Turkey) along with standard antifungal powders of ketoconazole (KET; Bilim Drug Company, Istanbul, Turkey) were obtained from the respective manufacturers and dissolved in phosphate buffer solution (pH 8.0, AMP, 0.1 mol/l), dimethylsulphoxide (DMSO) (KET), and water (OFX). The stock solutions of the agents were prepared in medium according to the NCCLS recommendations (National Committee for Clinical Laboratory Standards, 1996).

Microorganisms

Standard strains of bacteria, namely *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 10145), *Proteus mirabilis* (ATCC 7002), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae* (RSKK 574), *Acinetobacter baumannii* (RSKK 02026) (RSSK: Culture Collection of Refik Saydam Central Hygiene Institute, Ankara, Turkey) for the determination of antibacterial activity and a standard strain of the yeast-like fungus *Candida albicans* (ATCC 10231) for evaluation of the antifungal activity were employed.

Inoculum preparation

Mueller-Hinton broth (Difco) and Mueller-Hinton agar (Oxoid) were applied for growing and diluting of the bacteria. As for growing and diluting of the fungus, Sabouraud liquid medium (Oxoid) and Sabouraud dextrose agar (SDA) (Oxoid) were applied. The medium RPMI-1640 with L-glutamine was buffered, pH 7, with 3-(*N*-morpholino)-propansulfonic acid (MOPS). Prior to the tests, strains of bacteria and the fungus were cultured on media and passaged at least twice to ensure purity and viability at 35 °C for 24 to 48 h. Culture suspensions were prepared according to the NCCLS M27-A (Özçelik *et al.*, 2004). The bacterial suspensions used for inoculation were prepared at 10^5 cfu/ml by diluting fresh cultures at McFarland 0.5 density (10^8 cfu/ml). The fungus suspension was prepared by the spectrophotometric method of inoculum preparation at a final culture suspension concentration of 2.5×10^3 cfu/ml (National Committee for Clinical Laboratory Standards, 1996).

Antibacterial and antifungal tests

The microdilution method was employed for antibacterial and antifungal activity tests. Media were placed into each well of a microplate. Extract solutions at 1024 μg/ml were added into the first raw of microplates and two-fold dilutions of the compounds (512–0.25 μg/ml) were made by dispensing the solutions to the remaining wells. 10 μl of culture suspensions were inoculated into all the wells. The sealed microplates were incubated at 35 °C for 24 h and 48 h in a humid chamber. The lowest concentrations of the extracts that completely inhibited macroscopic growth and minimum inhibitory concentrations (MICs) were determined. Antibacterial activity of the extracts was tested against three Gram-positive and five Gram-negative bacterial strains, using AMP and OFX as the references. *C. albicans* was included in the antifungal screen with the reference KET (National Committee for Clinical Laboratory Standards, 2002; Özçelik *et al.*, 2005).

Cytotoxicity and antiviral tests

Cell line and growth condition

Vero cell line (African green monkey kidney) and Madin-Darby bovine kidney (MDBK) were obtained from the Department of Virology, Faculty of Veterinary, Ankara University (Turkey).

The cell cultures were grown in Eagle's Minimal Essential Medium (EMEM) enriched with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 100 mg/ml of streptomycin and 100 IU/ml of penicillin in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were harvested using trypsin solution (Bipco Life Technologies, London, UK).

Test viruses

In order to determine the antiviral activity of compounds **1**–**4**, *Herpes simplex* virus (HSV) and *Parainfluenza*-3 virus (PI-3), obtained from the Department of Virology, Faculty of Veterinary, Ankara University (Turkey), were employed.

Antiviral activity

Media (EMEM) were placed into each well of the 96-well microplates (Greiner, Essen, Germany). Stock solutions of compounds **1**–**4** were added into the first raw of microplates and two-fold dilutions of the compounds (512–0.25 μg/ml), which were prepared according to Log_2 on the microplates, were made by dispensing the solutions to the remaining wells. Acyclovir (Biofarma, Istanbul, Turkey) and oseltamivir (Roche, Istanbul Headquarters, Turkey) were used as the references. Strains of HSV and PIV titers were calculated by the Frey and Liess (1971) method as tissue culture infecting dose ($TCID_{50}$) and inoculated into all the wells. The sealed microplates were incubated in 5% CO_2 at 37 °C for 2 h to detect the possible antiviral activities of the samples. Following incubation, 50 μl of the cell suspension of 300,000 cells/ml, which were prepared in EMEM together with 5% fetal bovine serum, were put in each well and the plates were incubated in 5% CO_2 at 37 °C for 48 h. After that, the cells were evaluated, using a cell culture microscope, by comparing with treated and untreated control cultures and with acyclovir and oseltamivir. Consequently, maximum cytopathogenic effect (CPE) concentrations as the indicator of antiviral activities of the extracts were determined (Özçelik *et al.*, 2005).

