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Original article

# 4-Nerolidylcatechol and its synthetic analogues: Antioxidant activity and toxicity evaluation

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ABSTRACT

4-Nerolidylcatechol (**1**) is a secondary metabolite of plants and is described as a promising anti-inflammatory, antimalarial, antiulcerogenic, analgesic and cytotoxic compound possibly due to its antioxidant profile. In this study, we evaluated the pharmacologic activity and the antioxidant and toxicological profiles of compound (**1**) and its synthetic analogues (**2**–**6**). The synthetic analogues were designed from the lead compound, (**1**), using a molecular-simplification strategy. Compound **5** showed, by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and β-carotene systems, similar antioxidant activity when compared to compound (**1**). The oxidative stress in erythrocyte membrane demonstrated the highly protective effect of compounds (**4**), (**5**) and (**6**) and high antioxidant/pro-oxidant activity in relation to the concentrations of compounds (**1**) and (**3**). Compounds (**2**), (**4**), (**5**) and (**6**) were haemobiocompatible. All compounds (**1**–**6**) showed cytotoxic effects in 3T3 cells, but compounds (**2**) and (**6**) were highly cytotoxic in this lineage when compared to compound (**1**). Compound (**5**) had a lower myelosuppressive effect in haematopoietic progenitor cells compared to (**1**). Both compounds, (**1**) and (**5**), showed low genotoxic effects *in vitro*, on human lymphocyte cells. In addition, these compounds also showed low-toxicity *in vivo* as defined a $LD_{50} > 2000$ mg/kg. In this assay, we did not observe death in the animals exposed to treatment with (**1**) and (**5**) compound. In conclusion, the structural design of the analogues as validated once compound (**5**) was found to have an antioxidant profile that was as potent as the lead compound (**1**). In addition, considering the safety profile, these compounds are promising as preventive and/or therapeutic agents against oxidative damage.



## 1. Introduction

Plants have a vast diversity of defence chemicals, known as secondary metabolites. In general, these substances have specific functions including protection against oxidative or UV irradiation. Secondary metabolites such as catechol, flavanoids and lignins are potent antioxidants [1,2]. Supplementation with antioxidants in order to boost the production of endogenous antioxidants or scavenge excessive reactive oxygen species (ROS) production could be utilised to prevent some diseases by promoting the restoration of the oxidant/antioxidant balance [3–5]. In this context, ROS can result in mutagenesis and can contribute to the initiation, promotion and progression of cancer and other degenerative diseases such as heart attack, stroke, arthritis and cataracts [5,8]. Many reports have suggested that dietary phenolic compounds also exhibit pro-oxidant properties, depending on their concentration at the site of action [6–8]. In human cells, pro-oxidant activity can induce DNA damage and other pathophysiological processes [1,2]. These processes are associated with elevated levels of ROS, which may readily react with the surrounding biological tissues and damage lipids, nucleic acids and proteins [1,7,9].

4-Nerolidylcatechol (4-NRC) (**1**) is the main secondary metabolite found in Brazilian plants of the genus *Pothomorphe*, in special

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*Pothomorphe umbellata*. This species grows in the states of São Paulo, Minas Gerais, Espírito Santo and Bahia [10]. Several biological activities have been described for 4-NRC (**1**), including anti-inflammatory, antimalarial, antiulcerogenic, analgesic, and cytotoxic effects against MCF-7, B16 melanoma, HCT-8, CEM and HL-60 cells and, in special, antioxidant properties [10–16]. The antioxidant profile of the *P. umbellate* ethanolic extract was superior that of α-tocopherol, which was attributed to the presence of 4-NRC (**1**) in the extract [14,17,18]. Pohlit et al. (2004) showed that acetylated 4-NRC (**1**) loses its antioxidant properties, which may be related to the presence of a free catechol subunit [18].

With the goal of developing drugs for the treatment of cancer, we describe in the current study the synthesis, pharmacological evaluation, and the toxicological profiles of new analogues (**2**–**6**) of 4-NRC (**1**) (Fig. 1). These compounds (**2**–**6**) were designed using a molecular simplification strategy. Considering the structure of compound (**1**) we retained the aromatic ring or substituted with heteroaromatic ring and kept the lateral chain but without the chiral centre.

