Inhibition of Nitric Oxide Production in LPS-Stimulated RAW 264.7 Macrophages and 15-LOX Activity by Anthraquinones from *Pentas schimperi*

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Key words
- *Pentas schimperi*
- Rubiaceae
- anthraquinones
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- lipoxygenase

Abstract

The anti-inflammatory activity of a coumarin and nine anthraquinone derivatives, 3-hydroxy-1-methoxy-2-methylanthraquinone (**1**), 2-hydroxymethyl anthraquinone (**2**), schimperiquinone B (**3**), cleomiscosin A (**4**), damnacanthal (**5**), 1,2-dihydroxy anthraquinone (**6**), damnacanthol (**7**), 3-hydroxy-2-hydroxymethyl anthraquinone (**8**), 1-hydroxy-2-methoxyanthraquinone (**9**), and 2-hydroxymethyl-3-*O*-prenylanthraquinone (**10**), isolated from the roots of *Pentas schimperi* were determined. The anti-15-lipoxygenase activity and nitric oxide production inhibition on lipopolysaccharide-activated macrophages RAW 264.7 cells were determined as indicators of anti-inflammatory activity. The Griess assay was used to measure nitric oxide production and the ferrous oxidation-xylenol orange assay was used to determine the 15-lipoxygenase inhibitory activity. All the compounds significantly decreased nitrite + nitrate accumulation in lipopolysaccharide-stimulated RAW 264.7 cells in a concentration-dependent manner with 85.67% to 119.75% inhibition of nitrite + nitrate production at 20 µg/mL. Most of the compounds had a moderate inhibitory effect on 15-lipoxygenase activity. Compounds **8** and **10** were the most potent inhibitor both in nitrite + nitrate production with respective IC_{50} values of 1.56 µM and 6.80 µM. Compounds **2, 7**, and **8** had good anti-15-lipoxygenase activity with respective IC_{50} values of 13.80 µM, 14.80 µM, and 15.80 µM compared to quercetin, which was used as a standard lipoxygenase inhibitor (IC_{50} of 16.80 µM). Our study revealed 3-hydroxy-2-hydroxymethyl anthraquinone and damnacanthol as potent inhibitors of both 15-lipoxygenase activity and nitric oxide production. Further studies are needed in order to envisage its possible future use as a therapeutic alternative against inflammatory diseases.

Abbreviations

COX:	cyclooxygenase
iNOS:	inducible nitric oxide synthase
LOX:	lipoxygenase
LPS:	lipopolysaccharide
NO:	nitric oxide
NOx:	nitrite + nitrate

Supporting information available online at http://www.thieme-connect.de/products

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Introduction

Macrophages are the main proinflammatory cells responsible for producing various inflammatory mediators including cytokines, chemokines, lysozymes, proteases, growth factors, eicosanoids, and NO [1]. Eicosanoids encompass a wide array of hormones derived from polyunsaturated essential fatty acids containing 20 carbon atoms. The different classes of eicosanoids are prostaglandins, thromboxanes, leukotrienes, and lipoxins. Eicosanoid production is considerably increased during inflammation [2]. Arachidonic acid, the mother substance of the proinflammatory eicosanoids, is released from membrane phospholipids in the course of inflammatory activation and is metabolized to prostaglandins and leukotrienes. Various strategies have been evaluated to control the excessive production of lipid mediators on different levels of biochemical pathways, such as inhibition of COX and LOX pathways [3]. LOX enzymes are involved in a wide variety of inflammatory conditions, and represent an additional target for anti-inflammatory therapy. Another consequence of macrophage activation in the inflammation response is the induction of iNOS

expression, with a subsequent elevation of intracellular NO [4]. NO is an intercellular mediator produced in various mammalian cells by three forms of nitric oxide synthases (NOS), namely, endothelium NO synthase (eNOS), neural NO synthase (nNOS), and inducible NO synthase (iNOS). The neuronal (nNOS) and endothelial NOS (eNOS) are constitutive with the post-translational regulation of enzyme activity, whereas the inducible isoform (iNOS) is produced in response to certain stimuli such as cytokines and bacteria LPS [5]. One of the functions of NO is the enhancement of bactericidal and tumoricidal activities of activated macrophages [6]. However, overproduction of NO could potentially result in tissue damage and activation of proinflammatory mediators associated with acute and chronic inflammations. NO is believed to induce vasodilatation in the cardiovascular system and, furthermore, it is involved in immune responses by cytokine-activated macrophages, which release NO in high concentrations. Therefore, more attention is now being paid to the development of new anti-inflammatory drugs targeting the inhibition of iNOS [7].

