Original Article

Synthesis and *In vitro* Leishmanicidal Activities of Six Quercetin Derivatives

Abstract

Background: Today, leishmaniasis is a widespread, infectious parasitic disease caused by *Leishmania* spp. Natural-derived compounds are likely to provide a valuable source of new pharmaceuticals, and among them, quercetin derivatives may have antileishmanial effects. The antileishmanial activity of 3,5,7,3',4'-pentahydroxyflavonol (quercetin) derivatives is partly attributed to the position and pKa of phenolic or catechol hydroxyl groups. Therefore, to optimize their leishmanicidal effect, the structural features of quercetin and its derivatives were improved by acylation or alkylation of hydroxyl groups and changing their pKa and consequently their activities. **Materials and Methods:** In this study, during a regioselective method, quercetin derivatives were synthesized. The structures of synthesized compounds were confirmed by mass, IR, ^{1}H-, and ^{13}C-NMR spectral data. The antileishmanial activities of compounds 1–6 were compared with glucantime as the standard drug against promastigotes of *Leishmania major* using standard cell-based leishmanicidal assay. **Results:** In this study, during a regioselective method, two 7-O-quercetin derivatives (5 and 6), and three quercetin acetate derivatives (2, 3, and 4) were synthesized. In detail, the IC_{50} values found against *L. major* were (1) 2.5 ± 0.92; (2) 2.85 ± 0.99; (3) 15.5 ± 1.95; (4) 13.5 ± 3.5; (5) 2.6 ± 0.57; and (6) 1.3 ± 0.35 µM while IC_{50} value of glucantime as the standard drug was 88.5 ± 9.47 µM. **Conclusions:** The present study showed an effective antileishmanial activity of quercetin semisynthetic compounds (1–6) against *in vitro* promastigotes of *L. major*. Among them, quercetin analogs with more lipophilic and iron-chelating activity showed more antiparasite activity.

Keywords: *Leishmanicidal activity, quercetin derivatives, regioselective synthesis*

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Introduction

Leishmaniasis is a widespread, infectious parasitic disease caused by *Leishmania* spp. in both visceral and cutaneous manifestations. The World Health Organization has identified it as a major and increasing public health problem. Its visceral form, known as kala-azar, is widely distributed in Africa, Asia, Latin America, the Middle East, and the Mediterranean region.[1] About 350 million people are at risk of infection with *Leishmania* parasites, and over 12 million people are infected with different species of the parasite, with over 400,000 new cases each year.[1] Incidence increase is associated with urban development, forest devastation, environmental changes, and migrations of people to areas where the disease is endemic.[2,3]

Over time, different methods of local radiation therapy, burn lesions, cryotherapy, and cutaneous drugs such as five trivalent antimony compounds including pentostam (stibogluconate), glucantime, pentamidine, and amphotericin B were used to treat leishmaniasis. However, the problem was their limitations such as the long course of treatment, lack of response to treatment in about 10%–15% of the cases, and their side effects on the heart, liver, and kidneys.[4,5]

Natural-derived compounds are likely to have less side effects and be more tolerable. Among them, quercetin with flavonoid structure showed antileishmanial effects.[6] The antileishmanial activity of 3',4',3,5,7 -pentahydroxyflavonol (quercetin) derivatives is partly attributed to the position and pKa of phenolic or catechol hydroxyl groups. Therefore, the aim of this research is to improve quercetin leishmanicidal effects by acylation of hydroxyl group or regioselective 7-O-substitution of electron-donating or electron capture groups and changing their pKa and consequently their activities.

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Materials and Methods

General procedures

Progress of all the reactions was monitored by thin-layer chromatography (TLC) using natural product reagent as visualizing agent. The reaction mixtures were purified using polyamide SC6 column and hexane: acetone as solvent with increasing polarity in a gradient manner. Nuclear magnetic resonance analysis was done on a Bruker AV400 spectrometer using DMSO-D6 as solvent at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR. Mass spectra (ESI) were performed on Shimadzu 2010EV LC-MS system (Shimadzu, Japan) and are reported in m/z. The IR spectrum was recorded on a Rayleigh WQF-510 FTIR spectrophotometer.

