European Journal of Medicinal Chemistry 85 (2014) 615–620

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis of genistein coupled with sugar derivatives and their inhibitory effect on nitric oxide production in macrophages

Celso O.R. Junior [a], Sandra B.R. Castro [b], Adriane A. Pereira [a], Caio C.S. Alves [b], Erick E. Oliveira [b], Renata T. Rêgo [b], Ana Paula Ferreira [b], Mauro V. de Almeida [a, *]

[a] Department of Chemistry, Federal University of Juiz de Fora, Juiz de Fora, MG, Brazil
[b] Department of Parasitology, Microbiology and Immunology, Federal University of Juiz de Fora, Juiz de Fora, MG, Brazil

ARTICLE INFO

Article history:
Received 22 November 2013
Received in revised form
7 August 2014
Accepted 8 August 2014
Available online 10 August 2014

Keywords:
Genistein
J774A.1
RAW 264.7
Nitric oxide
Carbohydrate
TNF-α

ABSTRACT

The isoflavone genistein **1** and some derivatives modulate IL-12, TNF-α and NO production by macrophages and lung cancer cell lines, and improve the clinical signs of experimental autoimmune encephalomyelitis (EAE). Seven genistein derivatives connected at C-6 position of a sugar, such as D-glucose and D-galactose, were synthesized. The ability to modulate macrophage response was evaluated, showing variable inhibition capacity of NO and TNF-α production in J774.A1 and RAW 264.7. Five of the seven compounds were non-cytotoxic; compound **8** was more effective to inhibit NO and TNF-α production, without affecting cell viability.

1. Introduction

Innate immune response is an essential first line of defense against aggressors, and acts in the polarization of the adaptive response [1]. Macrophages are one of the components of innate immunity, and play an important role by releasing inflammatory mediators, such as nitric oxide (NO) and tumor-necrosis factor-alpha (TNF-α) [2,3].

Nitric oxide is a reactive molecule involved in several biological activities. Inducible nitric oxide synthase (iNOS), an enzyme formed in response to pathological conditions, controls NO production. The upregulation of iNOS is implicated in inflammatory processes [4]. Macrophages can be activated by addition of interferon-γ plus lipopolysaccharide, which promotes the release of large amounts of NO induced by iNOS [2,3].

TNF-α and its receptors (TNF-R1 and TNF-R2) are involved in autoimmune and inflammatory diseases characterized by excessive TNF-α production [5]. The blocking of the biochemical effects of TNF-α by soluble pre-ligand assembly domain (PLAD) of TNF receptors has been proved to inhibit arthritis in animal models [6].

Genistein **1** (4′,5,7-trihydroxyisoflavone) (Fig. 1) is a natural isoflavone that modulates IL-12, TNF-α and NO production by macrophages and lung cancer cell lines through the inhibition of the signaling pathway of nuclear factor kappa B (NF-κB) [7]. Moreover, genistein and its derivative 4′-*O*-tetradecanoyl-genistein **2** (Fig. 1) have been shown to reduce the clinical signs of experimental autoimmune encephalomyelitis (EAE), a murine autoimmune disease used in the study of multiple sclerosis (MS) [8,9].

Isoflavones are found in a number of plants in a glycosylated form [10]. Genistin **3** (Fig. 1), the glycoside form of genistein **1** [11], is metabolized to its aglycone form in the low gut by intestinal glycosidases [12,13]. However, it has been shown that genistin is partially absorbed without previous hydrolysis in the rat small intestine [14]. Furthermore, genistein bioavailability is greater when it is administered in the glycosylated form, in comparison with the aglycone form, due to the protection provided by the glycoside group and the lower solubility of aglycones [15]. Moreover, the transportation of the flavonoid quercetin 4′-β-glycoside by sodium-dependent glucose transporter was demonstrated in Caco-2 and G63 cells [16].

In order to obtain compounds with physicochemical properties similar to those exhibited by glycosides of genistein, this work

* Corresponding author. Department of Chemistry, Federal University of Juiz de Fora, 36036-900 Juiz de Fora, MG, Brazil.
E-mail address: mauro.almeida@ufjf.edu.br (M.V. de Almeida).

http://dx.doi.org/10.1016/j.ejmech.2014.08.032
0223-5234/

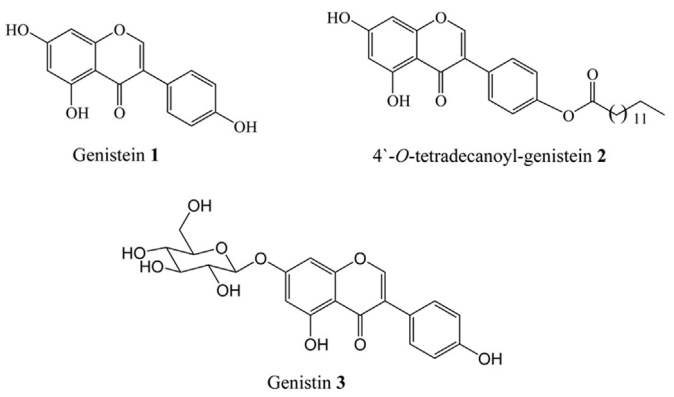


Fig. 1. Structure of **1**, **2** and **3**.

describes the synthesis of genistein derivatives attached to the C-6 position of D-galactose and D-glucose derivatives, and evaluates their effect on the production of NO and TNF-α by J774A.1 and RAW 264.7 cells, activated by LPS plus IFN-γ.

2. Results and discussion

2.1. Chemistry

The C7-OH group of genistein **1** exhibits a 100-fold increase in acidity, compared to the C4′-OH group. However, the C4′-phenolate or C4′-OH groups are better nucleophiles than the C7-phenolate or C7-OH groups, respectively. The C5-OH group is the less nucleophilic one of genistein. Because of this difference in reactivity, it is easy to make modifications at C4′-OH or/and C7-OH positions [17,18].