## 2. Materials and methods

### 2.1. Compound test

The $^{1}H$ and $^{13}C$ NMR measurements were acquired using a Bruker Avance III 500 instrument (operating at 500.13 MHz for $^{1}H$) equipped with a 5 mm tuneable multinuclear triple (TBI) resonance probe head equipped with a *z* gradient. To perform the $^{1}H$ and $^{13}C$ experiments, the samples containing 20 mg of substances typically (Table 1) in $CDCl_3$ and 1% tetramethylsilane as the internal standard were used. The 1D and 2D pulse sequences from the Bruker user library were used for the NMR experiments. Infrared (IR) spectra were obtained with a Nicolet-55a Magna spectrophotometer using potassium bromide plates. Mass spectra were obtained with an electrospray mass spectrometry (ESI) $[M + H]^+$. The progress of all reactions was monitored by thin layer chromatography (TLC), which was performed on 2.0–6.0 cm aluminium sheets precoated with silica gel 60 (Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under ultraviolet light (254–265 nm) and treated with iodine vapour. For column chromatography, we used Merck silica gel (70–230 mesh). The reagents aniline (Acros Organics, Belgium), 4-aminophenol (Acros Organics, Belgium), 3,4-(methylenedioxy) aniline (Acros Organics, Belgium), 1,3-dimethyl-1*H*-pyrazol-5-amine (Acros Organics, Belgium), $NaCNBH_3$ (Acros Organics, Belgium), $ZnCl_2$ (Acros Organics, Belgium), MsCl (Acros Organics, Belgium), $Et_3N$ (Acros Organics, Belgium), silica 60–200 μm and 70–230 mesh (Silicycle, Canada) and solvents as $CH_2Cl_2$ (Acros Organics, Belgium), MeOH (Vetec, Brazil), $CH_3CN$ (Vetec, Brazil), *n*-hexane (Vetec, Brazil), AcOEt (Vetec, Brazil) were purchased from commercial suppliers.

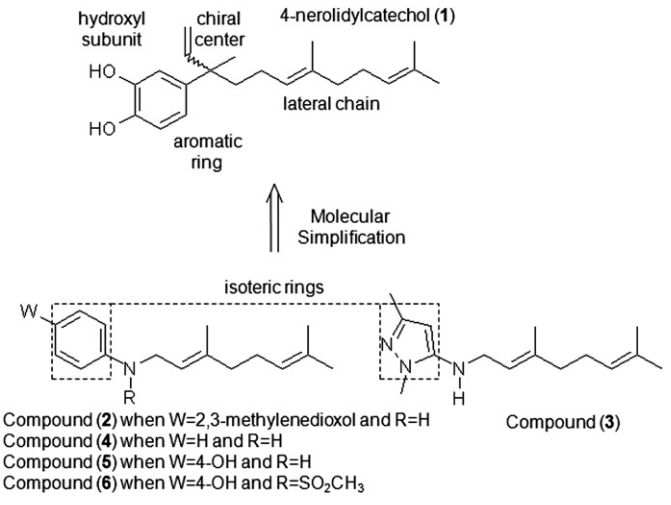


**Fig. 1.** Design of compounds (**2**–**6**) from the 4-NRC (**1**) lead compound.

**Table 1**
Balb/c 3T3-A31 ($1 \times 10^5$ cells) NRU cytotoxicity assay after 48 h exposure with (**1**–**6**) compounds using *in vitro* data to estimate the *in vivo* starting doses for acute toxicity.

| Compound test | Molecular weight | $IC_{50}$ (mM) | Estimated oral mouse $LD_{50}$ (mmol/kg) | Estimated oral mouse $LD_{50}$ (mg/kg) |
|---|---|---|---|---|
| 4-NRC (1) | 318 | 0.507 | 2.1170 | 673.220 |
| LQFM001 (2) | 137 | 0.0991 | 0.9264 | 126.91 |
| LQFM002 (3) | 247.2 | 0.200 | 1.3222 | 326.84 |
| LQFM014 (4) | 229 | 1.676 | 3.872 | 886.688 |
| LQFM015 (5) | 245 | 0.308 | 1.7239 | 422.373 |
| LQFM016 (6) | 279 | 0.158 | 1.173 | 327.43 |

#### 2.1.1. 4-NRC (*1*)

The 4-NRC was extracted from *P. umbellate* according to Rezende et al. (2004) [19].