Materials

Quercetin was obtained from Sigma (St. Louis, MO, USA). All solvents were analytical grade and purchased from Merck and used without purification unless specified.

Synthesis of quercetin-3,5,7,3′,4′-pentaacetate (2)

Quercetin (1, 2.0 g, 6.0 mmol), acetic anhydride (15 mm, 6.1 g, 60.0 mmol), and pyridine (15 mL) were heated to reflux under nitrogen atmosphere for 6 h. After cooling down to room temperature, the mixture was poured into 50 mL of ice water and filtered to give the product 2 in 85% yield. ^{1}H-NMR (DMSO-D6) δ: 7.96 (m, 2H, H-2', 6'), 7.75 (d, 1H, J = 2.2 Hz, H-8), 7.63 (d, 1H, J = 8.5 Hz, H-5'), 7.27 (d, 1H, J = 2.2 Hz, H-6), and 2.43 (s, 15H, 5 acetate); ^{13}C-NMR (DMSO-D6) δ: 169.3, 168.7, 168.3, 168.2, 168.0, 167.8, 156.3, 154.35, 153.4, 149.5, 144.42, 142.14, 133.9, 127.1, 126.7, 124.5, 123.7, 114.7, 114.05, 110.06, 20.9, 20.75, 20.4, 20.3, and 20.2; IR (KBr); ν_{max}: 1778, 1647, 1437, 1373, and 1194 cm^{-1}; and ESI-MS (*m*/z): 523 [M-H]$^-$.[7]

General procedure for the preparation of 3–4

To a solution of quercetin-3,5,7,3′,4′-pent aacetate (1.0 g, 2.13 mmol) in anhydrous N-methyl-2-pyrrolidone (NMP) (30 mL), imidazole (50 mg, 0.74 mmol) and thiophenol (0.22 mL, 2.13 mmol) were added. It was stirred at 0°C for 1 h until the disappearance of the starting material (monitored by TLC). Then, mixture was diluted with CH_2Cl_2 and washed successively with 1 N aqueous HCl and saturated sodium chloride. The organic phase was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by polyamide column chromatography (hexane/acetone, 40%, v/v) to give compound 3 as white powder in 70% yield and compound 4 in 30% yield.

Quercetin-2,5,3′,4′-tetraacetate (3)

^{1}H-NMR (DMSO-D6) δ: 7.94 (dd, 1H, J = 1.6 Hz, J = 8.4 Hz, H-6'), 7.90 (d, 1H, J = 1.7 Hz, H-2'), 7.61 (d, 1H, J = 8.4 Hz, H-5'), 7.02 (d, 1H, J = 2.6 Hz, H-8), 6.74 (d, 1H, J = 2.1 Hz, H-6), 2.44 (s, 3H, acetate), 2.43 (s, 3H, acetate), 2.40 (s, 3H, acetate), and 2.39 (s, 3H, acetate); ^{13}C-NMR (DMSO-D6) δ: 168.9, 168.8, 168.2, 168.0, 167.9, 157.6, 152.3, 150.2, 144.15, 142.1, 131.1, 127.4, 126.5, 124.5, 124.4, 123.7, 123.5, 100.95, 20.9, 20.4, 20.3, and 20.2; IR (KBr); ν_{max}: 3132, 2939, 1774, 1628, 1369, and 1171 cm^{-1}; and ESI-MS (*m*/z): 481 [M-H]$^-$.[8]

Quercetin-2,5,3′-triacetate (4)

^{1}H-NMR (DMSO-D6) δ: 7.75 (dd, 1H, J = 2.2 Hz, J = 8.6 Hz, H-6'), 7.69 (d, 1H, J = 2.2 Hz, H-2'), 7.21 (d, 1H, J = 8.6 Hz, H-5'), 7.01 (d, 1H, J = 2.2 Hz, H-8), 6.71 (d, 1H, J = 2.1 Hz, H-6), and 2.41 (s, 9H, 3 acetate); ^{13}C-NMR (DMSO-D6) δ: 168.9, 168.8, 168.7, 167.9, 157.5, 153.12, 150.09, 138.38, 126.98, 123.02, 119.57, 117.35, 109.25, 109.21, 108.5, 100.85, 20.87, 20.6, and 20.28.; and ESI-MS (*m*/z): 439 [M-H]$^-$.