The protected carbohydrates **4** and **5** were first prepared for the subsequent condensation with genistein **1**. Iodide **4** was obtained (58% yield) through the acetalization of the hydroxyl groups at C1, C2, C3 and C4 positions of D-galactose using dry acetone, zinc chloride and catalytic amounts of sulfuric acid followed by an iodination reaction at C-6 [19] (Scheme 1). Iodide **5** was obtained (62% yield) through the iodination at C-6 of methyl α-D-glucopyranoside followed by acetylation of the hydroxyl groups [20] (Scheme 1).

The treatment of genistein **1** with potassium carbonate followed by the addition of 3 equivalents of iodide **4** afforded to obtain the mono-ether **6** and di-ether **7** (30 and 9% yield, respectively; Scheme 2). The structure of compound **6** was confirmed by nuclear Overhauser effect enhancement spectroscopy (NOEDIFF)-NMR experiments: the presence of specific NOE signals between H-6″ and H-6 and H-8 of **6** proves that the ether was formed at the *O*-7 position of the genistein. Hydrolysis of the isopropylidene groups of compound **6** resulted in the formation of compound **8** (70% yield, Scheme 2).

The reaction of genistein with potassium carbonate and 3 equivalents of iodide **5** led to the formation of the ethers **9** and **10** (55 and 20% yield, respectively). Compounds **9** and **10** were deacetylated with sodium methoxide in MeOH to afford genistein derivatives **11** and **12** (95 and 87% yield, respectively; Scheme 3).

2.2. Biological evaluation

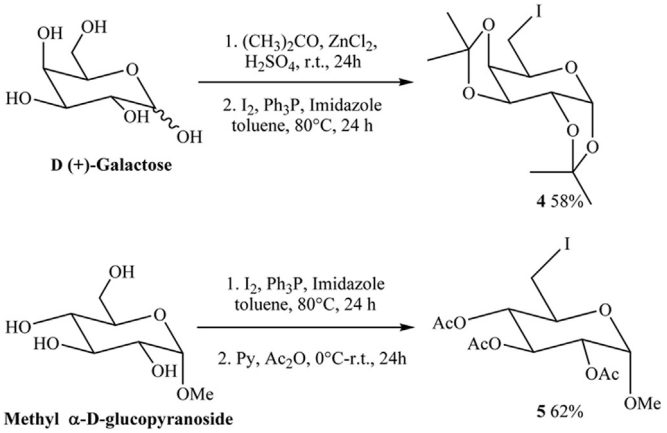
2.2.1. Cytotoxic activity

The inhibitory activity of genistein in the release of inflammatory mediators by macrophages has been previously described [2,21]. In the present work, seven genistein derivatives were synthesized and their ability to inhibit the production capacity of NO and TNF-α production in J774.A1 and RAW 264.7 macrophages was evaluated.

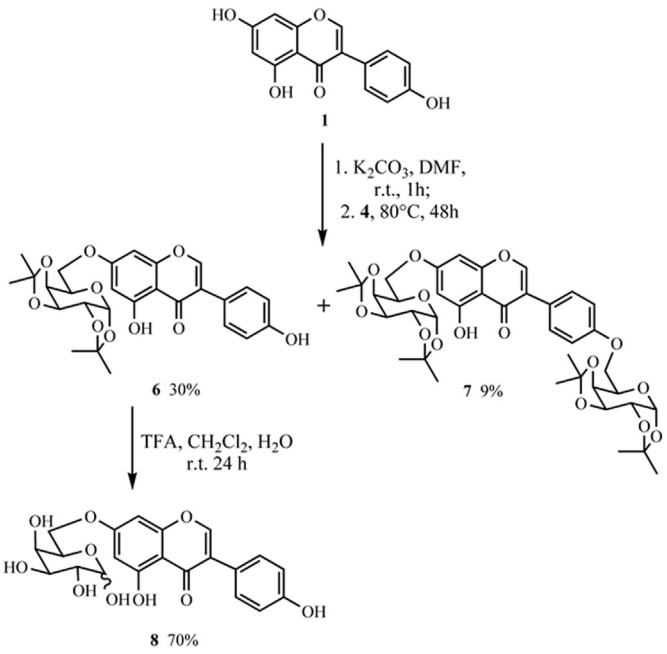
The cytotoxic activity of the synthesized compounds was evaluated by the determination of cell viability (Tables 1 and 2). None of these compounds was cytotoxic against RAW 264.7 macrophages. Monoether **11** was toxic against J774A.1 cells at all concentrations tested, and the protected monoether **6** was cytotoxic at 26 μM.

2.2.2. Inhibition of NO production

Nitric oxide has been implicated in several inflammatory diseases as septic shock, rheumatoid arthritis, and platelet aggregation. Inhibition of its production provides a useful therapy for inflammatory disorders [4,22]. The reduction of NO production after treatment of the cells with glycosylated flavonoids has been

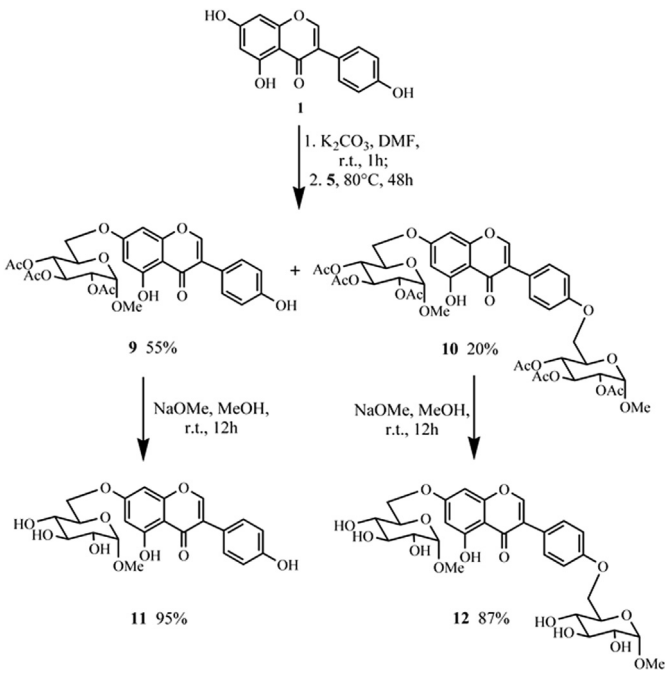


Scheme 1. Synthesis of iodide **4** and **5**.



