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ORIGINAL RESEARCH ARTICLE

Synthetic naphthoquinone derivatives suppressed nitric oxide and prostaglandin E2 production

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ABSTRACT

Objectives: To synthesize and evaluate the anti-inflammatory potential of fifteen naphthoquinones (NQs), determining their ability to inhibitory nitric oxide (NO•) and prostaglandin E2 (PGE2) production. **Methods:** NQs were screened for their inhibitory effect on NO• and PGE production using LPS-activated RAW 264.7 macrophages. **Results:** Three linear furanonaphthoquinones (4-6) were identified as potent inhibitors of NO• and PGE2 production, with IC_{50} values ranging from 0.45 to 2.86 μM (NO•) and 0.38 to 1.65 (PGE2), highlighting the potent activity showed by compound 5 with IC_{50} values lower than one and high selectivity indices. **Conclusions:** Synthesized furanonaphthoquinones constitute leading compounds for the design of new anti-inflammatory agents and provide a valuable tool for designing new NQs that might be useful to treat inflammation. The evaluation of compounds 4-6 using in vivo models is warranted.

Keywords: anti-inflammatory activity; synthesis; substituent effect; quinones; RAW 264.7 macrophages

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1. Introduction

Inflammation is one of the most important host defense mechanisms against tissue damage or foreign invasion, most notably microbial infections. It is a complex mechanism composed of cellular immunity and biochemical mediators with interrelated biological effects[1]. Therefore, inflammation is a beneficial process when it is limited and controlled; in contrast, chronic inflammation is a major contributor to the development of cardiovascular diseases, diabetes, rheumatoid arthritis, systemic lupus erythematosus, cancer, Alzheimer's, Parkinson's diseases, among others[2].

Macrophages play a crucial role in host immune defense mechanisms and tissue homeostasis, particularly in inflammatory response[3]. When activated by cytokines, lipopolysaccharide (LPS) of Gram-negative bacteria or with cell-wall teichoic acids and muramyl peptides, macrophages release nitric oxide (NO•), cytokines including interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNF-α), reactive oxygen species (ROS), and lipid mediators such as prostaglandins, which promote inflammation by directing cellular migration to the target site[4]. Thus, targeting the production of these inflammatory mediators provides tools to modulate the patterns of immunity in a

wide range of diseases, from autoimmunity to cancer.

Quinones, and particularly naphthoquinones (NQs), are widespread among the secondary metabolites of plants and microorganisms[5]. Their molecular structures endow them with redox properties, which confer activity in various biological oxidative processes[6]. Although these compounds have been studied mainly due to their cytotoxic and anti-cancer potential[7]. NQ structure is associated with antibacterial, anti-parasitic, antipyretic, and anti-inflammatory activities[8,9]. The anti-inflammatory effect of NQs is less explored by far, despite that the experimental evidence corroborates their promising potential and catalog them as useful lead compounds for new anti-inflammatory drugs[9–11]. The NQs, linearly or angularly anellated heterocyclic quinones, such as furanonaphthoquinones (FNQ) and indolenaphthoquinones (INQ), are recognized by their potent bioactivity. Indeed, several bioactive natural products and synthetic derivatives having these skeletons have demonstrated pronounced biological activities that positioned them as an interesting research area[12–14], highlighting their capacity to inhibit cytokine release[15–17], mast cell degranulation[18], and macrophages and neutrophil activation[19,20]. The present study aimed to identify active anti-inflammatory and less toxic NQ derivatives. For this, a series of fifteen derivatives consisting of the 1,4-NQ system either substituted with hydroxyl and alkyl side chains or fused with heterocyclic-rings (furan or indole) substituted with hydroxyl or alkyl side chains were prepared. Their bioactivity was assessed by screening the inhibition of NO• and PGE production in LPS-activated RAW 264.7 macrophages, a cell system used to mimic inflammation[21].