Nonsteroidal anti-inflammatory drugs (NSAIDs) work by inhibiting the COX pathway. Because of the significant side effect profiles of steroidal and NSAID medications, there is a greater interest in natural products. Alternatively to the COX pathway, many other inflammatory enzymes such as LOX and iNOS could be investigated as targets for new anti-inflammatory drugs. Because of their relatively few side effects, plant-derived preparations have been used for thousands of years to reduce various disorders, including pain and inflammation [8]. Plants of the genus *Pentas* have been reported in the prevention/treatment of various types of inflammatory conditions [9,10]. *Pentas schimperi* (Hook.f.) Verde (Rubiaceae) is a Cameroonian medicinal used in the treatment of viral infections and epilepsy [11]. Several bioactive compounds, including naphtoquinones, anthraquinones, coumarins, iridoids and terpenoids, have been isolated from the plant of the genus *Pentas* [12–14]. Based on the anti-inflammatory activity of *Pentas* extracts, the anti-inflammatory activities of isolated compounds were determined. Anthraquinones, the major group of naturally occurring quinones, are distinguished by a large structural variety, wide range of biological activity, and low toxicity. They exhibit a wide range of bioactivities such as antimicrobial, hypotensive, antitumor, antimalarial, analgesic, and anti-inflammatory [15,16]. Emodin, an anthraquinone derivative, has been reported as an inhibitor of iNOS [17,18]. To date, very few anti-inflammatory natural products have been reported to inhibit NO production and LOX activity. No previous study investigating the inhibitory effect of anthraquinone derivatives against 15-LOX has been reported. As part of our continuing investigation of natural products as sources of new drugs or new drug leads against pain and inflammatory conditions, one coumarin and seven naturally occurring anthraquinones derivatives isolated from *P. schimperi* and two obtained by chemical transformation were studied for their potential of inhibiting NO production and 15-LOX activity.

Results and Discussion

The structures of the isolated compounds and those from the chemical transformation (**Fig. 1**) were elucidated on the basis of spectroscopic data (EIMS, ^{1}H-NMR and ^{13}C-NMR, HSQC, HMBC). By comparison of the data with those reported in the literature, the compounds were identified as 3-hydroxy-1-methoxy-2-methylanthraquinone (**1**; $C_{16}H_{12}O_4$; m.p.: 290–292 °C; *m/z* 268) [14], 2-hydroxymethyl anthraquinone (**2**; $C_{15}H_{10}O_3$, m.p. 192–194 °C; *m/z* 238) [18], schimperiquinone B (**3**; $C_{29}H_{16}O_6$; m.p. 264–265 °C; *m/z* 460) [13], cleomiscosin A (**4**; $C_{20}H_{18}O_8$; m.p.: 249–250 °C; *m/z*: 386) [19], damnacanthal (**5**; $C_{16}H_{10}O_5$; m.p.: 214–215 °C; *m/z*: 282) [14], 1,2-dihydroxyanthraquinone (**6**; $C_{14}H_8O_4$; m.p.: 280–281 °C; *m/z*: 240) [20], damnacanthol (**7**; $C_{16}H_{12}O_5$; m.p.: 284–286 °C; *m/z*: 284) [14], and 3-hydroxy-2-hydroxymethyl anthraquinone (**8**; $C_{15}H_{10}O_4$; m.p.: 234–236 °C; *m/z* 254) [21] for the isolated compounds and as 1-hydroxy-2-methoxyanthraquinone (**9**; $C_{15}H_{10}O_4$; m.p.: 152–153 °C; *m/z* 254) [22] and 2-hydroxymethyl-3-*O*-prenylanthraquinone (**10**; $C_{15}H_{10}O_4$; m.p.: 198–199 °C; *m/z* 322) for the semisynthetic compounds. The derivative **10** was characterized here for the first time.

