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Antimicrobial and Anti-Lipase Activity of Quercetin and Its C2-C16 3-O-Acyl-Esters

Maria Teresa Gatto,[a] Serena Falcocchio,[a] Eleonora Grippa,[a] Gabriela Mazzanti,[a] Lucia Battinelli,[a] Giovanni Nicolosi,[b] Daniela Lambusta[b] and Luciano Saso[a,*]

[a]*Department of Pharmacology of Natural Substances and General Physiology, University of Rome "La Sapienza", P.le Aldo Moro 5, 00185 Rome, Italy*

[b]*Istituto CNR per lo Studio delle Sostanze Naturali di Interesse Alimentare e Chimico-Farmaceutico, Via del Santuario 110, 95028 Valverde (CT), Italy*

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Abstract—Neither quercetin (Q), nor 3-*O*-acylquercetines, up to 100 μg/mL, had any significant activity on selected gram-positive strains (*Staphylococcus aureus*, *Bacillus subtilis*, *Listeria ivanovi*, *Listeria monocytogenes*, *Listeria serligeri*), gram-negative strains (*Escherichia coli*, *Shigella flexneri*, *Shigella sonnei*, *Salmonella enteritidis*, *Salmonella tiphymurium*) and yeasts (*Candida albicans* and *Candida glabrata*). In addition, we confirmed the known anti-HIV activity of Q (80% inhibition at 40 μM), which might depend on the free hydroxyl in the C-3 position, as suggested by the lack of activity of the 3-*O*-acylquercetines. Finally, we described an interesting inhibitory activity on *Candida rugosa* lipase by Q ($IC_{16} = 10^{-4}$ M) and its esters (3-*O*-acylquercetines) which, in vivo, could play an important role against lipase producing microorganisms. In particular, 3-*O*-acyl-quercetines, being more active ($IC_{16} = 10^{-4}$–10^{-6} M) and more lipophilic, could be more effective than Q when applied to the skin or mucosae, and deserve to be studied further.

Quercetin (Q), one of the most studied flavonoids, has been reported to have antioxidant,[1] anti-cancer,[2] antithrombotic,[3,4] antimicrobial, antiviral and other activities. In particular, concerning the two latter properties:

1. Q, at high concentrations, appeared active against different microorganisms including *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus* and *Staphylococcus epidermidis*;[5] *Aspergillus flavus* and *Aspergillus parasiticus*.[6] Additionally, the in vivo antimicrobial activity of certain natural substances could be due to their ability to interfere with virulence factors such as lipase.[7,8] Indeed, the anti-lipase activity of a number of flavonoids,[9] but not of Q, has been described.
2. Q and other flavonoids appeared active against different viruses,[10] including HIV,[11] probably due to the inhibition of reverse transcriptase.[12]

Thus, the aim of the present study was to evaluate the effect of Q and its C2–C16 3-*O*-acyl derivatives on selected bacteria (five gram-positive and five negative strains), yeasts (four *Candida* strains) and HIV-1. Furthermore, we decided to examine whether these compounds could affect the activity of *Candida rugosa* lipase, using a chromatographic method that has recently been developed in this laboratory.[8]

Materials and Methods

Reagents

C. rugosa lipase (Crl, 700–1500 U/mg at pH 7.2 using olive oil, cat. No. L-1754), β-naphthyl-laurate (β-NL, essentially naphthol-free, cat. No. N-9375), β-naphthol (β-N, cat. No. N-1250), phenacetin (cat. No. A-2375), taurocholic acid (cat. No. T-4009), acetone (cat. No. A-4206), Tris (cat. No. T-1503), Quercetin (Q, cat. No. Q-0125), 3′-azido-3′-deoxythymidine (AZT, cat. No. A-2169) and tetracycline hydrochloride (T, cat. No. T-3383) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trifluoroacetic acid (cat. No.