2. Subjects and methods

2.1. Chemistry

All the reagents were ACS grade and purchased from Sigma-Aldrich (St. Louis, MO, USA); the solvents were obtained from J.T. Baker (Center Valley, PA, USA), and the Silica 60-230, analytical and preparative thin layer (TLC) chromatography plates from Merck (Merck Millipore, Billerica, MA, USA). Melting points were measured with a Fisher/Johns melting point apparatus (Thermo Fisher Scientific, Waltham, MA, USA) and are uncorrected. The IR spectra were determined on an FTIR-8400S spectrometer (Shimadzu Corporation, Kyoto, Japan) using KBr disks. The NMR spectra were acquired on Bruker spectrometers (300 MHz, 400 MHz, or 600MHz) (Bruker, Billerica, MA, USA, and Bruker, Rheinstetten, Germany) using deuterated chloroform as solvent, and trimethylsilane (TMS) as internal standard. The mass spectra were obtained from an Agilent HP 6890 gas chromatograph equipped with an HP 5973 selective mass detector (Agilent Technologies, Santa Clara, CA, USA). TLC, MS, NMR, and HPLC analytical data confirmed that purity of test compounds was >95%.

2.1.1. General procedure for the synthesis of hydroxynaphthalene-1,4-diones (1-3), naphtho[2,3-b]furan-4,9-diones (4-6) and naphtho[1,2-b]furan-4,5-diones (7-9)

Synthesis of test compounds was performed as previously detailed by us[22]. NQs and FNQs derivatives were obtained by condensation of 2-hydroxynaphthalene-1,4-dione with aldehydes (butanal, propanal and hexanal) in acidic condition as described by Hooker[23], to afford the intermediates 2-Hydroxy-3-[(1E)-prop-1-en-1-yl]naphthalene-1,4-dione (1), 2-[(1E)-But-1-en-1-yl]-3-hydroxynaphthalene-1,4-dione (2), and 2-[(1E)-Hex-1-en-1-yl]-3-hydroxynaphthalene-1,4-dione (3). Subsequently, these compounds were oxidized and cyclized by mercuric acetate as reported by Dudley[24], using harsh conditions (i.e. concentrated hydrochloric acid, 65 °C for 2 h) to afford 2-Methylnaphtho[2,3-b]furan-4,9-dione (4), 2-Ethylnaphtho[2,3-b]furan-4,9-dione (5), 2-Butylnaphtho[2,3-b]furan-4,9-dione (6). Alternatively, milder reaction conditions (i.e. diluted hydrochloric acid, 65 °C for 15 min) were used to obtain 2-Methylnaphtho[1,2-b]furan-4,5-dione (**7**), 2-Ethylnaphtho[1,2-b]furan-4,5-dione (8) and 2-Butylnaphtho[1,2-b]furan-4,5-dione (9).

2.1.2. General synthesis of benzo[f]indole-4,9-diones

Synthesis of test compounds was performed as previously detailed by us[22]. INQ were obtained by

subjecting naphthalene-1,4-dione to amination by means of Michael-type 1,4-conjugate addition to give 2-aminonaphthalene-1,4-dione, which was submitted to manganese (III)-based oxidative free radical cyclization with aldehydes or simple ketones to afford 1H-Benzo[f]indole-4,9-dione (10), 2-Methyl-1H-benzo[f]indole-4,9-dione (11), 2-Ethyl-1H-benzo[f]indole-4,9-dione (12), 2,3-Diethyl-1H-benzo[f]indole-4,9-dione (13), 3-Methyl-2-(propan-2-yl)-1H-benzo[f]indole-4,9-dione (14), 3-Methyl-2-propyl-1H-benzo[f]indole-4,9-dione (15).

2.2. Biological evaluation

2.2.1. Reagents

Dulbecco's Modified Eagle Medium (DMEM), L-glutamine, Antibiotics (Penicillin-Streptomycin), dimethyl sulfoxide (DMSO), Trypan Blue, lipopolysaccharide (LPS: Escherichia coli serotype 0127:B8), (N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride (1400W), sodium nitrate ($NaNO_2$), sodium nitroprusside (SNP), N-(1,1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, and Phosphate Buffer Saline tablets were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from GIBCO (Gaithersburg, MD, USA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Calbiochem® (San Diego, CA, USA). Organic solvents were analytical grade and obtained from Mallinckrodt Baker (San Diego, CA, USA).