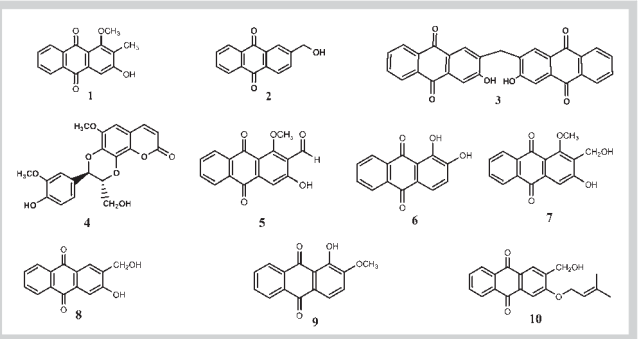

Fig. 1 Chemical structures of 3-hydroxy-1-methoxy-2-methylanthraquinone (**1**), 2-hydroxymethyl anthraquinone (**2**), schimperiquinone B (**3**), cleomiscosin A (**4**), damnacanthal (**5**), 1,2-dihydroxy anthraquinone (**6**), damnacanthol (**7**), 3-hydroxy-2-hydroxymethyl anthraquinone (**8**), 1-hydroxy-2-methoxyanthraquinone (**9**), and 2-hydroxymethyl-3-*O*-prenylanthraquinone (**10**).

In murine macrophage RAW 264.7 cells, LPS stimulation alone can induce iNOS transcription and protein synthesis, and subsequent NO production. Therefore, this cell system is an excellent model for screening potential inhibitors of the pathways that induce NO production [23]. It should be noted that NO intrinsically is difficult to quantify because of its short half-life and the presence of other scavenging molecules. Accumulation of the stable degradation products of NO, nitrite (NO_2^-) and nitrate (NO_3^-), are preferably measured as an index of NO production. Therefore, the Griess reagent is a combined measure of NOx. The effects of anthraquinone derivatives on NOx production in LPS-stimulated RAW 264.7 cells are shown in **Table 1**. All the compounds tested significantly decreased NOx accumulation in LPS-stimulated RAW 264.7 cells in a concentration-dependent manner. The percentage of NOx production inhibition ranged from 85.67% to 119.75% and from 6.67% to 53.83% at the highest (20 µg/mL) and lowest (0.5 µg/mL) concentrations, respectively. Compound **8** had the strongest inhibitory effect with an IC_{50} value of 1.57 µM, whilst compound **2** had the lowest inhibitory activity with an IC_{50} value of 23.57 µM. Since NOx is believed to be a major proinflammatory mediator, these results suggest that the anthraquinones tested, especially **8**, might have anti-inflammatory effects against the pathologic and excessive production of NOx. Several plant-derived components, including anthraquinones, have been reported to inhibit the NOx production in LPS-stimulated RAW 264.7 cells [24,25].

Table 1 Inhibitory effect of anthraquinones on NO production in LPS-activated RAW 264.7 macrophages at different concentrations (in µg/mL).