#### 2.1.2. Procedure general to reductive amination (Luo et al., 2004) [20]

To a mixture of the aromatic amines (1.05 equiv), citral (1.0 equiv), $NaBH_3CN$ (0.5 equiv) in 5 mL of MeOH was added $ZnCl_2$ (0.5 equiv) in one portion. The mixture was stirred at room temperature for 20 min. The residue was partitioned between saturated $NaHCO_3$ and $CH_2Cl_2$. The combined organic layers were dried ($Na_2SO_4$), concentrated *in vacuo*, and the residue was purified through column chromatography using *n*-hexane:AcOEt (6:4) as mobile phase and silica (60–200 μm, 70–230 mesh) as stationary phase.

*2.1.2.1. (E)-N-(3,7-Dimethylocta-2,6-dienyl)benzo[d][1,3]dioxol-5-amine.* Compound (**2**) was obtained as a brown oil, with a yield of 72%. $^{1}H$ NMR ($CDCl_3$) $\delta$: 1.61 (3H, s, $CH_3$-10), 1.69 (3H, s, $CH_3$-8), 1.74 (3H, s, $CH_3$-9), 2.11–2.32 (4H, m, H-4 and 5), 3.63 (2H, s, H-1), 5.08 (1H, m, H-6), 5.32 (1H, m, H-2), 5.86 (2H, s, $CH_2$), 6.13 (1H, dd, $J = 2.2$ and 8.2 Hz, H-6′), 6.29 (1H, d, $J = 2.2$ Hz, H-2′) and 6.62 (1H, d, $J = 8.2$ Hz, H-5′). $^{13}C$ NMR ($CDCl_3$) $\delta$: 17.2 (1C–C9), 19.6 (1C–C10), 25.6 (1C–C8), 26.4 (1C–C6), 39.0 (1C–C4), 39.5 (1C–C1), 98.0 (1C–C2′), 101.5 (1C–C1′), 106.8 (1C–C6′), 108.5 (1C–C5′), 120.0 (1C–C2), 123.5 (1C–C6), 132.1 (1C–C7), 135.6 (1C–C3), 141.1 (1C–C3′) and 148.1 (1C–C4′). IR (KBr) $cm^{-1}$: 3301 ($\nu$ N–H), 3100 ($\nu$ C–H), 2936 ($\nu$ $CH_3$), 1684 ($\nu$ C=C) and 1373 ($\nu$ C–N); ESI-MS calculated for $C_{17}H_{24}NO_2$ $[M + H]^+$: 274.18, found: 274.12.

*2.1.2.2. (E)-N-(3,7-Dimethylocta-2,6-dienyl)-1,3-dimethyl-1H-pyrazol-5-amine.* Compound (**3**) was obtained as a yellow oil, with a yield of 76%. $^{1}H$ NMR ($CDCl_3$) $\delta$: 1.61 (3H, s, $CH_3$-10′), 1.67 (3H, s, $CH_3$-8), 1.69 (3H, s, $CH_3$-9), 2.07–2.14 (4H, m, H-4 and 5), 2.16 (3H, s, $CH_3$-3′), 3.53 (3H, s, $CH_3$-1′), 3.59–3.66 (2H, m, H-1), 5.06–5.12 (1H, m, H-6), 5.27 (1H, H-4′) and 5.29–5.36 (1H, m, H-6). $^{13}C$ NMR ($CDCl_3$) $\delta$: 13.8 (1C–C3′), 16.4 (1C–C3), 17.8 (1C–C10), 25.8 (1C–C8), 26.4 (1C–C5), 39.7 (1C–C4), 43.9 (1C–C1), 88.1 (1C–C4′), 120.8 (1C–C2), 124.0 (1C–C6), 131.7 (1C–C7), 139.4 (1C–C3), 147.1 (1C–3), and 148.5 (1C–C5′). IR (KBr) $cm^{-1}$: 3218 ($\nu$ N–H), 1398 ($\nu$ C–N), 1268 ($\nu$ C–N) and 835 ($\nu$ N–H); ESI-MS calculated for $C_{15}H_{26}N_3$ $[M + H]^+$: 248.21, found: 248.18.