2.2.2. Preparation of test compounds

For biological evaluation, all of the test compounds were dissolved in DMSO to obtain a stock solution and stored as small aliquots at −20 °C. The compounds were diluted serially to the appropriate final concentration with supplemented DMEM, just before cell exposure with final doses ranging from 10 µM to 0.01 µM. The final percentage of DMSO was adjusted to 0.1% (v/v).

2.2.3. Cell culture

The murine RAW 264.7 macrophage-like cell line was obtained from the American Type Culture Collection (TIB-71; Rockville, MD, USA) and routinely cultured in DMEM supplemented with 2 mM L-glutamine, antibiotics (100 IU/mL of penicillin-100 µg/mL streptomycin) and 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2.4. MTT reduction assay

Cytotoxicity of test compounds was evaluated using the MTT assay[25]. RAW 264.7 macrophages at a confluence of 80–90% were scrapped out, adjusted to 2×10^5 cells/mL, plated into 96-wells plates and incubated for 48 h. Subsequently, the culture medium was discarded and replaced with various concentrations of test compounds (0–10 µM) or vehicle for 30 minutes, followed by stimulation with LPS (10 µg/mL). After 24 h, the supernatant was replaced by fresh medium containing MTT (0.2 mg/mL). Four hours later, the formazan crystals were dissolved in DMSO (100 µL), and optical Density at 550 nm (OD_{550}) was measured using Multiskan GO® microplate reader (Thermo Scientific, Waltham, MA, USA). Percentages of cell survival relative to the control group were calculated, as well as LC_{50} (concentration that reduces survival of the exposed sample to 50%) values. This assay was made by quadruplicate in three independent experiments ($n = 12$).

2.2.5. Production and scavenging of nitric oxide

RAW 264.7 macrophages were treated in a manner similar to that described for cell viability. In brief, cells were seeded in 24-well plates (2×10^5 cells/mL) and incubated for 48 h. After that, cells were treated for 30 min with various concentrations of test compounds (0–10 µM) or 1400 W (10 µM), as a positive control, and stimulated with LPS (10 µg/mL). Control cells were cultured under the same conditions but were not exposed to the effect of LPS. Twenty-four hours later, culture supernatants were collected and stored at −20 °C until use. Nitrite (NO_2^-) levels were quantified by the Griess method[26]. Briefly, 100 µL of supernatants were

mixed with 100 µL of Griess reagent (1:1 mixture of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4) and incubated at room temperature for 5 min. The OD_{550} was measured using a microplate reader (Multiskan GO®). The amount of nitrite was calculated from a standard curve (0–200 uM) of sodium nitrite ($NaNO_2$) freshly prepared in Phosphate Buffer Saline (PBS). Fresh culture media was used as the blank in all experiments. Inhibitory Rates (IR) were calculated against cells that were not treated but were induced with LPS.

To determine the effect of synthetic NQ on NO• in a cell-free system, we perform an assay of scavenging of NO•. Sodium nitroprusside (SNP) was used to generate NO•, which was detected by Griess reagent after reaction with dissolved oxygen to form NO_2^-. Scavengers of NO• compete with oxygen, leading to reduced production of NO_2^-[27]. Test compounds were incubated with 1 mL of SNP (5 mM) in PBS at 25 °C for 120 min. Samples (100 µL) were incubated at room temperature for 5 min with 100 µL of Griess reagent. The OD_{550} of the samples was measured using a microplate reader (Multiskan GO®) and compared with standard solutions of $NaNO_2$ (0–200 µM). Both the assays of production and scavenger were performed by triplicate in two independent experiments (n = 6).

2.2.6. PGE2 release

Levels of PGE2 in culture supernatants were determined using competitive ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Rofecoxib was used as positive control and evaluated under the same conditions. Final results were expressed as pg of the mediator/mL of supernatant, and present as mean ± SEM, from at least two independent experiments (n = 3–12).