Compounds	% of NO production inhibition				NO inhibition IC_{50}
	0.5	2	5	20	(µM)
1	15.00 ± 5.85[a]	30.00 ± 2.44[a]	65.50 ± 6.56[a]	95.50 ± 1.52[a]	13.66 ± 1.23[a]
2	23.42 ± 0.63[b]	30.67 ± 2.64[a]	53.58 ± 1.93[b]	88.08 ± 1.01[b]	23.57 ± 3.65[b]
3	21.75 ± 2.17[a, c]	31.42 ± 0.80[a]	60.75 ± 0.66[a]	94.67 ± 3.83[a, c]	8.39 ± 1.17[c]
4	17.92 ± 1.81[a]	33.17 ± 1.66[a]	51.00 ± 4.25[b, c]	85.67 ± 1.51[b, d]	14.30 ± 2.87[a]
5	18.25 ± 1.09[a]	34.33 ± 2.53[a]	61.33 ± 2.43[a]	96.08 ± 1.26[a, c]	13.37 ± 1.40[a]
6	17.58 ± 2.35[a]	33.50 ± 5.81[a, b]	62.83 ± 6.53[a, d]	95.58 ± 3.50[a]	15.42 ± 2.20[a, d]
7	16.25 ± 1.52[a]	42.50 ± 3.54[c]	85.08 ± 7.05[e]	98.83 ± 1.84[a, c, e]	9.15 ± 1.08[c, e]
8	53.83 ± 4.47[d]	103.92 ± 3.21[a]	108.33 ± 2.55[f]	119.75 ± 7.04[f]	1.57 ± 0.46[f]
9	8.33 ± 1.89[a, e]	27.00 ± 2.41[a, d]	59.42 ± 5.78[b, c, d]	94.92 ± 1.70[a, c]	16.38 ± 2.71[a, d, g]
10	6.67 ± 1.68[f]	43.83 ± 2.48[b, e]	90.25 ± 1.75[e, g]	100.08 ± 0.38[e]	6.80 ± 0.97[c, h]
Quercetin	46.83 ± 1.43[d]	90.38 ± 1.76[f]	96.17 ± 2.04[h]	97.44 ± 1.23[a, c]	8.80 ± 1.06[c, e, i]

Data represent the mean ± SD of three independent experiments; values with different letters (a, b, c, d, e, f, g, h, i) at the same concentration are significantly different at $p < 0.05$.

Table 2 Effect of anthraquinones on the viability of LPS-activated RAW 264.7 macrophages at different concentrations (in µg/mL).

Compounds	% of Cell viability			
	0.5	2	5	20
1	97.39 ± 2.49[a]	83.29 ± 4.14[a]	76.40 ± 2.89[a]	73.45 ± 2.13[a]
2	94.00 ± 10.61[a]	88.80 ± 8.04[a]	78.73 ± 6.47[a]	73.22 ± 2.81[a]
3	73.87 ± 2.28[b]	61.62 ± 1.93[b]	49.60 ± 1.07[b]	42.61 ± 1.48[b]
4	61.87 ± 5.78[c]	60.44 ± 5.36[b]	45.35 ± 1.05[c]	45.27 ± 1.91[b, c]
5	91.29 ± 3.32[a]	80.02 ± 1.91[a, c]	72.21 ± 5.32[a, d]	73.73 ± 3.88[a]
6	78.25 ± 2.36[b, d]	89.72 ± 6.67[a, d]	78.23 ± 3.93[a]	70.47 ± 7.43[a]
7	100.69 ± 2.95[a]	97.94 ± 3.63[a, d, e]	88.46 ± 3.79[a, e]	81.78 ± 3.24[d]
8	118.96 ± 4.89[e]	109.43 ± 5.55[f]	99.33 ± 7.98[e, f]	78.67 ± 4.48[a, d]
9	94.47 ± 3.06[a]	87.24 ± 4.67[a]	77.44 ± 6.10[a, d, g]	72.35 ± 2.48[a]
10	94.33 ± 5.63[a]	91.48 ± 3.33[a, d]	84.19 ± 3.51[a, d, e, g]	81.44 ± 1.10[d]
Quercetin	93.10 ± 1.09[a]	93.76 ± 3.15[a, d, e]	90.69 ± 2.43[e, h]	79.33 ± 2.03[a, d]

Data represent the mean ± SD of three independent experiments; values with different letters (a, b, c, d, e, f, g, h) at the same concentration are significantly different at $p < 0.05$.