Scheme 2. Synthesis of **6**, **7** and **8**.

described: it has been shown that quercetin 7-*O*-β-D-



Scheme 3. Synthesis of **9**, **10**, **11** and **12**.

glucopyranoside reduces iNOS expression [23], and that luteolin 5-*O*-glucopyranoside suppressed the expression of the same enzyme by LPS-stimulated RAW 264.7 cells [24]. Almost all tested compounds reduced NO release more intensely than genistein, especially by deprotected derivatives **8** and **12** in both cell lineages (Tables 1 and 2).

2.2.3. Inhibition of TNF-α production

TNF-α is a potent mediator of inflammation produced mainly by monocytes and macrophages, in response to pathogens and inflammatory processes. Like NO, it has an important role in the pathogenesis of inflammatory diseases such as rheumatoid arthritis and septic shock [6]. It has been shown that glycosylated flavonoids are able to inhibit TNF-α production by THP-1 cells stimulated with LPS [25]. However, the quercetin 7-O-β-D-glucopyranoside failed to inhibit TNF-α production by RAW 264.7 cells [26].

In the present study, only compounds **8**, **9** and **10** significantly inhibited TNF-α production by J774A.1 in relation to the non-treated stimulated cells, at 6.5 μM (38.3 ± 6.7 pg/mL; 51.7 ± 13.7 pg/mL and 51.9 ± 1.7 pg/mL *vs.* 89.2 ± 17.1 pg/mL, respectively $p < 0.05$). Genistein was not effective in the tested concentrations, and none of the tested compounds was able to inhibit the TNF-α production by RAW 264.7.

3. Conclusion

The variability in the responses observed in this work can be explained by the fact that different cell lines present distinct inhibition patterns [2,21,26,27], and more studies are needed to establish a relationship between structure and activity. However, the galactosylated genistein derivative **8** appears to be promising anti-inflammatory agent, as it displayed the highest capacity to inhibit NO and TNF-α production, without affecting cell viability. Further studies should be conducted to investigate the beneficial effect of this compound *in vivo*.

4. Experimental section

4.1. General

Melting points (mp) were determined on a Microquímica MQAPF apparatus and were uncorrected. IR spectra were recorded using a BOMEM-FTIR MB102 spectrometer. Optical rotations were measured in a Bellingham Stanley ADP410 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded in a Bruker Advance DRX300 spectrometer. Chemical shifts (δ) are quoted in parts per million (ppm), and compared to the appropriate residual solvent peak reference ($CDCl_3$, CD_3OD, DMSO or C_5D_5N), with the abbreviations s, br s, d, t and m denoting singlet, broad singlet, doublet, triplet and multiplet, respectively. *J* represents the coupling constants (in Hz). Proton and carbon spectra were obtained at room temperature. Thin layer chromatography was carried out on silica gel plates and visualized under ultraviolet light and/or by treatment with a solution of sulfuric acid stain followed by heating. For column chromatographic purification, column grade silica gel (0.063–0.200 mm mesh size) was employed using Hexane-EtOAc or CH_2Cl_2–MeOH mixtures of increasing polarity as eluent. Solvents were purchased from Vetec Química and were distilled prior to use. Reagents were purchased from Aldrich and used without further purification. High-resolution mass spectra (HRMS) were recorded in a Micromass LCT spectrometer at the Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France. Compounds **4** and **5** were prepared as reported in references 19 and 20, respectively.

4.2. Synthesis of 7-O-(1″,2″:3″,4″-di-O-isopropylidene-6″-deoxy-α-D-galactopyrane-6″-yl)-genistein (**6**) and 7,4′-di-O-(1″,2″:3″,4″-di-O-isopropylidene-6″-deoxy-α-D-galactopyrane-6″-yl)-genistein (**7**)

Potassium carbonate (1.33 mmol) at room temperature was added to a solution of genistein **1** (0.75 mmol) in 10 mL of DMF. The mixture was stirred for 1 h. Next, 6-deoxy-6-iodo-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (**4**, 2.25 mmol) was added to the solution. The mixture was stirred at 80 °C for 48 h, DMF was removed under reduced pressure, and the residue was extracted with diethyl ether and water. The organic phase was dried with sodium sulfate and the solvent was concentrated under reduced pressure. The residue was purified by column chromatography (hexane/AcOEt) to afford **6** and **7**.

6 (30%); mp: 48–50 °C; $[\alpha]_D = -120$ (*c* 0.1, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.36 (s, 6H), 1.48 (s, 3H), 1.56 (s, 3H), 4.19–4.20 (m, 3H), 4.35–4.38 (m, 2H), 4.68 (dd, 1H, $J = 2.4$ and 7.8 Hz), 5.59 (d, 1H, $J = 5.0$ Hz), 6.40 (d, 1H, $J = 2.2$ Hz), 6.43 (d, 1H, $J = 2.2$ Hz), 6.83 (d, 2H, $J = 8.6$ Hz), 7.31 (d, 2H, $J = 8.6$ Hz), 7.82 (s, 1H) and 12.77 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 24.4, 24.9, 26.0, 26.1, 66.2, 67.3, 70.5, 70.6, 70.9, 93.1, 96.3, 98.9, 106.4, 108.9, 109.7, 115.6, 122.5, 123.7, 130.2, 152.9, 156.3, 157.9, 162.5, 164.5 and 180.9; IR (KBr): 3155–3427, 2854–2988, 1656 and 1071 cm^{-1}; HRMS calculated for $[M+H]^+$ was 513.1755, the value found was 513.1745.