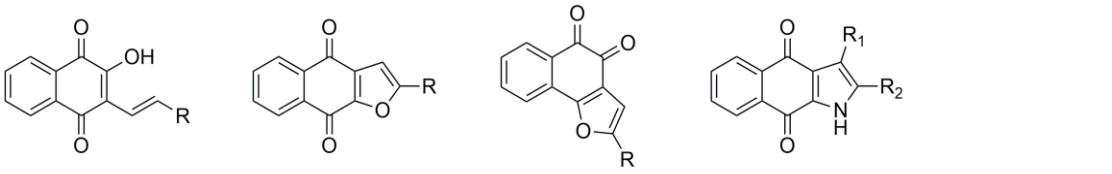
2.2.7. Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM) for each group. The concentration that inhibited the production of NO•/PGE2 by 50% (IC_{50}) was calculated using nonlinear regression analysis and expressed as mean and its 95% confidence interval. The efficacy of NO•/PGE2 inhibition was estimated by the selectivity index (SI), which was calculated as the ratio LC_{50}/IC_{50}. One-way analysis of variance (ANOVA), followed by Dunnett's post hoc test, was used to determine differences between groups. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Chemistry

In the present study, we prepared a series of 15 synthetic naphthoquinones derivatives. The synthesis of test compounds was performed as previously detailed by us[22]. The structures of the synthesized compounds are summarized in **Figure 1**. The analytical analysis revealed that the purity of compounds was >95%, and their structures, physicochemical characteristics, as well as IR, MS, and NMR spectra coincided with those previously reported[22,28–31].



(1) R= CH_3
(2) R= C_2H_5
(3) R= n-C_4H_9

(4) R= CH_3
(5) R= C_2H_5
(6) R= n-C_4H_9

(7) R= CH_3
(8) R= C_2H_5
(9) R= n-C_4H_9

(10) R_1= R_2= H
(11) R_1= H; R_2= CH_3
(12) R_1= H; R_2= C_2H_5
(13) R_1= R_2= C_2H_5
(14) R_1= CH_3; R_2= $CH(CH_3)_2$
(15) R_1= CH_3; R_2= n-C_3H_7

Figure 1. Chemical structures of the NQ derivatives synthesized and evaluated in this study.

3.2. Effect of NQ derivatives on RAW 264.7 cell viability

As can be seen in **Figure 2**, most of the NQ derivatives did not show cytotoxicity against RAW 264.7 macrophages at the highest tested concentration (10 µM); therefore, they were employed at this concentration in the anti-inflammatory assays. In contrast, compounds 4–9, 14, and 15 showed a significant effect on cell viability at this concentration. Therefore, these derivatives were evaluated to determine their highest non-toxic concentration, defined as the concentration where cell viability was superior to 80%. Consequently, compounds 7 and 8 were further evaluated at concentrations lower than 0.1 µM, compounds 5 and 9 at concentrations lower than 1 µM, and compounds 4, 6, 14, and 15 at concentrations lower than 5 µM. Furthermore, the determination of LC_{50} values allowed us to classify the compounds according to their toxicity. Under our assay conditions, NQ derivatives **7**, 8 and 9 showed a high cytotoxic effect on RAW 264.7 macrophages, with LC_{50} values of 1.04 (0.75–1.42) µM, 1.17 (0.91–1.47) µM, and 2.18 (1.69–2.85) µM, respectively; while compound 5 showed a less toxic effect with LC_{50} of 7.25 (6.31–8.11) µM.