*2.1.2.3. (E)-N-(3,7-Dimethylocta-2,6-dienyl) benzenamine.* Compound (**4**) was obtained as a brown oil, with a yield of 77%. $^{1}H$ NMR

($CDCl_3$) $\delta$: 1.60 (3H, s, H-10), 1.68 (3H, s, H-8), 1.70 (3H, s, H-9), 2.01–2.06 (2H, m, H-5), 2.07–2.13(2H, m, H-4), 3.67 (1H, d, $J = 6.8$ Hz, H-1), 3.79 (1H, d, $J = 6.8$ Hz, H-1), 5.07–5.13 (1H, m, H-6), 5.31–5.36(1H, m, H-2), 6.59–6.63 (2H, m, H-2′ and 6′), 6.68–7.72 (1H, m, H-4′) and 7.15–7.19 (2H, m, H-3′ and 5′). $^{13}C$ NMR ($CDCl_3$) $\delta$: 16.4 (1C–C9), 17.5 (1C–C10), 25.5 (1C–C8), 26.4 (1C–C5), 39.4 (1C–C4), 43.2 (1C–C1), 113.7 (2C–C 2′ and 6′), 117.7 (1C–C4′), 121.2 (1C–C2), 123.0 (1C–C6), 130.4 (1C–C3′ and 5′), 131.0 (1C–C7), 139.0 (1C–C3), 148.3 (1C–C1′). IR (KBr) $cm^{-1}$: 3558 ($\nu$ N–H), 3100 ($\nu$ C–H), 2906 ($\nu$ $CH_3$), and 1375 ($\nu$ C–N); ESI-MS calculated for $C_{16}H_{24}N$ $[M + H]^+$: 230.19, found: 231.06.

*2.1.2.4. (E)-N-4-(3,7-Dimethylocta-2,6-dienylamino) phenol.* Compound (**5**) was obtained as a black solid, with a yield of 60%. $^1H$ NMR ($CDCl_3$) $\delta$: 1.60 (3H, s, H-10), 1.66 (3H, s, H-8), 1.68 (3H, s, H-9), 1.96–2.04 (4H, m, H-5), 2.04–2.11(2H, m, H-4), 3.62 (1H, d, $J = 6.8$ Hz), 3.65 (1H, d, $J = 6.8$ Hz), 5.02–5.13 (1H, m, H-6), 5.28–5.36 (1H, m, H-2), 6.56 (2H, d, $J = 8.6$ Hz, H-2′ and 6′), 6.70 (2H, d, $J = 8.6$ Hz, H-3′ and 5′). $^{13}C$ NMR ($CDCl_3$) $\delta$: 16.5 (1C–C9), 17.5 (1C–C10), 26.0 (1C–C8), 26.6 (1C–C5), 38.7 (1C–C5), 39.7 (1C–C1), 115.6 (1C–C2′ and 6′), 116.6 (1C–C3′ and 5′), 121.9 (1C–C2), 124.2 (1C–C6), 131.7 (1C–C7), 139.0 (1C–C3), 141.8 (1C–C1′), 149.0 (1C–C4′). IR (KBr) $cm^{-1}$: 3427 ($\nu$ O–H), 3300 ($\nu$ N–H), 2946 ($\nu$ $CH_3$), 1686 ($\nu$ C=C) and 1606 ($\nu$ N–H); ESI-MS calculated for $C_{16}H_{24}NO$ $[M + H]^+$: 246.19, found: 246.19.

*2.1.3. Procedure to produce (E)-N-(3,7-dimethylocta-2,6-dienyl)-N-(4-hydroxyphenyl)methanesulfonamide compound (**6**)(Vogel, 1989) [21]*

To a mixture of compound (**5**) (1.0 mmol, 111 mg), MsCl (1.0 mmol, 77 μL mg) in 5 mL of acetonitrile was added triethylamine (1.0 mmol, 138 μL). The mixture was stirred at room temperature for 1 h. The residue was then partitioned between saturated $NaHCO_3$ and $CH_2Cl_2$. The combined organic layers were dried ($Na_2SO_4$), concentrated *in vacuo* and the residue was purified by through column chromatography using *n*-hexane:AcOEt (5:5) as mobile phase and silica (60–200 μm, 70–230 mesh) as stationary phase to generate (*E*)-*N*-(3,7-dimethylocta-2,6-dienyl)-*N*-(4-hydroxyphenyl)methanesulfonamide, which was obtained as a black oil with a yield of 50%.