7 (9%); mp: 73–76 °C; $[\alpha]_D = -80$ (*c* 0.1, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.36 (s, 6H), 1.47 (s, 9H), 1.54 (s, 6H), 1.55 (s, 3H), 4.13–4.26 (m, 6H), 4.34–4.39 (m, 4H), 4.64–4.69 (m, 2H), 5.58 (d, 2H, $J = 4.9$ Hz), 6.41 (d, 1H, $J = 3.0$ Hz), 6.45 (d, 2H, $J = 3.0$ Hz), 7.02 (d, 2H, $J = 8.6$ Hz), 7.45 (d, 2H, $J = 8.6$ Hz), 7.85 (s, 1H) and 12.82 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 24.5, 24.9, 26.0, 29.7, 66.2, 66.7, 67.3, 70.6, 70.7, 70.9, 93.1, 96.4, 98.9, 108.8, 108.9, 109.5, 109.6, 115.0, 130.0, 152.7, 157.9, 158.9, 162.7 and 180.9; IR (KBr): 3140–3413, 2853–2988, 1657 and 1072 cm^{-1}; HRMS calculated for $[M+Na]^+$ was 777.2729, the value found was 777.2717.

Table 1
Nitric oxide production and cellular viability of J774 A.1 stimulated by LPS/IFN-γ and treated with genistein derivatives.[a]

Compounds[b]	NO production (μM)			Cellular viability[d] (%)		
	6.5[c]	13[c]	26[c]	6.5[c]	13[c]	26[c]
6	17.7 ± 2.3▲	12.8 ± 0.7*,▲	0.7 ± 0.1*	102.1 ± 4.3	85.5 ± 1.4	30.3 ± 17.1
7	9.8 ± 1.5*,▲	11.8 ± 0.9*,▲	12.3 ± 0.7*,▲	89.7 ± 2.1	87.3 ± 0.9	83.6 ± 8.1
8	9.7 ± 0.5*,▲	10.9 ± 1.7*,▲	9.1 ± 0.6*,▲	93.6 ± 1.9	95.2 ± 1.6	88.9 ± 2.1
9	12.7 ± 1.5*	13.1 ± 0.8*	10.4 ± 2.1*,▲	94.7 ± 2.8	92.7 ± 1.1	87.4 ± 1.5
10	11.5 ± 0.4*,▲	12.1 ± 0.2*	11.7 ± 0.2*,▲	82.1 ± 8.3	86.7 ± 5.8	87.9 ± 2.0
11	7.2 ± 0.6*,▲	11.3 ± 0.5*,▲	14.3 ± 0.6*,▲	68.1 ± 4.9	64.3 ± 6.9	44.9 ± 0.1
12	8.7 ± 0.6*,▲	9.1 ± 0.2*,▲	8.5 ± 0.3*,▲	99.9 ± 0.4	96.6 ± 2.1	94.8 ± 0.4
Genistein	13.4 ± 0.4*	13.8 ± 0.6*	14.5 ± 0.6*	111.7 ± 17.7	103.9 ± 2.9	110.1 ± 1.4

*$p < 0.05$ to NO production by cells treated *versus* cells stimulated not treated, (NO production = 16.5 ± 1.4 μM).
▲ $p < 0.05$ of genistein *versus* genistein derivative.
[a] The results represent at least three independent experiments and are presented as the mean ± SEM.
[b] (**6**): *7-O-(1″,2″:3″,4″-di-O-isopropylidene-6″-deoxy-α-D-galactopyrane-6″-yl)-genistein*, (**7**): *7,4′-di-O-(1″,2″:3″,4″-di-O-isopropylidene-6″-deoxy-α-D-galactopyrane-6″-yl)-genistein*, (**8**): *7-O-(6″-deoxy-α-D-galactopyrane-6″-yl)-genistein*, (**9**): *7-O-(1″-O-methyl-2″,3″,4″-tri-O-acetyl-6″-deoxy-α-D-glicopyrane-6″-yl)-genistein*, (**10**): *7,4′-di-O-(1″-O-methyl-2″,3″,4″-tri-O-acetyl-6″-deoxy-α-D-glicopyrane-6″-yl)-genistein*, (**11**): *7-O-(1″-O-methyl-6″-deoxy-α-D-glicopyrane-6″-yl)-genistein*, (**12**): *7,4′-di-O-(1″-O-methyl-6″-deoxy-α-D-glicopyrane-6″yl)-genistein*.
[c] Concentration of compounds (μM).
[d] Not treated cells viability 100 ± 1.1%.

Table 2
Nitric oxide production and cellular viability of RAW 264.7 stimulated by LPS/IFN-γ and treated with genistein derivatives.[a]