General procedure for the preparation of 4a–4b

K_2CO_3 (95 mg, 0.69 mmol) and appropriate benzyl chloride (50 mg, 0.35 mmol) were added to a solution of 3 (200 mg, 0.23 mmol) in acetone (15 mL). The reaction mixture was stirred for 3 h at room temperature and then filtered. The filtrate was concentrated under reduced pressure to offer the crude product 5–6 for the next step directly without further purification.

General procedure for the preparation of 5–6

The solution of crude compound 4a–4b in NH3/MeOH (10 mL) was stirred for 1 h at 0°C and concentrated under reduced pressure. This residue was purified by reversed-phase chromatography (RPC) (water/EtOH, 30%, v/v) to give the desired compounds 5–6.

7-O-paranitrobenzylquercetin (5)

Yield 60%; ^{1}H-NMR (DMSO-D6) δ: 8.32 (d, 2H, J = 8.8. Hz, 4-nitrobenzyl-H-3'',5''), 7.81 (d, 2H, J = 8.8. Hz, 4-nitrobenzyl-H-2'', 6''), 7.66 (d, 1H, J = 2.0 Hz, H-2'), 7.63 (dd, 1H, J = 2.0 Hz, J = 8.8 Hz, H-6'), 7.00 (d, 1H, J = 8.4 Hz, H-5'), 6.9 (d, 1H, J = 2.0 Hz, H-8), 6.56 (d, 1H, J = 2.0 Hz, H-6), and 5.51 (s, 2H, 4-nitrobenzyl-CH_2). ^{13}C-NMR (DMSO-D6) δ: 175.9, 163.3, 160.4, 155.9, 147.9, 147.4, 147.1, 145.05, 144.05, 136.06, 128.3, 128.3, 123.7, 123.7, 123.5, 121.75, 119.98, 115.56, 115.2, 104.3, 97.97, 92.85, 68.58, 39.84, 39.63, 39.4, 39.2, and 39; IR (KBr); ν_{max}: 3404, 2922, 1655, 1595, 1522, 1346, and 1165 cm^{-1}; and ESI-MS (*m*/z): 436 [M-H]$^-$.[9]

7-O-paramethylbenzylquercetin (6)

Yield 50%; ^{1}H-NMR (DMSO-D6): 7.81 (bs, 1H, H-2'), 7.65 (bd, 1H, J = 8.4 Hz, H-6'), 7.45 (d, 2H, J = 8.1 Hz, 4-methylbenzyl-H-3'',5''), 7.31 (d, 2H, J = 8.0 Hz, 4-methylbenzyl-H-2'',6''), 6.99 (dd, 1H, J = 2.2 Hz, J = 8.1 Hz, H-5'), 6.87 (bs, 1H, H-8), 6.50 (bs, 1H, H-6), 5.27 (s, 2H, 4-methylbenzyl-CH_2), and 2.40 (s, 3H, 4-methylbenzyl-Me).; ^{13}C-NMR (DMSO-D6) δ: 175.9,

163.9, 160.3, 155.9, 147.8, 147.3, 145.04, 137.4, 136, 133.1, 129.06, 127.9, 121.8, 120, 115.5, 115.2, 104.03, 97.99, 92.7, 69.8, and 20.75; IR (KBr); ν_{max}: 3253, 2922, 1655, 1589, 1498, 1350,1323, and 1165 cm^{-1}; and ESI-MS (*m*/z): 405 $[M-H]^-$.[10]