$^1H$ NMR ($CDCl_3$) $\delta$: 1.60 (3H, s, H-10), 1.68 (3H, s, H-8), 1.70 (3H, s, H-9), 1.94–2.13 (4H, m, H-4 and 5), 3.07 (3H, s, $SO_2CH_3$), 3.65 (1H, d, $J = 6.6$ Hz, H-1), 3.68 (1H, d, $J = 6.6$ Hz, H-1), 5.02–5.13 (1H, m, H-6), 5.27–5.34(1H, m, H-2), 6.57 (2H, d, $J = 9.3$ Hz, H-3′ and 5′), 7.08 (2H, d, $J = 9.3$ Hz, H-2′ and 6′). $^{13}C$ NMR ($CDCl_3$) $\delta$: 16.3 (1C–C9), 17.6 (1C–C10), 25.6 (1C–C8), 26.1 (1C–C5), 36.7 (1C–$SO_2CH_3$), 39.6 (1C–C4), 41.9 (1C–C1), 113.0 (2C–C2′ and 6′), 120.9 (1C–C2), 122.6 (2C–C3′ and 5′), 123.7 (1C–C6), 131.0 (1C–C7), 141.0 (1C–C3), 140.2 (1C–C1′), 147.4 (1C–C4′). IR (KBr) $cm^{-1}$: 3322 ($\nu$ O–H), 3200 ($\nu$ C–H) and 1307 ($\nu$ N–H); ESI-MS calculated for $C_{17}H_{26}NO_3S$ $[M + H]^+$: 324.16, found: 324.13.

### 2.2. Cell lines and culture conditions

The mouse Balb/c 3T3 fibroblast cell line was donated by Dr. Mari Cleide Sogayar, Biochemistry Department of the Chemistry Institute of University of São Paulo, São Paulo, Brazil. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum and were routinely grown as a monolayer in 75-$cm^2$ tissue culture flasks at 37 °C, 90% humidity, 5.0% $CO_2$/air.

### 2.3. Animals

The mice used in this study were supplied by IQUEGO (Industria Química do Estado de Goiás) and raised under specific pathogen-free conditions. The female Swiss mice (eight weeks old) were maintained at 22 °C and given food and water *ad libitum* for an acclimatisation period of five days. This study was approved by the Ethical Committee of this University (protocol number 37/2009).

### 2.4. Antioxidant activity

#### 2.4.1. β-Carotene and linoleic acid system

The antioxidant activities of the compounds (**1**–**6**) at concentrations of 200–12.5 μg/mL were evaluated using the β-carotene/linoleic acid model system according to Miller (1971) [22]. Briefly, 5.0 mL of the solution of the β-carotene/linoleic acid solution (20 mg/mL) was mixed with 0.4 mL of the test compound. Trolox (400 μg/mL) was used as the positive control. The absorbance was read at 470 nm at 37 °C, 2 min after exposure and then every 15 min for 2 h total. The assays were carried out in duplicate. Oxidation inhibition was measured using the equation: Absorbance reduction (ABS reduction) = $ABS_{start} - ABS_{finish}$. The percentage of the oxidation and the protective effect of the compounds were measured using the equations:

$$\%\ \text{Oxidation} = \frac{[(\text{ABS reduction})_{test} \times 100]}{(\text{ABS reduction})_{system}}$$

% Protective effect = 100 − (% Oxidation).

#### 2.4.2. DPPH system

The capability of these compounds (**1**–**6**) to scavenge the DPPH free radical was assayed according to the method of Duarte-Almeida et al. (2006) [23]. Each test compound (500–0.48 μg/mL), 2.5 mL, was mixed with 1 mL of DPPH radical ethanolic solution (0.3 mM). The mixture was then incubated for 30 min at 25 °C in the dark. The reduction of the DPPH was determined by measuring the absorbance at 517 nm. The antioxidant activity (AA%) was calculated as demonstrated in the equation: $AA\% = 100 - \{[(ABS_T - ABS_B) \times 100]/ABS_C\}$, where AA% = Antioxidant activity; $ABS_T$ = Test absorbance (compound + DPPH); $ABS_B$ = Blank absorbance (compound + ethanol); and $ABS_C$ = Negative control Absorbance (DPPH + ethanol).