The numbers of viable activated RAW 264.7 macrophages were relatively altered by most of the tested compounds as determined by the MTT cell viability assay (**Table 2**). At the 20 µg/mL concentration, the percentage of cell viability in the presence of anthraquinones for 24 h varied between 42.61% and 81.78%, indicating that the inhibition of NOx synthesis by the compounds was not due simply to cytotoxic effects. However, the lowest cell viability percentages (42.61% and 45.27%) were recorded, respectively, with compounds **3** and **4**. Although the two compounds had activity against the NOx production with respective IC_{50} values of 23.57 µM and 8.39 µM, their inhibitory effect seems to be more likely related to their cytotoxic effect towards the RAW 264.7 cells. According to our results, compound **8** strongly inhibits LPS-induced nitric oxide production without notable cytotoxicity, and therefore appears as a promising NOx production inhibitor candidate. However, taking into account the fundamental differences between macrophages from mice and humans regarding NO synthase activity, more attention should be paid in the development of compound **8** as new potent drug inhibitors of NO production in relation to the treatment of chronic inflammatory diseases.

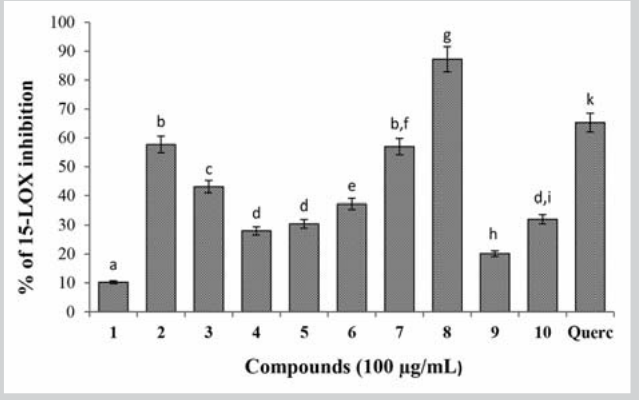

Fig. 2 Percentage of 15-lipoxygenase inhibition by anthraquinone derivatives from *P. schimperi* (Querc: quercetin). Data represent the mean ± SD of three independent experiments; values with different letters at the same concentration are significantly different at $p < 0.05$.

The anti-LOX activity of the compounds was measured as inhibition of linoleic acid's peroxidation, a reaction which is catalyzed by soybean LOX. All the compounds were initially screened at a single concentration of 100 µg/mL and the results were determined as a percentage of inhibition. As shown in **Fig. 2**, all the compounds inhibited the activity of 15-LOX with the percentage of inhibition varying from 10.25% to 87.22%. Most of the compounds had moderate anti-LOX activity. According to the classification suggested by Pinto et al. [26], high or significant activity against 15-LOX (more than 70% inhibition) was recorded for

Table 3 IC_{50} of the three active anthraquinone derivatives from *P. schimperi* against 15-lipoxygenase.

Compounds	IC_{50} (μM)
(**2**)	13.80 ± 0.33[a]
(**7**)	14.80 ± 1.54[a, b]
(**8**)	15.80 ± 1.87[b, c]
Quercetin	16.80 ± 1.32[c, d]

Data represent the mean ± SD of three independent experiments; values with different letters (a, b, c, d) are significantly different at $p < 0.05$.

compound **8**, moderate activity (41–70% inhibition) for compounds **2, 3**, and **7**, and low or insignificant activity (0–40% inhibition) was recorded for the other six compounds. The moderate anti-LOX activity of anthraquinone compounds observed in this study is in agreement with previous findings reported by Ngoc et al. [27], who found that chrysophanol, physcion, emodin, chrysophanol-8-*O*-*β*-D-glucopyranoside, and emodin-8-*O*-glucopyranoside, which possessed anthraquinone skeletons, had weak or no inhibitory activity.

Nonetheless, three out of the ten anthraquinone derivatives tested had more than a 50% inhibitory effect; these compounds were tested in a concentration-response study and the IC_{50} values were determined. As shown in **Table 3**, the three compounds **2, 7**, and **8** inhibited the activity of 15-LOX with respective IC_{50} values of 13.80 μM, 14.80 μM, and 15.80 μM. This result could be considered good anti-15-LOX activity compared to quercetin used as a standard LOX inhibitor (IC_{50} of 16.80 μM) [28]. Therefore, our result clearly indicates that compounds **2, 7**, and **8** could be considered promising 15-LOX inhibitors. To date, few anti-inflammatory natural products have been reported to inhibit LOX activity. No previous study reporting the inhibitory effect of anthraquinone derivatives against 15-LOX was found in the literature. Our results demonstrate that these compounds might be regarded as potential compounds or lead compounds for development of a 15-LOX-targeted anti-inflammatory agent.