Leishmanicidal activity

Promastigotes of *Leishmania major* were obtained in M-199 medium and HEPES buffer from Pasteur Institute in Tehran, Iran. They maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 25°C. Promastigotes were seeded in 96-well plates (5.6×10^6 parasites/well) with fresh RPMI medium supplemented with 10% FBS and serially diluted concentrations of compounds 1–6 (1, 10, 100 μM), blank (wells without parasites), negative controls (untreated parasites), and glucantime as positive control, and incubated at 25°C. Using a neobar lam on an inverted microscope, survived parasites were identified and counted.[11]

Results

Chemistry

As is shown in Figure 1, quercetin (1) was acetylated by acetic anhydride in pyridine at 180°C for 6 h (85%). After purification of quercetin pentaacetate, by polyamide column using hexane: acetone (70:30), it was 7-O-regioselectively deacetylated using thiophenol and imidazole in NMP as solvent at −10°C for 1 h. After purification, it yielded quercetin-2,5,3′,4′-tetraacetate (3) with free hydroxyl group on C-7 (60%) and quercetin-2,5,3″-triacetate (4) with free hydroxyl groups on C-7 and C-4' (40%). Compound 3 was arylated with 4-methylbenzyl chloride and 4-nitrobenzyl chloride, separately. Reaction mixture was then deacetylated by treatment with ammonia (2M) in acetone at 0°C for 5 min. Excess ammonia was neutralized by adding HCl (0.5 M). Finally, 7-O-arylquercetins 5 and 6 were purified by RP-18 RPC using water: methanol (30:70) as solvent.[10]

Biological evaluation

Quercetin derivatives were tested to determine their leishmanicidal activities after 48 h of treatment. They showed a dose-dependent effect on *L. major* promastigote growth. In detail, the IC_{50} values found against L. major were (1) 2.5 ± 0.92; (2) 2.85 ± 0.99; (3) 15.5 ± 1.95; (4) 13.5 ± 3.5; (5) 2.6 ± 0.57; and (6) 1.3 ± 0.35 μM, and IC_{50} value of glucantime as the standard drug suppressed promastigotes was 88.5 ± 9.47 μM [Figure 2].

Discussion

There are three possible domains in quercetin analogs which can chelate metal ions. These are between ortho 3' and 4'-dihydroxyl groups on the B ring, 3-hydroxy and 4-oxo in the C ring, and 5-hydroxy and 4-oxo in the A ring. Protection of these chelating sites is expected