*Corresponding author. Tel.: +39-06-4991-2479; fax: +39-06-4991-2480; e-mail: luciano.saso@uniroma1.it

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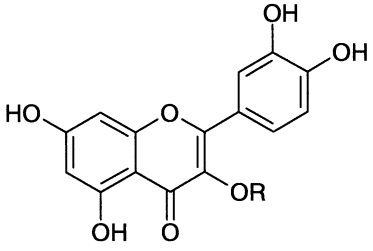
108262) and sodium chloride were from Merck (Darmstadt, Germany). Methanol, ethanol, acetonitrile, and water for high performance liquid chromatography (HPLC), were purchased from Lab-Scan (Dublin, Ireland). Ethyl acetate (EA) was obtained from Carlo Erba (Milan, Italy). All reagents were used without further purification.

Preparation of 3-*O*-acylquercetines

Q esters (Fig. 1) have been prepared as previously reported for the acetyl derivative[13] and the following procedure is representative: lipase from *Mucor miehei* (immobilised, Lipozyme® IM, 0.50 g) was added to a solution of the peracylated quercetine of choice (0.50 g) in *t*-BME containing *n*-butanol (10 equiv). The suspension was shaken (300 rpm) at 45 °C until analysis of the reaction mixture by thin layer chromatography showed the presence of a single product (12–96 h). The reaction was then quenched, filtering off the catalyst and the filtrate evaporated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel using a gradient of acetone in petroleum ether as the eluent to give the relevant 3-*O*-acylquercetine.

Antimicrobial activity

Microbiological assays were performed using different clinically isolated microorganisms, including five gram-positive strains (*S. aureus*, *B. subtilis*, *Listeria ivanovi*, *Listeria monocytogenes*, *Listeria serligeri*), five gram-negative strains (*Escherichia coli*, *Shigella flexneri*, *Shigella sonnei*, *Salmonella enteritidis*, *Salmonella tiphymurium*) and four yeasts (two strains of *Candida albicans* and two strains of *Candida glabrata*). Q, Q-Ac-1, Q-Bu-1, Q-Is-1 and Q-DE-1 were dissolved at the concentration of 100 μg/mL in Triton 5% and serially diluted and filtered through a 0.22 μm filter. The minimal inhibitory concentration (MIC) was determined by the classical microdilution method using 96-well culture plates, inoculated with the microorganisms suspended (10^5 cells/mL) in Muller–Hinton broth (Becton-Dickinson, Oxnard, CA, USA). The plates were incubated for 24 h (or 48 h for the yeasts) at 37 °C. The cultures that did not present growth were used to inoculate plates of solid medium in order to determine the minimal bactericidal concentration (MBC). Proper blanks were assayed simultaneously. All samples were tested in triplicate.



	R	
1	Acetyl	Q-Ac
2	Butyryl	Q-Bu
3	Iso-Butyryl	Q-Is
4	Caproyl	Q-Ca
5	Decanoyl	Q-De
6	Lauroyl	Q-La
7	Myristoyl	Q-My
8	Palmitoyl	Q-Pa

Figure 1. Chemical structure of quercetin and abbreviations used for 3-*O*-acylquercetines.

Anti-HIV activity

A primary strain of HIV-1 was isolated from plasma of an HIV-infected patient and expanded in peripheral blood mononuclear cells (PBMC) according to standard protocols.[14] Briefly, PBMC from healthy donors were isolated by Ficoll-Hypaque centrifugation, suspended (1×10^6 cells/mL) in RPMI 1640 complete medium [10% fetal calf serum, penicillin-G (50 U/mL), streptomycin (50 mg/mL) and L-glutamine (2 mM)] containing phytohemagglutinin (5 mg/mL) and incubated for 72 h at 37 °C in a 5% CO_2 atmosphere. Then, cells were centrifuged, resuspended in complete medium, infected with HIV-1 by addition of 500 tissue culture infective dose (TCID) $50/10^6$ PBMC (multiplicity of infection = 0.05), incubated for 2 h, suspended (1×10^6 cells/mL) in complete medium containing IL-2 (5 U/mL), and seeded in 48-well plates in the presence or not of AZT (3.7 μM), Q (40 μM), Q-Ac (40 μM) and Q-Pa (40 μM). After 72 h, the viral production was determined by HIV-1 p24 antigen ELISA (Immunogenetics).