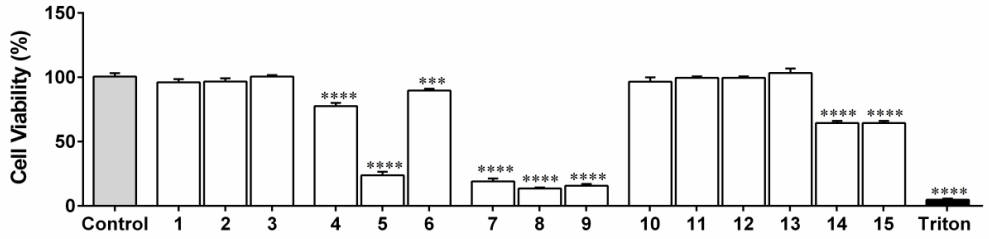


Figure 2. Effect of synthetic NQ derivatives on cell viability of RAW 264.7 macrophages. Each value represents mean ± SEM. from at least two independent experiments (n = 12).
$^{**}P < 0.01$, $^{****}P < 0.0001$ compared with control group.

3.3. Effect of NQ derivatives on NO• production in LPS-induced RAW 264.7 macrophages

RAW 264.7 macrophages, in resting state, released trace amounts of nitrite during 24 h of incubation, whereas upon exposure to LPS, the macrophages increased nitrite production, of up to a concentration of 33.08 ± 2.02 µM. Initially, we evaluated the capacity of NQ synthetic derivatives to inhibit NO• production at their highest non-toxic concentration. The effect of tested compounds, in terms of their inhibitory rates (IR), is reported in **Table 1**. Compounds were classified by their activity as highly active (IR ≥ 60%, +++), moderately active (60% > IR ≥ 40%, ++), weakly active (40% > IR ≥ 20%, +), and inactive (IR ≤ 20%, −).

Seven derivatives (1–6 and 9) suppressed significantly the LPS-induced production of NO•. However, only three compounds (4–6) inhibited the production of this inflammatory mediator by more than 60% and were classified as highly active compounds (IR > 90%) and comparable activity to that exhibited by 1400 W at 10 µM (IR = 83.88 ± 1.07%), a selective inhibitor of inducible nitric oxide synthase (iNOS), employed as a positive control.

Sodium nitroprusside (SNP), releases large amounts of NO• at physiological pH in aqueous solution. As expected, caffeic acid showed a significant effect as a scavenger of NO• (59.58%). The co-incubation of SNP with tested compounds at 10 µM did not diminish the levels of nitrite in the medium, indicating that suppression of NO• release shown by compounds 4–6 can be directly attributed to blocking NO• production in LPS-stimulated RAW 264.7 macrophages (Data not shown).

Table 1. Effect of NQ derivatives on NO• production in RAW 264.7 macrophages.

Compound	Concentration	Viability (%)	Inhibitory rates (IR)	Rank[a]
1	10 μM	96.15 ± 2.35	27.34 ± 1.99****	+
2	10 μM	96.65 ± 2.61	23.44 ± 2.01****	+
3	10 μM	102.14 ± 1.39	27.77 ± 4.23****	+
4	5 μM	97.35 ± 3.55	100.51 ± 1.01****	+++
5	1 μM	101.87 ± 2.54	91.32 ± 5.08****	+++
6	5 μM	103.65 ± 2.57	99.18 ± 0.65****	+++
7	0.1 μM	98.54 ± 2.03	6.19 ± 4.02	-
8	0.1 μM	104.04 ± 1.01	3.71 ± 1.79	-
9	1 μM	95.72 ± 1.91	26.99 ± 1.22****	+
10	10 μM	96.54 ± 3.44	8.97 ± 2.33	-
11	10 μM	99.54 ± 1.12	7.59 ± 3.52	-
12	10 μM	103.40 ± 3.51	12.25 ± 2.14	-
13	10 μM	103.41 ± 3.50	10.37 ± 1.54	-
14	5 μM	104.40 ± 2.18	9.34 ± 2.09	-
15	5 μM	108.30 ± 3.20	5.77 ± 3.78	-
1400 W	10 μM	N.D.	83.88 ± 1.07****	+++

****$P < 0.0001$ compared with LPS-treated only group. [a] Compounds were classified as highly active (IR ≥ 60%, +++), moderately active (60% > IR ≥ 40%, ++), weakly active (40% > IR ≥ 20%, +), and inactive (IR ≤ 20%, −). ***$P < 0.001$ compared with LPS-treated group.