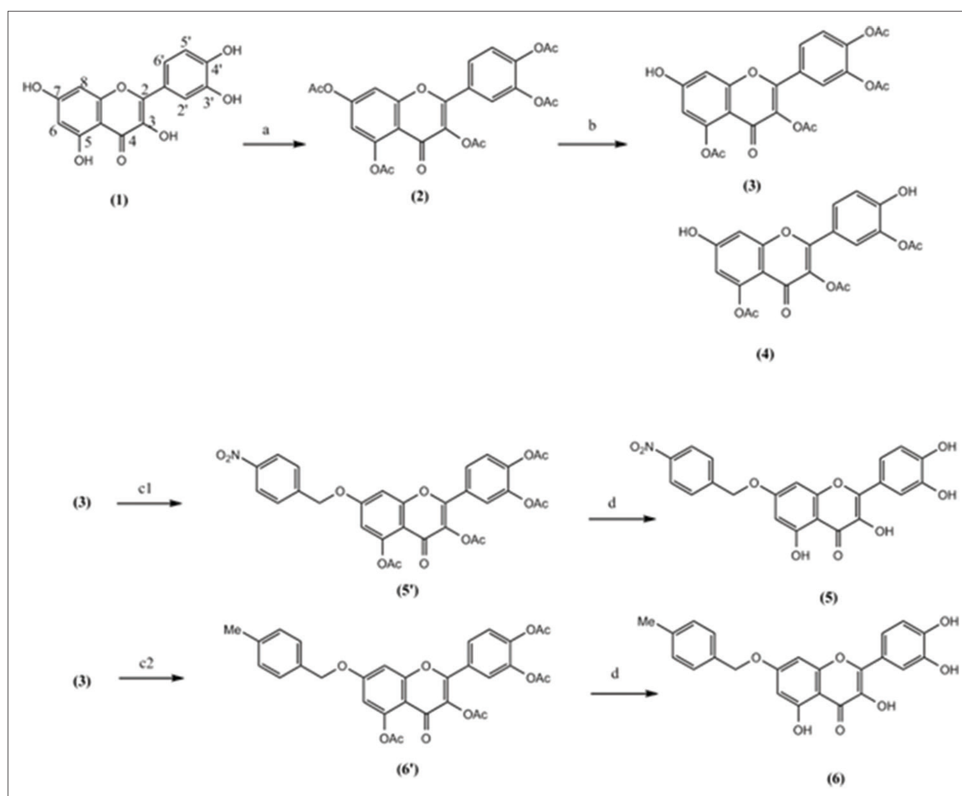

Figure 1: Reagents and conditions: (a) Ac_2O, pyridine, reflux, 6 h, 85%. (b) PhSH, imidazole, NMP, 0°C, 1 h, 80%. (c_1) NO_2-$PhCH_2Cl$, Na_2CO_3, acetone, 3 h. (c_2) CH_3-$PhCH_2Cl$, Na_2CO_3, acetone, 3 h. (d) 2M NH3/acetone, 0°C, 5 min (40%)

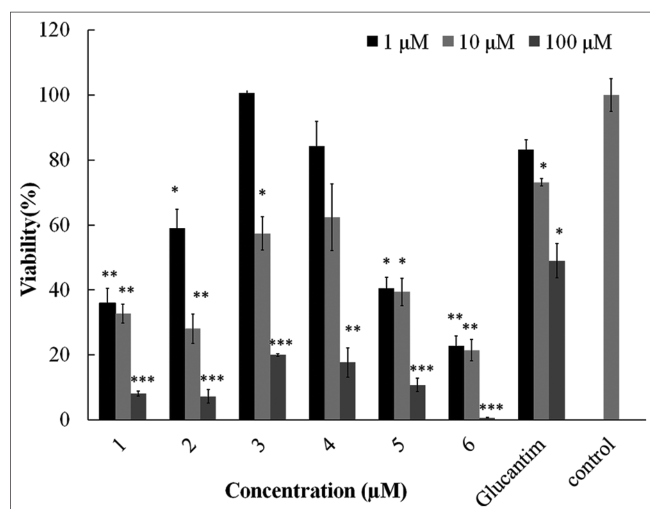

Figure 2: leishmanicidal activity of six quercetin derivatives: Quercetin derivatives were tested to determine their leishmanicidal activity after 48 h of treatment against *Leishmania major* promastigotes. (P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. placebo)**

to reduce the interaction of ligand with Fe ions. In this agreement, compounds 3 (quercetin-2,5,3′,4′-tetraacetate) and 4 (quercetin-2,5,3′-triacetate) with no free chelating group were less active than compounds 1 (quercetin), 5 (7-O-paranitrobenzylquercetin), and 6 (7-O-paramethylbenzylquercetin) with free chelating groups localized on C-5, C-3, and B ring. Therefore, 5-OH, 3-OH, and ortho 3’ and 4’-dihydroxyl groups in ring B are involved in leishmanicidal activity in quercetin derivatives. In comparision with compound 5 with paranitrobenzyl and 6 with paramethylbenzenyl on C-7, compound 6 showed more antileishmanial activity probably because of increased chelating activity of 5-hydroxy and 4-oxo in A ring, and also more parasite membrane penetration.