Anti-lipase activity

Lipase activity in presence or not of the substances under study was evaluated by HPLC as previously described.[8] Briefly, β-NL was dissolved in acetone at 3 mg/mL and this solution was diluted 1:20 in lipase buffer [50 mM Tris at pH 7.4 (at 22 °C), containing 3.5 mM NaCl, 1.5 mM $CaCl_2$, and 1 mM sodium taurocholate] to obtain a final volume of 2 mL of a stable substrate suspension. Then Crl, dissolved at 0.1% w/v in 100 mM Tris pH 7.4 (at 22 °C), was added to the substrate suspension to a final concentration of 0.001% w/v and incubated for 30 min at 37 °C under mild mixing. Then, 20 μL of the internal standard phenacetin, dissolved in methanol at the concentration of 15 mg/mL, were added and the enzymatic reaction was ended by adding 2 mL of the immiscible solvent (EA), which efficiently extracted the product of the reaction β-naphthol (β-N) and the internal standard phenacetin. Afterwards a 500 μL portion of the organic phase was withdrawn, evaporated under a nitrogen stream at room temperature and redissolved in 1 mL of methanol. Hence, aliquots of 50 μL were analyzed by HPLC using a C-18 reversed-phase column (4.6×250 mm; 5-μm particle size; 90 Å pore size; Vydac cat. No. 201HS54) equilibrated at the flow rate of 1 mL/min, with a mobile phase consisting of 40:60

Table 1. Effect of quercetine and its esters on *Candida rugosa* lipase

	S_{MAX}[a] [mg/mL (M)]	IC_{16} (M)[b]	IC_{50} (M)[b]
Tetracycline	1.2 (2.5×10^{-3})	1.6×10^{-4}	2.5×10^{-4}
Q	2.7×10^{-1} (8.0×10^{-4})	1×10^{-4}	($\sim S_{MAX}$)
Q-Ac	1.5×10^{-1} (4.3×10^{-4})	2.9×10^{-4}	4×10^{-4}
Q-Bu	1.5×10^{-1} (4.0×10^{-4})	1×10^{-4}	2.6×10^{-4}
Q-Is	1.5×10^{-1} (4.0×10^{-4})	2.7×10^{-5}	2×10^{-4}
Q-Ca	1.0×10^{-1} (2×10^{-4})	9.7×10^{-7}	2×10^{-5}
Q-De	1.5×10^{-1} (3.7×10^{-4})	1.1×10^{-5}	1.8×10^{-4}
Q-La	1.5×10^{-2} (3.3×10^{-5})	5.7×10^{-6}	($\sim S_{MAX}$)
Q-Mi	1.5×10^{-2} (3.1×10^{-5})	4.5×10^{-6}	($\sim S_{MAX}$)
Q-Pa	1.1×10^{-2} (1×10^{-5})	1.3×10^{-6}	5.2×10^{-6}

[a]Highest concentration at which each substance was tested expressed both as mg/mL and mol/L (M).
[b]Inhibitory concentrations (IC) 16 and 50% calculated from the inhibition versus concentration curves, according to Tallarida and Murray.[15]