Compounds[b]	NO production (μM)			Cellular viability[d] (%)		
	6.5[c]	13[c]	26[c]	6.5[c]	13[c]	26[c]
6	2.4 ± 0.3*,▲	2.5 ± 0.2*,▲	3.1 ± 0.4▲	87.6 ± 1.3	88.1 ± 4.5	71.3 ± 5.5
7	2.6 ± 1.2▲	2.7 ± 1.0▲	2.4 ± 1.0*,▲	98.9 ± 1.9	89.4 ± 6.2	94.4 ± 2.7
8	1.4 ± 1.1*,▲	0.0 ± 0.4*,▲	0.0 ± 0.4*,▲	95.9 ± 2.9	98.2 ± 0.8	95.8 ± 2.8
9	2.1 ± 0.1*,▲	4.2 ± 0.2▲	2.4 ± 0.4*,▲	103.0 ± 3.2	103.5 ± 2.6	102.8 ± 1.2
10	1.9 ± 0.3*,▲	2.5 ± 0.3*,▲	4.1 ± 0.3▲	102.8 ± 0.8	98.4 ± 2.4	101.3 ± 0.8
11	1.4 ± 0.4*,▲	1.0 ± 0.1*,▲	1.7 ± 0.5*,▲	91.3 ± 1.0	88.1 ± 2.4	78.3 ± 2.8
12	0.0 ± 0.6*,▲	0.0 ± 1.0*,▲	0.0 ± 1.0*,▲	93.6 ± 1.1	99.2 ± 1.2	92.2 ± 2.5
Genistein	6.3 ± 0.8*	6.3 ± 0.7*	6.1 ± 0.4*	128.3 ± 13.8	102.9 ± 5.2	107.9 ± 2.8

*$p < 0.05$ of NO production by cells treated *versus* cells stimulated not treated, (NO production = 6.4 ± 0.5 μM).
▲ $p < 0.05$ of genistein *versus* genistein derivative.
[a] The results represent at least three independent experiments and are presented as the mean ± SEM.
[b] (**6**): *7-O-(1″,2″:3″,4″-di-O-isopropylidene-6″-deoxy-α-D-galactopyrane-6″-yl)-genistein*, (**7**): *7,4′-di-O-(1″,2″:3″,4″-di-O-isopropylidene-6″-deoxy-α-D-galactopyrane-6″-yl)-genistein*, (**8**): *7-O-(6″-deoxy-α-D-galactopyrane-6″-yl)-genistein*, (**9**): *7-O-(1″-O-methyl-2″,3″,4″-tri-O-acetyl-6″-deoxy-α-D-glicopyrane-6″-yl)-genistein*, (**10**): *7,4′-di-O-(1″-O-methyl-2″,3″,4″-tri-O-acetyl-6″-deoxy-α-D-glicopyrane-6″-yl)-genistein*, (**11**): *7-O-(1″-O-methyl-6″-deoxy-α-D-glicopyrane-6″-yl)-genistein*, (**12**): *7,4′-di-O-(1″-O-methyl-6″-deoxy-α-D-glicopyrane-6″yl)-genistein*.
[c] Concentration of Compounds (μM).
[d] Not treated cells viability 100 ± 1.8%.

*4.3. Synthesis of 7-O-(6″-deoxy-α-D-galactopyrane-6″-yl)-genistein (**8**)*

One milliliter of trifluoroacetic acid at room temperature was added to a solution of **6** (0.2 mmol) in 2 mL of CH_2Cl_2 and 1 mL of H_2O. The mixture was stirred at room temperature for 24 h and evaporated under reduced pressure. The residue was purified by column chromatography (CH_2Cl_2/MeOH) to afford **8** (70% yield).

mp: 175–178 °C; ^{1}H NMR (300 MHz, C_5D_5N): δ 4.24–4.40 (m, 2H), 4.55–4.84 (m, 4H), 5.05 (s, 0.5H), 5.34 (d, 0.5H, J = 7.3 Hz), 6.15 (br s, $OH_{aliphatic}$), 6.69 (s, 1H), 6.74 (s, 1H), 7.30 (d, 2H, J = 8.4 Hz), 7.72 (d, 2H, J = 8.4 Hz), 8.18 (s, 1H), and 13.56 (s, 1H); ^{13}C NMR (75 MHz, C_5D_5N): δ 56.3, 66.0, 70.8, 71.0, 71.2, 71.7, 72.1, 72.3, 72.5, 75.3, 76.5, 85.2, 94.4, 95.8, 100.6, 108.0, 117.6, 129.9, 130.7, 132.2, 155.0, 159.6, 160.6, 164.4, 166.7 and 182.6; IR (KBr): 3118–3380, 2921–2986, 1669 and 1055 cm^{-1}; HRMS calculated for $[M+H]^+$ was 433.1129, the value found was 433.1122.

*4.4. Synthesis of 7-O-(1″-O-methyl-2″,3″,4″-tri-O-acetyl-6″-deoxy-α-D-glicopyrane-6″-yl)-genistein (**9**) and 7,4′-di-O-(1″-O-methyl-2″,3″,4″-tri-O-acetyl-6″-deoxy-α-D-glicopyrane-6″-yl)-genistein (**10**)*

Potassium carbonate (1.33 mmol) at room temperature was added to a solution of genistein **1** (0.75 mmol) in 10 mL of DMF. The mixture was stirred for 1 h, and methyl 2,3,4-tri-*O*-acetyl-6-deoxy-6-iodo-α-D-glucopyranoside (**5**, 2.25 mmol) was added to the solution. The mixture was stirred at 80 °C for 48 h, DMF was removed under reduced pressure, and the residue was extracted with diethyl ether and water. The organic phase was dried with sodium sulfate, and the solvent was concentrated under reduced pressure. The residue was purified by column chromatography (hexane/AcOEt) to afford **9** and **10**.

9 (55% yield); mp: 89–92 °C; $[\alpha]_D$ = +108 (*c* 0.1, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.00 (s, 3H), 2.02 (s, 3H), 2.06 (s, 3H), 3.42 (s, 3H), 4.06–4.14 (m, 3H), 4.88–4.97 (m, 2H), 5.14 (t, 1H, J = 9.0 Hz), 5.51 (t, 1H, J = 9.0 Hz), 6.32 (d, 1H, J = 3.0 Hz), 6.67 (d, 1H, J = 3.0 Hz), 6.84 (d, 2H, J = 9.0 Hz), 7.31 (d, 2H, J = 9.0 Hz), 7.81 (s, 1H) and 8.80 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.9, 55.8, 67.4, 67.6, 69.4, 70.3, 71.0, 93.3, 96.9, 98.7, 106.8, 115.8, 122.0, 122.6, 124.0, 130.4, 153.0, 156.7, 158.0, 162.9, 164.3, 170.1, 170.4 and 181.1; IR (KBr): 3154–3446, 2852–2956, 1751, 1656 and 1045 cm^{-1}; HRMS calculated for $[M+Na]^+$ was 595.1430, the value found was 595.1409.