Quercetin analogs probably exert their effect by their antioxidant and reductant properties. They react with ferric ions (Fe 3+) and make quercetin quinones and ferrous ions. Ferrous ions are chelated by quercetin derivatives lead to depletion of Fe in cells. Therefore, we suggest that iron depletion is one of the mechanisms of quercetin analogs against *Leishmania* infection.

In this agreement, in the study directed by Sen *et al.*, they showed that quercetin as a lipophilic iron chelator interferes with iron metabolism in *Leishmania donovani* and targets ribonucleotide reductase to exert leishmanicidal activity.[12] In fact, ribonucleotide reductase activity, which regulates cell proliferation, is dependent to the iron supply, and iron deprivation by this mechanism may exert its antileishmanial effect.[13] Ben-Othman *et al.* reported that iron chelators such as 8-hydroxyquinoline inhibit the expression of genes in *Leishmania* that are related to the infectivity and/or virulence of the parasites.[14] In the study conducted by Soteriadou *et al.,* they showed that *in vitro* incubation of *L. major* promastigotes with iron-chelating compounds such as deferoxamine (DFO), L1, and 1,2-diethyl-3-hydroxypyridin-4-one (CP94) in a dose-dependent manner suppressed parasite growth. In a reverse manner, addition of referrated iron chelators like FeDFO, Fe $(Ll)_{3,}$ and Fe $(CP94)_{3}$ to cultured media restored parasite growth, which means that proliferation inhibition is caused by the iron chelator.[15]

However, because of this point that quercetin analogs with protected chelating groups (compounds 2, 3, and 4) and those with iron-chelating activity (compounds 1, 5, and 6) both inhibit *L. major* growth *in vitro*, it seems that reduction in promastigote growth is not dependent to iron chelation alone, and other mechanisms are incorporated. In this regard, Virginia Iniesta team founded that cellular *Leishmania* infection is dependent to arginase enzyme, which generates L-ornithine essential for *Leishmania* growth.[16] Afterward, Manjolin *et al.* showed that quercetin exerted its antileishmanial effect by inhibitory effect on arginase enzyme, which is essential for parasite proliferation.[17] In a different study, Fonseca-Silva *et al.* reported another mechanism for the antileishmanial activity of quercetin against *L. amazonensis* promastigotes. They founded that reactive oxygen species production and mitochondrial respiratory dysfunction induced parasite death (IC_{50} value of 31.4 μM) through collapse of mitochondrial membrane potential.[18] Mittra *et al*. reported also that quercetin inhibited the growth of *L. donovani* promastigotes by interacting with DNA topoisomerases and promoting site-specific DNA cleavage leading to cell cycle arrest and apoptosis with IC_{50} value of 45.5 μM.[19]

In fact, quercetin mechanism of action is not only related to its chelating property but also because of multiple quercetin targets that have been identified for the treatment of leishmaniasis include: arginase, ribonucleotide reductase, topoisomerase II, and mitochondrial dysfunction made quercetin analogs interesting for new antileishmanial drug designs.

Conclusions

The present study showed an effective antileishmanial activity of quercetin semisynthetic compounds (1–6) against *in vitro* promastigotes of *L. major*. Among them, quercetin analogs with more lipophilic and iron-chelating activity showed more antiparasite activity. Based on calculated pKa values in quercetin, 5-OH in A ring is the strongest chelator site in quercetin analogs, which because of the intermolecular hydrogen bond between 4-oxo and 5-OH is relatively inactive. In this study, we investigated the effects of electron withdrawing or electron donor substituents addition on C-7 on pKa of 5-OH and its chelating activity. The results of this paper showed that 7-O-paramethylbenzyl substitution with electron donating and more lipophilic properties increased antileishmanial activity *in vitro* against *L. major* promastigotes ($IC_{50} = 1.3 \pm 0.35$ μM) more than

7-O-paranitrobenzyl substitution with electron-withdrawing effects (2.6 ± 0.57) or quercetin alone (2.5 ± 0.92).

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Conflicts of interest

There are no conflicts of interest.

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