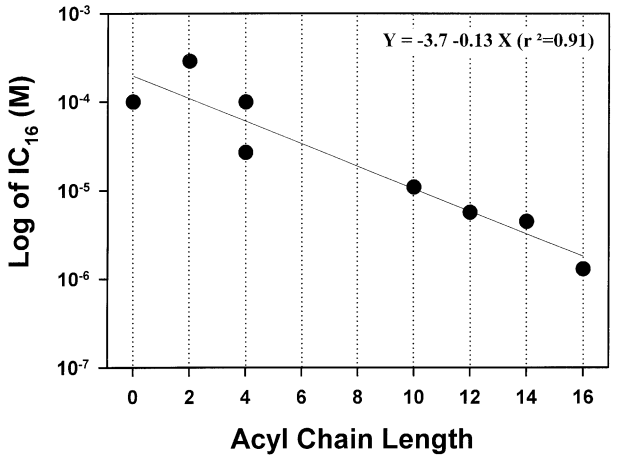


Figure 2. Anti-lipase activity of quercetin its esters. The 3-*O*-acylquercetines examined here were more active (IC_{16} = inhibitory concentration 16) against *Candida rugosa* lipase than the parent compound quercetin (Q). In particular, the inhibitory activity of the 3-*O*-acylquercetines increased with the length of the acyl chain, as indicated by the good linear correlation ($r^2 = 0.91$).

acetonitrile/water, containing 0.1% trifluoroacetic acid. The eluate was monitored at the wavelength of 230 nm with a sensitivity of 0.8 A.U.F.S. using a recorder chart speed of 0.5 cm/min. All chromatographic analyses were done at room temperature. The chromatographic system consisted of a controller (Pharmacia-LKB, model LC 2152), a precision pump (Pharmacia-LKB, model 2150), a high pressure mixer, a variable wavelength monitor (Pharmacia-LKB, model 2151), and a peak area integrator (Waters, model 749).

Analysis of lipase inhibitors. The substances under evaluation were dissolved at their maximum solubility in methanol, serially diluted and analyzed as described above. Lipase inhibition (*I*) was calculated by measuring free β-N resulting from the enzymatic reaction, according to the following formula:

$$[I(\%)]_{X,c} = 1 - \frac{AUC_c - AUC_{blank}}{AUC_0 - AUC_{blank}} \times 100$$

where AUC is the area under the chromatographic peak of β-N extracted from suspensions containing Crl, incubated with (AUC_c), or without (AUC_0) the substance *X* at the concentration *c*, or devoid of the enzyme (AUC_{blank}). All experiments were done at least in duplicate.

The concentrations yielding a lipase inhibition of 16% (IC_{16}) and 50% (IC_{50}) were calculated from the *I* (%) versus concentration curves according to Tallarida and Murray.[15]

Statistics

Linear regression analysis was performed using the software Sigma-Plot 6.0 (SPSS, Chicago, IL, USA).

Results

Preparation of 3-*O*-acylquercetines

3-*O*-Acetylquercetine (**1**). Isolated in 94%. Anal. calcd for $C_{17}H_{12}O_8$: C, 59.31; H 3.51, found: C 59.71; H 3.70. 3-*O*-Butirrylquercetine (**2**). Isolated in 90%. Anal. calcd for $C_{19}H_{16}O_8$: C, 61.29; H 4.33, found: C 61.70; H 4.60. 3-*O*-iso-Butirrylquercetine (**3**). Isolated in 92%. Anal. calcd for $C_{19}H_{16}O_8$: C, 61.29; H 4.33, found: C 61.72; H 4.58. 3-*O*-Hexanoylquercetine (**4**). Isolated in 90%. Anal. calcd for $C_{21}H_{20}O_8$: C, 63.00; H 5.03, found: C 63.31; H 5.20. 3-*O*-Decanoylquercetine (**5**). Isolated in 76%. Anal. calcd for $C_{25}H_{28}O_8$: C, 65.78; H 6.18, found: C 65.99; H 6.32. 3-*O*-Lauroylquercetine (**6**). Isolated in 75%. Anal. calcd for $C_{27}H_{32}O_8$: C, 66.93; H 6.66, found: C 67.20; H 6.85. 3-*O*-Miristoylquercetine (**7**). Isolated in 78%. Anal. calcd for $C_{29}H_{36}O_8$: C, 67.95; H 7.08, found: C 68.22; H 7.29. 3-*O*-Palmitoylquercetine (**8**). Isolated in 82%. Anal. calcd for $C_{31}H_{40}O_8$: C, 68.87; H 7.46, found: C 69.16; H 7.66.