10 (20% yield); mp: 102–103 °C; $[\alpha]_D$ = +28 (*c* 0.1, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.99 (s, 6H), 2.01 (s, 6H), 2.06 (s, 6H), 3.42 (s, 6H), 4.04–4.15 (m, 6H), 4.89–4.96 (m, 4H), 5.15 (m, 2H), 5.51 (t, 2H, J = 9.0 Hz), 6.33 (d, 1H, J = 3.0 Hz), 6.38 (d, 1H, J = 3.0 Hz), 6.94 (d, 2H, J = 9.0 Hz), 7.41 (d, 2H, J = 9.0 Hz), 7.84 (s, 1H) and 8.81 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.9, 55.7, 67.2, 67.4, 67.6, 67.9,

69.4, 69.5, 70.2, 70.4, 71.0, 71.1, 93.4, 96.9, 98.7, 106.8, 115.1, 123.7, 130.3, 153.0, 158.0, 158.9, 163.0, 164.3, 169.9, 170.3 and 181.0; IR (KBr): 3124–3430, 2850–3002, 1756, 1656 and 1049 cm^{-1}; HRMS calculated for $[M+Na]^+$ was 897.2424, the value found was 897.2407.

4.5. Synthesis of 7-O-(1″-O-methyl-6″-deoxy-α-D-glicopyrane-6″-yl)-genistein (**11**)

A solution of sodium methoxide (1.6 mmol in 5 mL of MeOH) was added to a solution of **9** (0.18 mmol) in 7 mL of MeOH. The mixture was stirred at room temperature for 24 h and neutralized with hydrochloric acid (1 mol L^{-1}) to pH = 7. The mixture was evaporated under reduced pressure and the residue was purified by column chromatography (CH_2Cl_2/MeOH) to afford **11** (95% yield).

mp: 116–119 °C; $[\alpha]_D$ = +187.5 (*c*: 0.1; MeOH); ^1H NMR (300 MHz, C_5D_5N): δ 3.48 (s, 3H), 4.17–4.23 (m, 2H), 4.45 (br s, 1H), 4.54–4.59 (m, 1H), 4.65–4.70 (m, 1H), 4.80–4.83 (m, 1H), 5.18 (s, 1H), 6.75 (d, 2H), 7.27 (d, 2H, *J* = 8.4 Hz), 7.68 (d, 2H, *J* = 8.4 Hz), 8.12 (s, 1H) and 13.50 (s, 1H); ^{13}C NMR (75 MHz, CD_3OD): δ 55.8, 69.5, 71.7, 71.8, 73.6, 75.3, 94.1, 99.9, 101.5, 107.3, 116.4, 123.3, 125.0, 131.5, 155.2, 159.0, 159.6, 163.7, 166.5 and 182.5; IR (KBr): 3149–3421, 2852–2922, 1657 and 1049; HRMS calculated for $[M+Na]^+$ was 469.1105, the value found was 469.1095.

4.6. Synthesis of 7,4′-di-O-(1″-O-methyl-6″-deoxy-α-D-glicopyrane-6″yl)-genistein (**12**)

A solution of sodium methoxide (0.5 mmol in 1 mL of MeOH) was added to a solution of **10** (0.05 mmol) in 2 mL of MeOH. The mixture was stirred at room temperature for 24 h and neutralized with hydrochloric acid (1 mol L^{-1}) to pH = 7. The mixture was evaporated under reduced pressure and the residue was purified by column chromatography (CH_2Cl_2/MeOH) to afford **12** (87% yield).

mp: 119–121 °C; $[\alpha]_D$ = +120.0 (*c*: 0.1; CD_3OD) ^1H NMR (300 MHz, C_5D_5N): δ 3.48 (d, 6H), 4.24–4.26 (m, 4H), 4.46 (br s, 2H), 4.60–4.83 (m, 6H), 5.19 (s, 2H), 6.77 (br s, $OH_{aliphatic}$), 7.69 (d, 2H, *J* = 8.4 Hz) and 8.17 (s, 1H); ^{13}C NMR (75 MHz, C_5D_5N): δ 51.3, 56.8, 66.1, 70.5, 71.2, 73.1, 73.4, 75.3, 77.0, 77.1, 95.1, 101.1, 103.1, 106.3, 116.8, 132.3, 155.6, 159.9, 161.5, 164.8, 167.3 and 182.7; IR (KBr): 3159–3422, 2853–2961, 1663 and 1049; HRMS calculated for $[M+Na]^+$ was 645.1790, the value found was 645.1680.

4.7. Cell line and culture

J774A.1 and RAW 264.7 macrophages were placed in 96-well plates containing RPMI-1640 supplemented (2 mM L-glutamine, 100 μg mL^{-1} of streptomycin and penicillin, 5% fetal bovine serum) at 2 × 10^5 cells mL^{-1}. Cells were incubated at 37 °C in 5% CO_2 atmosphere in the presence of genistein or its derivatives (6.5, 13 and 26 μM) for 1 h and subsequently stimulated with LPS (10 μg mL^{-1}) and IFN-γ (9 ng mL^{-1}) at 10% of culture volume. TNF-α production was measured after 24 h of culture, and NO was measured after 48 h. For the cellular viability assay (MTT assay), J774A.1, and RAW 264.7 macrophages were placed in 96-well plates, at 2 × 10^5 cells mL^{-1}, in supplemented RPMI-1640. Cells were treated with genistein or its derivatives (6.5, 13, and 26 μM) for 48 h at 37 °C in 5% CO_2 atmosphere. Genistein (Sigma, St. Louis, MO, USA) and its derivatives were solubilized in dimethyl sulfoxide (DMSO – Sigma St. Louis, MO, USA), never exceeding 0.1% (v/v), and diluted in RPMI-1640 (Gibco, Grand Island, USA) before use. The concentration of DMSO was determined in order to allow the solubilization of the compounds, but without interfering with macrophage activity. Genistein and its derivatives were tested using concentrations defined in previous studies, which showed the effect of isoflavonoids on macrophage cultures [21,28,29].