Based on these results, we decided to study a concentration-response effect of 4–6 derivatives on LPS-activated RAW 264.7 macrophages, we found that compounds 4–6, inhibited significantly NO• production in a concentration-dependent manner, (**Figure 3A**). So, their IC_{50} was calculated, revealing values lower than those obtained for 1400 W (**Table 2**). Furthermore, compounds 4–6, showed selectivity index (SI) higher than 3, which indicates their potential as safe anti-inflammatory drugs.

Table 2. Effect of 4-6 derivatives on cell viability and accumulation of NO• and PGE2 in the culture media of RAW 264.7 macrophages in response to LPS exposure.

Compound	Cell Viability LC50 (μM)	NO production		PGE2 production	
		IC50 (μM)	SI[a]	IC50 (μM)	SI[a]
4	>10	2.86 (2.59–3.12)	>3.50	1.65 (1.29–2.09)	>6.06
5	7.25 (6.31–8.11)	0.45 (0.37–0.54)	16.11	0.38 (0.18–0.78)	19.08
6	>10	2.68 (2.39–2.96)	>3.73	1.29 (0.97–1.72)	>7.76
1400 W	>40	3.72 (2.98–4.57)	>10.75	-	-
Rofecoxib	>10	-	-	<10	~1

[a] Selectivity index.