It is noteworthy that compounds **7** and **8** inhibited the NOx production at much lower concentrations than 15-LOX (IC_{50} values of 1.57 μM in NOx inhibition and 15.80 μM in 15-LOX inhibition). This finding might indicate that the iNOS could be more target specific than 15-LOX.

Although no definite structure-activity relationship could be determined, some structural features that might have influenced the inhibitory activity can be drawn from the comparison of the chemical structures of the compounds with different activities. Compounds **7, 8**, and **10**, all bearing a hydroxymethyl at C-2, were the most potent in NOx production inhibition. Therefore, it appears that the hydroxymethyl group at C-2 of the anthraquinone scaffold might be essential for NOx production inhibitory activity. The activity decreases slightly with compound **10**, which bears a hydroxymethyl at C-2 but does not have a hydroxyl group. However, this activity decreases further with compound **3**, which does not bear a hydroxymethyl group, but instead bears a hydroxyl at C-3. This observation indicates that the inhibition of NOx production due to the hydroxymethyl seems to be more marked than that due to the hydroxyl group. Moreover, the activity decreases even more with compound **7**, which, in addition to the hydroxymethyl at C-2 and the hydroxyl at C-3, bears a methoxy at C-1. In the 15-LOX inhibitory assay, the hydroxymethyl group at C-2 seems to be necessary for the activity, since compounds **2, 7**, and **8** that were the most active, all bearing a hydroxymethyl at C-2. Therefore, the structure-activity relationship analysis suggests that the hydroxymethyl at the C-2 position of the anthraquinone scaffold might be involved in the anti-inflammatory activity of this class of compounds.

The anthraquinone derivatives tested had an inhibitory effect on NOx production with low toxicity towards RAW 264.7 cells and a relative weak inhibitory effect on 15-LOX activity. However, compounds **8** and **7** strongly inhibited both the NOx production and 15-LOX activity, thus they could be regarded as potential compounds or lead compounds for development of a NOx synthesis and 15-LOX-targeted anti-inflammatory agent.

Materials and Methods

Plant material

The roots of *P. schimperi* were collected at Mount Bamboutos, West Region, Cameroon, in February 2011. The plant material was authenticated by Mr. Tadjouteu Fulbert, a botanist of the National Herbarium of Cameroon (Yaounde), where a voucher specimen (22 547 SRF/Cam) is deposited.

Extraction and isolation

The air-dried and fine powdered roots of *P. schimperi* (2.2 kg) were extracted with EtOH (3 × 6 L) at room temperature for 72 h to yield a crude extract (82 g) after filtration and evaporation under vacuum. A portion of this extract (80 g) was subjected to chromatography (8 cm diameter by 60 cm long) on silica gel (0.200–0.500 mm, 500 g) and eluted with a gradient system of hexane-EtOAc and EtOAc-MeOH to afford 74 fractions of 300 mL each. These fractions were combined on the basis of their TLC profiles into four major fractions A–D: A (18 g, 1–17), B (13 g, 18–34), C (15 g, 35–56), and D (36 g, 57–74). Fraction A (18 g) contained mostly fatty material and was not further investigated. Fraction B (13 g) was purified on silica gel (0.063–0.200 mm) column chromatography (2 cm × 30 cm) with a gradient system of hexane-EtOAc to obtain 3-hydroxy-1-methoxy-2-methylanthraquinone (**1**, 14 mg), 2-hydroxymethyl anthraquinone (**2**, 11 mg), schimperiquinone B (**3**, 7 mg), and cleomiscosin A (**4**, 10 mg). Fraction C (15 g, 35–56) was subjected to silica gel (0.063–0.200 mm) column chromatography (2 cm × 30 cm) eluted with a gradient system of CH_2Cl_2-EtOAc to afford damnacanthal (**5**, 14 mg), 1,2-dihydroxyanthraquinone (**6**, 17 mg), damnacanthol (**7**, 14 mg), and 3-hydroxy-2-hydroxymethyl anthraquinone (**8**, 16 mg).