#### 2.4.3. Oxidative stress in erythrocyte membranes

The antioxidant activities of compounds (**1**–**6**) at the 100, 50, 25 and 12.5 μM concentrations were evaluated using the erythrocyte membrane model of oxidative stress [23]. The blood samples were obtained from different blood banks and diluted in saline phosphate containing 1 mM $NaN_3$ at 4 °C; the plasma and white cells were removed. The isolated erythrocytes were then incubated for 30 min at 37 °C with compounds test. Next, PBS was added until the haematocrit was stable at 2%. Oxidative stress was induced by adding $H_2O_2$ (300 μM). The test compounds in the erythrocyte solution were incubated at 37 °C for 3 h. The absorbances were measured at 540 nm.

### 2.5. Biocompatibility

The biocompatibility of compounds (**1**–**6**) (50–0.419 μg/mL) was studied using the haemolytic assay according to Laranjeira et al. (2010) [24]. The blood samples were obtained from different blood banks. After separation, the erythrocytes were prepared as a suspension in a 2% saline solution. The erythrocytes were incubated with the test sample for 1 h. Triton X (5 mg/mL) was used as the positive control. The absorbance was then read at 450 nm.

### 2.6. Toxicity profile

#### 2.6.1. Cytotoxicity in 3T3 cells using the neutral red uptake assay

The cytotoxic effects of compounds (**1**–**6**) at concentrations of 4000–20 μM were evaluated according to ECVAM (2003) [25].

Balb/c 3T3-A31 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum at 37 °C, 90% humidity, 5.0% $CO_2$/air. 3T3 cells were incubated with the synthetic analogues, and the control wells (blanks) received culture medium supplemented with 10% bovine serum. After 48 h, the solutions were removed and 250 μL of neutral red (NR) solution was added to all wells and incubated for 3 h (37 °C, 90% humidity, 5.0% $CO_2$/air). After 3 h, the NR medium (250 μg/mL of VN diluted in DMEM culture medium) was removed and the cells were carefully rinsed with 250 μL/well of pre-warmed PBS. The PBS was decanted from the plate and 100 μL of NR solvent (50 EtOH: 1 acetic acid: 49 water) solution were added to all wells, including the blanks. The plates were rapidly shaken on a microplate shaker for 20 min to extract NR from the cells and form a homogeneous solution. The absorption was measured at 550 nm in a microtiter plate reader (spectrophotometer). The optical density (OD) was calculated as the difference between the absorbance at the test wavelength and that at the reference wavelength. For each concentration tested, wells containing all the reagents used but no cells served as reference blanks.

#### 2.6.2. Clonal culture haematopoietic progenitor cell assay

The potential myelosuppressive effect of the ethanolic solutions of compounds (**1**) and (**5**) (534–4.17 μM) was evaluated using $1 \times 10^5$ bone marrow cells obtained from Swiss mouse according to Parchment et al. (1998) [26]. The incubation medium was prepared with agar (bacto agar), 2× DMEM, mouse GM-CSF and bovine serum. Next, the cells and compounds (**1**) and (**5**) were added to the incubation medium. The negative control contained only the incubation medium and cells. The cells were incubated with the test compounds for seven days. A criterion for scoring this test was read in glass with a 32× increase.

#### 2.6.3. Comet assay for measuring DNA damage

The DNA damage caused by 100, 50 and 25 μM of compounds (**1**) and (**5**) dissolved in DMSO was assessed using the comet assay.

The gel to electrophoresis (agarose solution to 1.5%), was in the glass laminate and briefly melt agarose and added the blood with heparin maintained the suspension at 37 °C. Next, the test compound was added and incubated for 1 h. Electrophoresis was performed at 25 V and 300 mA for 25 min. Then, the gel was fixed with ethanol, and the DNA was visualised with 25 μL ethidium bromide (0.5 μg/mL). The DNA damage was observed using a fluorescence microscope set to 515–560 nm (400–600×). After analysing 50 cells, these cells were classified as either without genomic damage (WGD) or with genomic damage (GD).