Antimicrobial activity

Q and its esters, up to the concentration of 100 μg/mL, did not show any activity on the microorganisms examined.

Anti-HIV activity

Q, but not its acetyl or palmitoyl esters, at the concentration of 40 μM, inhibited about 80% of HIV-1 reproduction, similarly to AZT at 3.7 μM.

Anti-lipase activity

Q and selected 3-*O*-acylquercetines inhibited *C. rugosa* lipase with IC_{16} in the range 10^{-4}–10^{-6} M (Table 1). When the IC_{16} were plotted against the length of the acyl chain, a linear correlation was found (Fig. 2).

Discussion

In the present study, a good antiviral (against HIV-1) but not antimicrobial activity of quercetin (Q) was observed.

- Q was reported by other authors to inhibit different microorganisms such as *E. coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *A. parasiticus*, *A. flavus*,[6] *S. aureus*, *S. epidermidis*, *B. subtilis*, *M. luteus* and *E. coli*.[5] However, these authors used higher concentrations (100–200 and 500 μg/mL, respectively) than the maximum concentration tested here (100 μg/mL), at which no significant inhibition of five gram-positive strains (*S. aureus*, *B. subtilis*, *L. ivanovi*, *L. monocytogenes*, *L. serligeri*), five gram-negative strains (*E. coli*, *S. flexneri*, *S. sonnei*, *S. enteritidis*, *S. tiphymurium*) and four yeasts (two strains of *C. albicans* and two strains of *C. glabrata*) was observed. Besides, our attempt to augment the activity by introducing an acyl group on the C-3 position, thus generating highly lipophilic derivatives, did not succeed.
- In addition, we confirmed the known anti-HIV activity of Q,[11] which might depend on the presence of a free hydroxyl in the C-3 position, as suggested by the lack of activity of the 3-*O*-acylquercetines examined.

Moreover, for the first time to our knowledge, we described the inhibition of *C. rugosa* lipase by Q and its esters (3-*O*-acylquercetines), which appeared even more active (Table 1, Fig. 2). The reason for the latter phenomenon is not clear yet, but in our opinion the trend observed in the experiments reported in Table 1 could be explained in terms of lipophilicity degree of the substrates considered. It is plausible that, as observed for other lipases,[16] the inhibitory effect of Q may be related to the phenolic hydroxyls located on its flavane framework. The introduction of an acyl group on the C-3 position did not affect the inhibitory faculty, improving the lipophilicity. Consequently, by elongating the alkyl chain in the acyl group, a better affinity is favored for the space around the active site of the lipase, whose nature is hydrophobic.[17]

In vivo, the anti-lipase properties of Q could increase its efficacy against lipase producing microorganisms. In fact, it is known that the production of extracellular lipases improves the ability of certain microorganisms to colonize the external surfaces of the host: (a) free fatty acids derived from the hydrolysis of endogenous lipids can increase the adherence of *S. epidermidis*[18] and *Propionibacterium acnes*[19] to the skin; (b) lipase-producing fungal dermatophytes can efficiently colonize the keratinized layers of the skin.[20,21] Thus, lipase inhibitors such as Q could be useful adjuvants in the therapy of infective diseases due to lipase-producing microorganisms. In particular, the 3-*O*-acyl-quercetines not only appeared more active than both Q and the reference compound tetracycline[22–25] but being more hydrophobic than Q and tetracycline they could have more favorable pharmacokinetic properties, when applied to the skin or mucosae, which deserve to be further studied.

In conclusion, we have described here for the first time the anti-lipase activity of Q, and its C2–C16 3-*O*-acylesters, which could be of pharmacological interest for the development of new antimicrobic agents.

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