4.8. MTT assay

Cellular viability was measured using the MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay in non-stimulated cell cultures. After 48 h of culture, the supernatant was removed and cells were incubated with 100 μL of supplemented RPMI medium and 10 μL of MTT (5 mg mL^{-1}) for 4 h at 37 °C in 5% CO_2. After purple formazan crystal formation, the supernatant was gently removed and crystal products were solubilized and incubated with DMSO. Complete solubilization was obtained by shaking the plates for 10 min. Optical density (OD) values were determined in the microplate reader (Spectramax 190; Molecular Devices, Sunnyvale, CA, USA) at 560 nm [30]. Cellular viability was calculated using the formula $(\overline{x}_1/\overline{x}_2)$*100, considering $\overline{x}_1$ the mean OD of treated cells and $\overline{x}_2$ the mean OD of untreated cells. Compounds were considered cytotoxic when viability was lower than 70%.

4.9. Griess method

Nitrites were quantified in supernatants from J774A.1 cells and RAW 264.7 cultures according to the Griess method. Aliquots of supernatants were plated with 1% of sulfanilamide and 0.1% of *N*-(1-naphthyl) ethylenediamine. Nitrite production was quantified by comparison with a standard curve, created using different concentrations of $NaNO_2$. Readings were carried out in the microplate reader (Spectramax 190) at 540 nm [2,3]. The differences in production of treated cells were related to NO production by stimulated and not treated J774A.1 cells (16.5 ± 1.4 μM). Nitric oxide production by stimulated and non-treated RAW 264.7 cells was 6.4 ± 0.5 μM. Inhibition of NO production was not considered for compounds that showed cytotoxicity higher than 30%, due to cell death and reduced viable cells in the well.

4.10. ELISA assay

Supernatants of J774A.1 and RAW 264.7 cells were analyzed for the quantification of TNF-α. Concentrations were assayed by ELISA using commercially available antibodies and according to the procedures supplied by the manufacturer (BD Biosciences Pharmingen, San Diego, USA).

4.11. Statistical analysis

Results represent at least three independent experiments and are presented as mean ± SEM. All data were analyzed using two-way ANOVA followed by Bonferroni posttests (GraphPad Prism 5.00), and the differences were considered significant at $p < 0.05$.

Acknowledgment

This work was supported by grants from CNPq (306575/2012-4; 503376/2012-4), CAPES (PNPD-2882/2011) and FAPEMIG. The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.08.032.

References

[1] K. Hoebe, E. Janssen, B. Beutler, The interface between innate and adaptive immunity, Nat. Immunol. 5 (2004) 971–974.

[2] S.B. Castro, C.O.R. Junior, C.C. Alves, A.T. Dias, L.L. Alves, L. Mazzoccoli, M.T. Zoet, S.A. Fernandes, H.C. Teixeira, M.V. de Almeida, A.P. Ferreira, Synthesis of lipophilic genistein derivatives and their regulation of IL-12 and TNF-α in activated J774A.1 cells, Chem. Biol. Drug Des. 79 (2012) 347–352.

[3] C.C.S. Alves, C.F. Costa, S.B.R. Castro, T.A. Corrêa, G.O. Santiago, R. Diniz, A.P. Ferreira, M.V. de Almeida, Synthesis and evaluation of cytotoxicity and inhibitory effect on nitric oxide production by J774A.1 macrophages of new anthraquinone derivatives, Med. Chem. 9 (2013) 812–818.

[4] N. Paesano, S. Marzocco, C. Vicidomini, C. Saturnino, G. Autore, G. Martino, G. Sbardella, Synthesis and biological evaluation of 3-benzyl-1-methyl- and 1-methyl-3-phenyl-isothioureas as potential inhibitors of iNOS, Bioorg. Med. Chem. Lett. 15 (2005) 539–543.

[5] G. Kollias, TNF pathophysiology in murine models of chronic inflammation and autoimmunity, Semin. Arthritis Rheum. 34 (2005) 3–6.

[6] G.M. Deng, L. Zheng, F.K. Chan, M. Lenardo, Amelioration of inflammatory arthritis by targeting the pre-ligand assembly domain of tumor necrosis factor receptors, Nat. Med. 11 (2005) 1067–1072.

[7] S.M. Gadgeel, S. Ali, P.A. Philip, A. Wozniak, F.H. Sarkar, Genistein enhances the effect of epidermal growth factor receptor tyrosine kinase inhibitors and inhibits nuclear factor kappa B in nonsmall cell lung cancer cell lines, Cancer 115 (2009) 2165–2176.

[8] S.B.R. Castro, C.O.R. Junior, C.C.S. Alves, A.T. Dias, L.L. Alves, L. Mazzoccoli, F.P. Mesquita, N.S.V. Figueiredo, M.A. Juliano, M.C.M.N. Castañon, J. Gameiro, M.V. de Almeida, H.C. Teixeira, A.P. Ferreira, Immunomodulatory effects and improved prognosis of experimental autoimmune encephalomyelitis after *O*-tetradecanoyl-genistein treatment, Int. Immunopharmacol. 12 (2012) 465–470.

[9] M.L.D. Paula, D.H. Rodrigues, H.C. Teixeira, M.M. Barsante, M.A. Souza, A.P. Ferreira, Genistein down-modulates pro-inflammatory cytokines and reverses clinical signs of experimental autoimune encephalomyelitis, Int. Immunopharmacol. 8 (2008) 1291–1297.