#### 2.6.4. Acute oral toxicity evaluation

The starting dose for the acute oral systemic toxicity assays in Swiss mice ($LD_{50}$) was estimated by using the prediction model described in the ICCVAM (2001) validation study, and these results were compared to the results obtained by *in vivo* testing performed according to OECD Test Guideline 423 (2001) [27]. After the cytotoxic tests, the estimated $LD_{50}$ values were calculated based on the equation:

$$\text{Log}\,(LD_{50}) = 0.506 \times \log\,(IC_{50}) + 0.475.$$

For the *in vivo* tests, 2000 mg/kg solutions of compounds (**1**) and (**5**) were incorporated in sunflower oil under agitation, according to OECD Test guideline 423. The test compounds (**1**) and (**5**) were administered in Swiss mice ($n = 3$) as a single dose by oral gavage. These animals were observed individually after dosing at least once during the first 30 min and periodically during the first 24 h, with special attention given during the first 4 h. Daily observations were continued for 14 days.

### 2.7. Statistical analysis

The data were analysed using the GraphPad Prism program (version 5.00 for Windows XP, GraphPad Software, San Diego, California, USA). All compounds were compared to the control using analyses of variance (ANOVA). In cases of significant differences, the Tukey test was used. Statistical significance was set at $p < 0.05$.

## 3. Results and discussion

### 3.1. Chemistry

The compounds (**2**–**5**) were produced through a reductive amination reaction, which was carried out using aromatic amines (**7**, **9**–**11**), citral (**8**), zinc chloride, and sodium cyanoborohydride in a methanol medium. Compounds (**2**–**5**) were formed, yielding among 60% and 77%, after 20 min (Fig. 2) [20]. Compound (**5**) was mesylated by using methanesulphonyl chloride, triethylamine and acetonitrile as the solvent. This procedure resulted in the formation of compound (**6**) at a yield of 50% after 1 h at room temperature (Fig. 2) (Vogel, 1989) [21]. We mesylated the compound (**5**) since some sulphonamides like nimesulide have anti-inflammatory profile as demonstrated by 4-nerolidilcathecol (**1**).

### 3.2. Antioxidant activity

#### 3.2.1. DPPH and the β-carotene and linoleic acid system

The effective concentration for the antioxidant activity of compounds (**1**–**6**) was evaluated using the β-carotene/linoleic acid system (200–12.5 μg/mL) and the DPPH system (500–0.48 μg/mL) as shown in Fig. 3. Using the DPPH assay, as reported previously, compound (**1**) showed approximately 75% antioxidant activity. A similar result was only observed with compound (**5**). Compounds (**2**), (**3**) and (**4**) showed antioxidant activities that were lower than 4-NRC (**1**). In the β-carotene/linoleic acid assay, better antioxidant activity results were observed with compounds (**1**), (**4**), (**5**) and (**6**).

As expected, these behaviours can be explained by the absence of phenolic subunit in compound structures [28]. In this regard, Ujo et al. (2001) showed that the antioxidant activity of compound (**1**) was high but was limited by the formation of semiquinone [28]. On the other hand, the high antioxidant activities of compounds (**5**) and (**6**) were, at least in part, due to the presence of the phenolic subunits. The introduction of the methylsulphonamide subunit in (**6**), such as nimesulide, which is an anti-inflammatory drug with antitumour properties, did not produce significant changes in the compounds' antioxidant profiles [29,30].

#### 3.2.2. Oxidative stress in erythrocyte membranes

In the erythrocyte membrane model of oxidative stress, all compounds (**1**–**6**) showed protective antioxidant effects. Our results showed that compounds (**2**), (**4**), (**5**) and (**6**) were highly protective at all concentrations tested than to (**1**) compound. Compound (**1**) demonstrated a protective effect at approximately 35 μM. However, when higher concentrations of (**1**) and (**3**) compounds were tested, they did not show any protective effect on erythrocyte membranes and also were able to increase the haemolysis as shown in Fig. 4. Sakihama et al. (2002) reported that when phenolic compounds accumulate inside cells, they can increase the formation of ROS and trigger cellular damage [8]. It has been reported that high concentrations of some commercial antioxidants can demonstrate a pro-oxidant profile, *e.g.*, gallic acid (3,4,5-trihydroxybenzoic acid) [31]. Yamamoto (2001) reported that α-tocopherol is associated with the first defence system to protect the cellular membrane from oxidative stress [32]. Usually,