Semisynthesis of 9 and 10

1,2-Dihydroxyanthraquinone (10 mg, 4.16×10^{-3} mmol) was dissolved in DMF (1 mL) and dry NaOH (0.5 mg) was added, followed by MeI (5 mg). The mixture was allowed to reflux over a water bath at 60 °C for 24 h. The solvent was distilled off and the residue poured into water. The organic phase was extracted with EtOAc, then washed, dried, and separated onto a column of silica gel (hexane-Me_2CO, 9 : 1) to yield 1-hydroxy-2-methoxyanthraquinone (**9**, 3 mg, 28%).

A mixture of 3-hydroxy-2-hydroxymethylanthraquinone (4 mmol), K_2CO_3 (12 mmol), and acetone (100 mL) was stirred for 5 min at room temperature. Allyl bromide, 2.384 g, was added and the mixture was allowed to reflux over a water bath at 65 °C for 6 h. The solid phase was filtered, dried, and separated onto a silica gel column (hexane-EtOAc, 95 : 5) to afford 2-hydroxymethyl-3-*O*-prenylanthraquinone (**10**, 0.8 mg).

General experimental procedure

Melting points were determined on an Electrothermal IA9000 series digital melting point apparatus and are uncorrected. NMR spectra were recorded at room temperature on a Bruker AVANCE-400 with a solvent signal or TMS as the internal references (δ in ppm and J in Hz). EIMS were carried out on Joel DX-303 mass spectrometer. Column chromatography was run on Merk silica gel 60 (0.063–0.200 mm, 0.200–0.500 mm) and Sephadex LH-20, while TLC was carried out on silica gel GF_{254} precoated plates with detection accomplished by visualization with a UV lamp at 254 and 365 nm, followed by spraying with 50% H_2SO_4 and then heating at 100 °C. Solvents were distilled prior to use.

Chemicals for bioassays

Ferric chloride and linoleic acid were purchased from Merck and Schuchardt, respectively. Xylenol orange was obtained from Searle Company. Sodium carbonate was obtained from Holpro Analytic. Fetal calf serum (FCS) and DMEM were provided by Highveld Biological. PBS and trypsin were purchased from Whitehead Scientific. Quercetin (≥ 95% HPLC), Griess reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and DMSO, lipopolysaccharides from *Escherichia coli* 0111:B4, and 15-LOX from *Glycine max* (L.) Merr. (Fabaceae) were purchased from Sigma.

Assay of nitric oxide production and cytotoxicity of LPS-activated RAW 264.7 macrophages

Cell culture: The RAW 264.7 macrophages cell lines obtained from the American Type Culture Collection were cultured in a plastic culture flask in DMEM containing L-glutamine supplemented with 10% FCS and 1% penicillin/streptomycin/fungizone (PSF) solution under 5% CO_2 at 37 °C, and were split twice a week. Cells were seeded in 96-well microtiter plates and were activated by incubation in medium containing LPS (1 µg/mL) and various concentrations of the compounds (20, 5, 2, and 0.5 µg/mL) dissolved in DMSO. The final concentration of DMSO in the well was less than 0.2% [preliminary analysis with 0.5% (v/v) affected neither the growth of the cells nor the change of color due to this growth].

Quantification of NO released: NO concentration in culture medium was determined by the Griess reaction assay as previously described [28].

Soybean LOX inhibition assay: The assay was performed according to a previously described procedure of Pinto et al. [26] with slight modifications, as previously described [29].

Statistical analysis

All results are presented as the means of triplicate experiments. All experiments were conducted in triplicate and values are expressed as the 0mean ± standard deviation. Statistical analysis was performed with GraphPad InStat Software. Differences between values were assessed for significance using analysis of variance and results were compared using the Fisher's least significant difference (LSD) at a 5% significance level.

Supporting information

Spectral data for compounds **1**–**10** are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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