Cytotoxicity

The maximum non-toxic concentration (MNTC) of each sample was determined by the method described beforehand by Özçelik *et al.* (2005) based on cellular morphologic alteration. Several concentrations of each sample were placed in contact with a confluent cell monolayer and incubated in 5% CO_2 at 37 °C for 48 h. MNTCs were determined by comparing treated and controlling untreated cultures.

Results and Discussion

The results of antibacterial, antifungal, and antiviral activity tests of four flavonoid derivatives, scandenone (also known as warangalone) (**1**), tiliroside (**2**), quercetin-3,7-*O*-α-L-dirhamnoside (**3**), and kaempferol-3,7-*O*-α-L-dirhamnoside (**4**), are presented in Tables I and II. Antibacterial and antifungal activities of the compounds **1**–**4** were screened against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus au-*

Table I. Antimicrobial activity of the extracts and references as minimum inhibitory concentrations (MICs) (μg/ml).

Compound	*E. coli*	*P. aeruginosa*	*P. mirabilis*	*K. pneumoniae*	*A. baumannii*	*S. aureus*	*B. subtilis*	*E. faecalis*	*C. albicans*
Scandenone (**1**)	2	32	16	4	8	0.5	8	0.5	1
Tiliroside (**2**)	2	32	16	4	8	0.5	8	0.5	1
Quercetin-3,7-*O*-α-L-dirhamnoside (**3**)	2	32	16	4	8	0.5	8	0.5	1
Kaempferol-3,7-*O*-α-L-dirhamnoside (**4**)	2	32	16	4	8	0.5	8	0.5	1
AMP[a]	2	–[d]	2	2	2	0.12	0.5	0.5	–
OFX[b]	0.12	1	< 0.12	0.12	0.12	0.5	1	1	–
KET[c]	–	–	–	–	–	–	–	–	1

[a] AMP, ampicilline.
[b] OFX, ofloxacine.
[c] KET, ketoconazole.
[d] No activity observed.

Table II. Antiviral activity of the extracts and references.

Compound	MDBK cells			Vero cells		
	MNTC[a] [μg/ml]	CPE[b] inhibitory concentration [μg/ml]		MNTC [μg/ml]	CPE inhibitory concentration [μg/ml]	
		HSV			PI-3	
		Max.	Min.		Max.	Min.
Scandenone (**1**)	64	–[c]	–	64	–	–
Tiliroside (**2**)	64	–	–	64	–	–
Quercetin-3,7-*O*-α-L-dirhamnoside (**3**)	64	–	–	64	32	8
Kaempferol-3,7-*O*-α-L-dirhamnoside (**4**)	64	–	–	64	–	–
Acyclovir	16	16	< 0.25	–	–	–
Oseltamivir	–	–	–	32	32	< 0.25

[a] MNTC, maximum non-toxic concentration.
[b] CPE, cytopathogenic effect.
[c] No activity observed.

reus, *Bacillus subtilis*, and *Enterococcus faecalis* as well as the fungus *Candida albicans* by the microdilution method. As seen in Table I, all four compounds were found to be the most active against *S. aureus* and *E. faecalis* with a MIC value of 0.5 μg/ml, followed by *E. coli* (2 μg/ml), *K. pneumoniae* (4 μg/ml), *A. baumannii* and *B. subtilis* (8 μg/ml). *P. mirabilis* and *P. aeruginosa* were the most resistant bacteria against compounds **1**–**4** (16 and 32 μg/ml, respectively). Notably, antibacterial activity of the compounds was as potent as AMP and OFX towards *S. aureus* and *E. faecalis*. These compounds also possessed a quite remarkable antifungal activity against *C. albicans* which was the same as for ketoconazole (1 μg/ml).

As shown in Table II, none of the compounds had the ability to inhibit the DNA virus HSV, while only quercetin-3,7-*O*-α-L-dirhamnoside (**3**) had inhibitory activity against the RNA virus PI-3 in the range of 8–32 μg/ml of minimum and maximum CPE inhibitory concentrations, respectively. The inhibitory concentration range of this compound is on a vast scale, which resembles to that of oseltamivir (32 to < 0.25 μg/ml). Besides, the MNTC value of compound **3** (64 μg/ml) was observed to be better than that of oseltamivir (32 μg/ml).