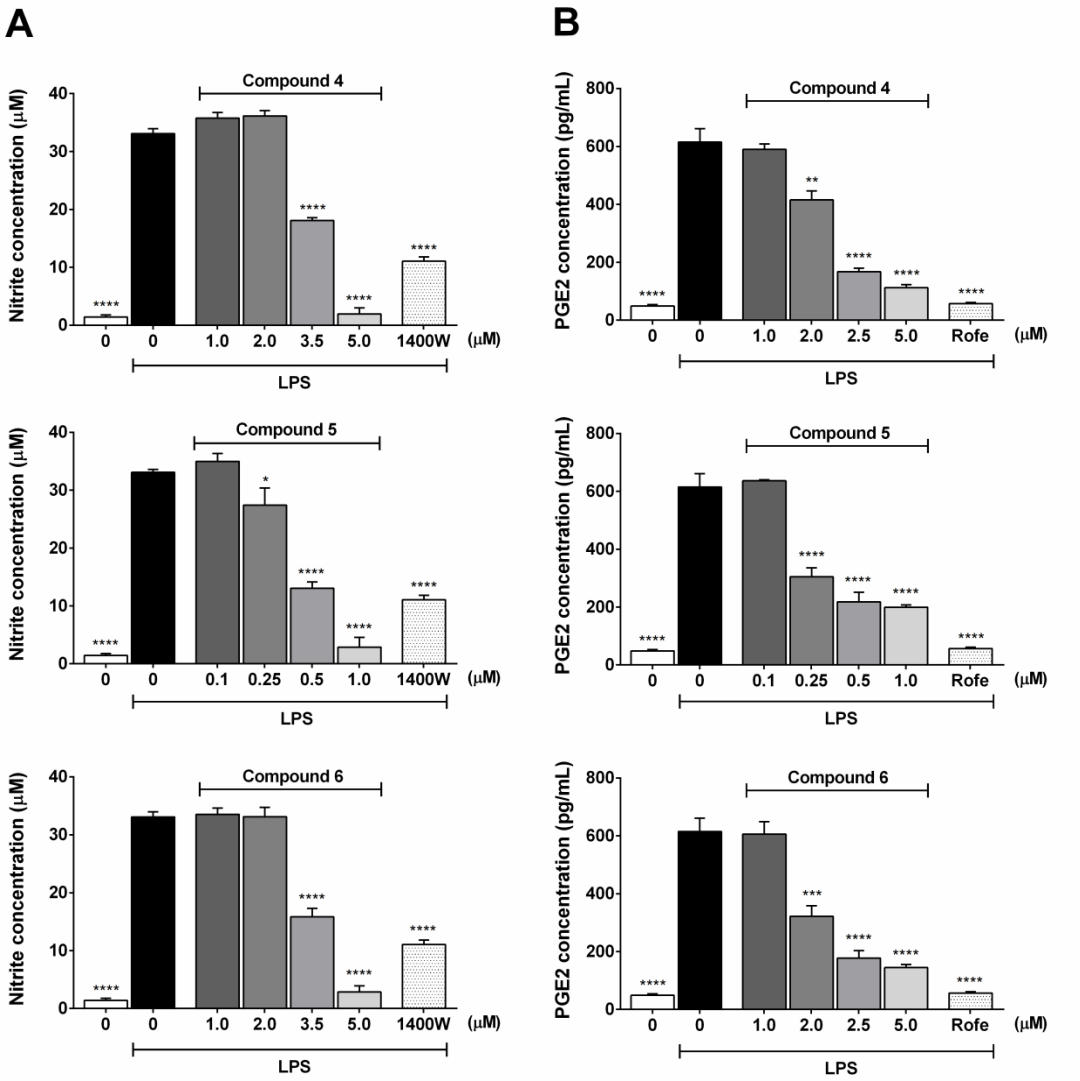


Figure 3. Inhibitory effect of compounds 4, 5 and 6 on the NO (A) and PGE2 (B) production. Results show the mean ± SEM. from at least two independent experiments (n = 3–6). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, compared with LPS treated group.

3.4. Effect of compounds 4-6 on PGE2 production

Unstimulated macrophages RAW 264.7 synthesized 48.79 ± 4.78 pg/mL of PGE2, during incubation for 24 h, whereas LPS-stimulated cells showed a significant increase in PGE2 production (615.23 ± 46.17 pg/mL). Rofecoxib, one of the most potent inhibitors of COX-2-catalyzed prostaglandin biosynthesis, was used as a positive control and significantly inhibited the production of PGE2 at 87.6 ± 1.6% at a concentration of 10 μM. Similarly, compounds 4–6 inhibited LPS-induced PGE2 production in a concentration-dependent manner (**Figure 3B**), so their values of IC_{50} were calculated (**Table 2**). Neither Rofecoxib nor compounds 4–6 at the effective concentrations showed any cytotoxic effect in RAW 264.7 cells, which can be confirmed by their high SI (>6.06, 19.08, and >7.76, respectively).

4. Discussion

Nitric oxide is widely recognized as an important and unique messenger and effector molecule in a variety of biological processes such as vasodilation, nonspecific host defense, and acute or chronic inflammation. NO• is synthesized from the amino acid L-arginine by nitric oxide synthase (NOS) isoforms in a nicotinamide adenine dinucleotide phosphate (NADPH)-and O_2-dependent process[32]. The constitutively expressed, and Ca^{2+}-calmodulin-dependent enzymes are neuronal (nNOS) and endothelial NOS (eNOS) and are responsible

for physiological NO• production. An inducible nitric oxide synthase (iNOS or NOS-2), Ca^{2+}-calmodulin-independent, is synthesized in response to inflammatory signals and is responsible for the prolonged and profound production of NO•[33]. The aberrant release of NO• can lead to amplification of inflammation by promoting oxidative stress, as well as tissue injury, and has been proven to be a contributing factor in the pathophysiology of several chronic inflammatory conditions[34,35].

Several studies have demonstrated the inhibitory effect of NQs on the production of NO• by LPS-stimulated RAW 264.7 macrophages, leading to the identification of plumbagin, naphthazarin, juglone, menadione, diosquinone, and shikonin, as promising anti-inflammatory compounds[36–38]. Therefore, the assessment of the ability of quinone derivatives to reduce the production of this mediator has been a useful tool for exploring their anti-inflammatory potential. The selection and use of this mediator have been based on its crucial role in the inflammatory process and the ease and affordability of its quantification.