[10] A. Rusin, Z. Grynkiewicz, A. Gogler, J.Z. Puchalka, W. Szeja, Synthetic derivatives of genistein, their properties and possible applications, Acta Biochim. Pol. 57 (2010) 23–34.

[11] C.D. Allred, Y.H. Ju, K.F. Allred, J. Chang, W.G. Helferich, Dietary genistin stimulates growth of estrogen-dependent breast cancer tumors similar to that observed with genistein, Carcinogenesis 22 (2001) 1667–1673.

[12] A.J. Day, M.S. DuPont, S. Ridley, M. Rhodes, M.J.C. Rhodes, M.R.A. Morgan, G. Williamson, Deglycosylation of flavonoid and isoflavonoide glycosides by human small intestine and liver β-glucosidase activity, FEBS Lett. 436 (1978) 71–75.

[13] M.K. Piskula, J. Yamakoshi, Y. Iwai, Daidzein and genistein but not their glucosides are absorbed from the rat stomach, FEBS Lett. 447 (1999) 287–291.

[14] W. Andlauer, J. Kolb, P. Furst, Absorption and metabolism of genistein in the isolated rat small intestine, FEBS Lett. 475 (2000) 127–130.

[15] K.D.R. Setchell, N.M. Brown, P. Desai, L. Zimmer-Nechemias, B.E. Wolfe, W.T. Brashear, A.S. Kirschner, A. Cassidy, J.E. Heubi, Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone, J. Nutr. 131 (2001) 1362S–1375S.

[16] R.A. Walgren, J.T. Lin, R.K.H. Kinne, T. Walle, Cellular uptake of dietary flavonoid quercetin 49-β-glucoside by sodium-dependent glucose transporter SGLT11, J. Pharmacol. Exp. Ther. 294 (2000) 837–843.

[17] P.T. Lewis, K. Wahala, A. Hoikkala, I. Mutikainen, Q.H. Meng, H. Adlercreutz, M.J. Tikkanen, Synthesis of antioxidant isoflavone fatty acid esters, Tetrahedron 56 (2000) 7805–7810.

[18] L. Ho, D.M. Chang, H.Y. Shiau, C.H. Chen, T.Y. Hsieh, Y.L. Hsu, C.S. Wong, J.H. Lai, Aspirin differentially regulates endotoxin-induced IL-12 and TNF-α production in human dendritic cells, Scand. J. Rheumatol. 30 (2001) 346–352.

[19] A.L. Raymond, E.F. Schroeder, Synthesis of some iodo-sugar derivatives, J. Am. Chem. Soc. 70 (1948) 2785–2791.

[20] M. Zief, R.C. Hockett, Methyl 6-iodo-6-deoxy-α-D-glucopyranoside, J. Am. Chem. Soc. 67 (1945) 1267–1268.

[21] M. Comalada, I. Ballester, E. Bailón, S. Sierra, J. Xaus, J. Gálvez, F.S. Medina, A. Zarzuelo, Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship, Biochem. Pharmacol. 72 (2006) 1010–1021.

[22] F.R. Cochran, J. Selph, P. Sherman, Insights into the role of nitric oxide in inflammatory arthritis, Med. Res. Rev. 16 (1996) 547–563.

[23] J. Legault, P. Tommy, V. Mshvildadze, K. Girard-Lalancette, S. Perron, C. Laprise, P. Sirois, A. Pichette, Antioxidant and anti-inflammatory activities of quercetin 7-*O*-*β*-D glucopyranoside from the leaves of *Brasenia schreberi*, J. Med. Food 14 (2011) 1127–1134.

[24] H.A. Jung, S.E. Jin, B.S. Min, B.W. Kim, J.S. Choi, Anti-inflammatory activity of Korean Thistle Cirsium maackii and its major flavonoid, luteolin 5-*O*-glucoside, Food Chem. Toxicol. 50 (2012) 2171–2179.

[25] I. Chung, A. Ahmad, M. Ali, O. Lee, M. Kim, J. Kim, D. Yoon, C.A.M. Peebles, K.Y. San, Flavonoid glucosides from the hairy roots of *Catharanthus roseus*, J. Nat. Prod. 72 (2009) 613–620.

[26] Y. Kawai, N. Takasuka, K. Inoue, K. Akagawa, M. Nishijima, Ornithine-containing lipids stimulate CD14-dependent TNF-α production from murine macrophage-like J774.1 and RAW 264.7 cells, FEMS Immunol. Med. Microbiol. 28 (2000) 197–203.

[27] T.A. Heming, D.M. Tuazon, S.K. Davé, A.K. Chopra, J.W. Peterson, A. Bidani, Post transcriptional effects of extracellular pH on tumour necrosis factor-alpha production in RAW 246.7 and J774 A.1 cells, Clin. Sci. 100 (2001) 259–266.

[28] M. Hämäläinen, R. Nieminen, P. Vuorela, M. Heinonen, E. Moilanen, Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF κappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NFκappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages, Mediat. Inflamm. (2007) 45673.

[29] C. Chon, H. Cho, J. Park, C. Cho, Y. Song, Suppressive effects of genistein on oxidative stress and NFκappaB activation in RAW 264.7 macrophages, Biosci. Biotechnol. Biochem. 67 (2003) 1916–1922.

[30] T.A. Corrêa, E.F.C. Reis, L.L. Alves, C.C.S. Alves, S.B.R. Castro, A.T. Dias, A.F. Taveira, M. Le Hyaric, M.R.C. Couri, A.P. Ferreira, M.V. de Almeida, Preparation of amino alcohols condensed with carbohydrates: evaluation of cytotoxicity and inhibitory effect on NO production, Chem. Biol. Drug Des. 76 (2010) 451–456.