Antimicrobial activity of pure flavonoid derivatives or flavonoid-containing plant extracts has been extensively studied so far and summarized in some recent excellent reviews (Perez, 2003; Erlund, 2004; Cushni and Lamb, 2005; Rios and Recio, 2005; Khan *et al.*, 2005). Quercetin and kaempferol are known to be the most common flavonols present in many plants in different glycosidic forms. In many studies, they or their various glycosides have been proved to possess antimicrobial activity or, in other words, antimicrobial activity of plant extracts (*e.g. Rubus ulmifolius*, *Combretum erythrophyllum*, *Morus alba*, *Trollius chinensis*, and propolis) has been attributed to quercetin and kaempferol, which is in accordance with our data (Panizzi *et al.*, 2002; Gatto *et al.*, 2002; Li *et al.*, 2002; Du *et al.*, 2003; Martini *et al.*, 2004; Kosalec *et al.*, 2005). In the review of Bylka *et al.* (2004), it was suggested that the antibacterial effect towards Gram-negative bacteria is higher with flavones, while flavonoids containing two or three hydroxy groups in rings A and B are more active on inhibition of Gram-positive bacteria. However, in our study, all four flavonoid derivatives, consisting of one prenylated isoflavone and three flavonol glycosides, exhibited an equal strength of antibacterial and antifungal activities, independent of their structural substitutions. In contrast to our study Mitrokotsa *et al.* (1993) stated that tiliroside isolated from *Plantanus orientalis* was found to be weakly active or inactive to *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*, and *K. pneumaniae*. However, tiliroside was reported usually to be a mixture of *cis* and *trans* isomers from *Rosa canina* and since its *cis* isomer has been

concluded to have an antibacterial effect in general, its antibacterial activity was attributed solely due to the *cis* isomer form (Liu *et al.*, 1999; Kumarasamy *et al.*, 2003). In the report of Picman *et al.* (1995), among the 25 flavonoids they screened against the mycelial growth of *Verticillium alboatrum*, it was stated that the unsubstituted flavonoids had stronger growth inhibitory activity and, in most cases, increasing the number of substitutions such as hydroxylation, methoxylation, and glycosylation resulted in the loss of antifungal activity. However, our compounds were equally active against *C. albicans*, whereas they did not share the same substitional patterns. In one of the previous studies, kaempferol-3-*O*-α-L-rhamnopyranoside was shown to inhibit the replication of HSV, whereas its 3,7-dirhamnoside in our study was observed to be inactive against the same virus as speculated by Bylka *et al.* (2004). They stated that flavonoid glycosides are less active in regard to antimicrobial activity (Almeida *et al.*, 1998).

On the other hand, quercetin has been shown to be effective against divergent virus types by many researchers, which supports our present data on quercetin-3,7-*O*-α-L-dirhamnoside (**3**). In one of the earliest studies, oral application of quercetin in mice was found to display a protective effect towards intraperitoneal encephalomyocarditis, $Mengo_{M,L}$ and $Mengo_{M}$ virus infections, but not against intracerebral challenge with $Mengo_{M}$ virus, and it was not virucidal and did not interfere with *Mengo* virus replication in L cells (Veckenstedt and Puszhai, 1981). Veckenstedt *et al.* (1987) also proved the potentiative interaction of quercetin with murine alpha/beta interferon (MuIFN-α/β) in mice against *Mengo* virus infection. Moreover, quercetin was reported to enhance greatly the antiviral effect of tumor necrosis factor (TNF) that produces a dose-dependent inhibition of vesicular stomatitis virus (VSV), *Encephalomyocarditis* virus (EMCV), and *Herpes simplex* virus type 1 (HSV-1) replication in WISH cells (Ohnishi and Bannai, 1993).

In contrary, the aqueous extract of *T. argentea* was also examined for its antimicrobial activity against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa*, together with the fungus *C. albicans* by a disk diffusion method, but, interestingly, the extract did not exhibit any antimicrobial activity (Yildirim *et al.*, 2000). This difference might be resulting from the methods used or solubility problems. It was also speculated that the microdilution is the best manner for determining the actual potency of pure compounds, which the solubility is generally requisite for crude extracts (Rios and Recio, 2005).

On the other hand, prenyl substitution of isoflavonoids has been suggested to increase the antifungal activity (Tahara and Ibrahim, 1995). In Mahmoud's study (1981), two isoflavones, namely osajin and pomiferin, isolated from *Maclura pomifera* were reported to exhibit interesting antibacterial activity against *E. coli* (25 and 12.5 μg/ml, respectively) and *Salmonella gallinarum* (25 μg/ml for each) and to moderately inhibit *Mycobacterium smegmatis* (6.25 μg/ml for each) as compared to streptomycin sulphate, where scandenone (**1**), a prenylated isoflavone also obtained from *M. pomifera*, revealed strong antibacterial and antifungal activities against *E. coli* (2 μg/ml), *K. pneumoniae* (4 μg/ml), *E. faecalis* (0.5 μg/ml), and *C. albicans* (1 μg/ml).

It is evident that structure-activity relationship exists between the various flavonoids and their antimicrobial activity in most cases. In conclusion, our results demonstrate that scandenone (**1**), tiliroside (**2**), quercetin-3,7-*O*-α-L-dirhamnoside (**3**), and kaempferol-3,7-*O*-α-L-dirhamnoside (**4**) possess severe antibacterial and antifungal activities, whereas only quercetin-3,7-*O*-α-L-dirhamnoside (**3**) exert noticeable antiviral activity against PI-3 virus. This is the first report describing the antimicrobial potentials of scandenone (**1**), quercetin-3,7-*O*-α-L-dirhamnoside (**3**), and kaempferol-3,7-*O*-α-L-dirhamnoside (**4**), except for tiliroside (**2**).

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