In the present study, we report for the first time the effect of fifteen synthetic NQs on the production of NO• by LPS-stimulated macrophages**.** First, a cytotoxicity test (MTT assay) was performed to guarantee the viability of macrophages when treated with NQs derivatives. This allowed us to identify the angular FNQ, Naphtho[1,2-b]furan-4,5-diones (compounds 7–9), showed the higher cytotoxic effect, which is consistent with previous studies[29,39]; this bioactivity might be further explored in the design of new anti-cancer drugs. When the non-toxic concentration of test compounds was established, their anti-inflammatory effect was assessed.

The results showed that linear furanonaphthoquinones, Naphtho[2,3-b]furan-4,9-diones (compounds 4–6), are prominent inhibitors of NO• production, indicating that the fusion with furan rings favors the bioactivity when a linear conformation is obtained. In addition, it is observed that increasing the number of carbons in the 2-position on the furan ring does not generate significant differences in its activity. Moreover, the bioisosteric replacement of the furan oxygen of the basic linear furanonaphthoquinones structure with nitrogen was detrimental to activity, as the equivalent indolenaphthoquinones derivatives were inactive (4 Vs. 11 or 5 Vs. 12). The findings obtained in this work, concerning some aspects of the structure-activity relationship of naphthoquinones as anti-inflammatory agents, represent a significant contribution for other researchers who wish to design naphthoquinone-type drugs with anti-inflammatory activity.

Taking into account that NO• can induce prostaglandin synthesis and the expression of inducible cyclooxygenase-2 (COX-2)[40–42], in this work, we also assessed the effect of active linear FNQs (4–6) on the production of PGE2, an essential homeostatic factor that plays an important role in the modulation of the inflammatory and immune response through the regulation of cytokine production, leukocyte migration, proliferation, and differentiation. In macrophages, large amounts of PGE2 are generated during the inflammatory process through the oxygenated conversion of arachidonic acid from membrane glycerophospholipids to the intermediate PGH2 by COX-2 and isomerization by membrane-associated PGE synthases-1 (mPGES-1)[43,44]. Altered prostanoid production is associated with a variety of illnesses, including acute and chronic inflammation, cardiovascular disease, cancer, and allergic diseases[45].

Our results demonstrated that derivatives 4, 5, and 6 were highly active (IC_{50}<2.9 μM) as inhibitors of NO• and PGE2 production in LPS-activated macrophages. This remarkable inhibition of both NO• and PGE2 by compounds 4–6 might be achieved by the suppression of the nuclear factor-kB (NF-κB), a transcription factor involved in the expression of iNOS and COX-2, as previously reported for other furanonaphthoquinones[36,46,47]. Therefore, further studies to evaluate the mRNA and protein expression of iNOS and COX-2 or NF-κB phosphorylation should be performed.

Compound 5 presented the best anti-inflammatory profile and selectivity index, comparable to 1400W and Rofecoxib, selective inhibitors of iNOS and COX-2, respectively. Taking into account the high incidence

of undesirable side-effects induced by available anti-inflammatory drugs and considering the selectivity index of this compound, it appears as a lead compound for the development of new, more active, and selective therapeutic agents capable of inhibiting the overproduction of inflammatory mediators in related diseases and as a target for further experimental investigations. Moreover, the differences in the bioactivity of the NQ series allowed us to identify some structural elements that might be essential for their anti-inflammatory activity as the addition of a heterocyclic ring within a linear conformation is favorable while the bioisosteric replacement of furan oxygen by nitrogen is detrimental.

Finally, the identification of a new leading compound (compound 5) and the discussion of some aspects of the structure-activity relationship of naphthoquinones as anti-inflammatory agents is an important contribution to the field of medicinal chemistry and provide a valuable tool for the design of new naphthoquinones that might be useful to treat inflammation.

Authors contributions

Conceptualization, LF and RG; methodology, LF and RG; validation, LF, RG, YO, JC and JP; formal analysis, LF and RG; investigation, YO, JC and JP; resources, LF and RG; data curation, LF and RG; writing—original draft preparation, YO, JC and JP; writing—review and editing, LF and RG; visualization, YO, JC and JP; supervision, LF; project administration, LF; funding acquisition, LF and RG. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflicts of interest.

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