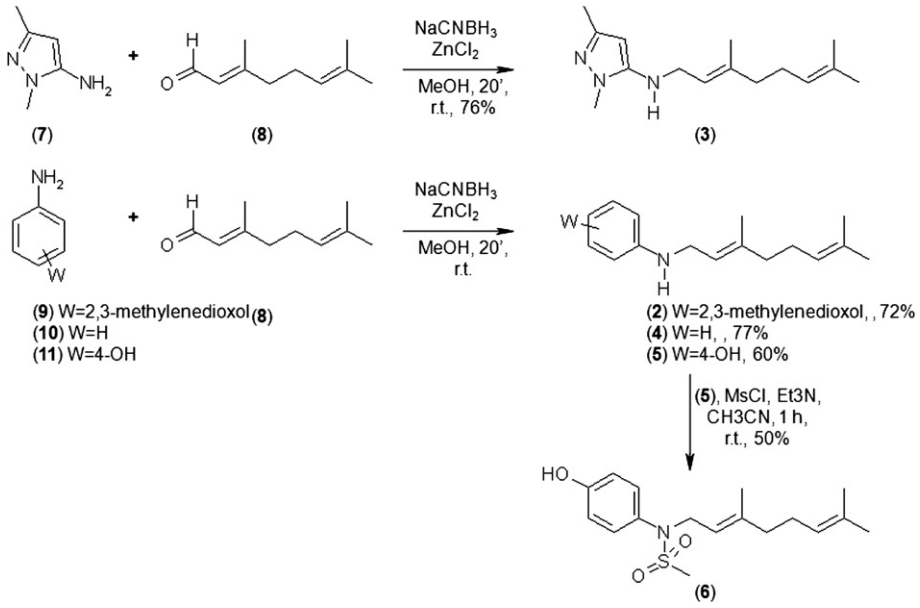


**Fig. 2.** General route of synthesis for compounds (**2**–**6**) production.

this compound inhibits the formation of lipid hydroperoxide, in early stage of oxidative process, producing the radical α-tocopherol. In general, this radical can be reverted to α-tocopherol by endogenous and exogenous antioxidants. However, when this process is blocked, lipoperoxidation occurs if the concentration of α-tocopherol is higher or similar to hydroperoxide. Considering that compound (**1**) has a chemical structure that is related to α-tocopherol, it is possible that antioxidant or pro-oxidant effects occur in a concentration-dependent manner. The same profile was observed for compound (**3**).

### 3.3. Biocompatibility

The haemolytic test is frequently used to evaluate the haemocompatibility of compounds by detecting the disruption of the membrane of the human erythrocytes. The haemolytic potentials of compounds (**1**–**6**) (50–0.419 μg/mL) are illustrated in Fig. 5. Compounds (**1**) and (**3**) showed $IC_{50}$ values of approximately 3.76 μg/mL and 1.76 μg/mL, respectively. These compounds were more toxic to human erythrocytes, with haemolytic potentials of approximately 75%. Our results showed that compounds (**1**) and (**3**) both induced haemolytic rates higher than 5% at almost all concentrations studied. Compounds (**2**), (**4**), (**5**) and (**6**) demonstrated low toxicity in erythrocyte cells in comparison to the lead compound (**1**), resulting in less than 5% haemolysis. These results indicated haemo/biocompatibility.

### 3.4. Toxicology profile

#### 3.4.1. Cytotoxicity in 3T3 cells using the neutral red assay

The cytotoxic effects of compounds (**1**–**6**) (1068–16.6 μM) using 3T3 cells are shown in Fig. 6. In this assay, all compounds (**1**–**6**) showed cytotoxic effects; however (**2**), (**3**), (**5**) and (**6**) were more cytotoxic when compared to (**1**). Compound (**4**) demonstrated the lowest cytotoxicity. The estimated $LD_{50}$ values for compounds (**1**) and (**2**–**6**) were found to be higher than 300 mg/kg (Table 1).

In general, phenolic compounds can demonstrate a toxic profile due to the possible formation of reactive intermediates, such as

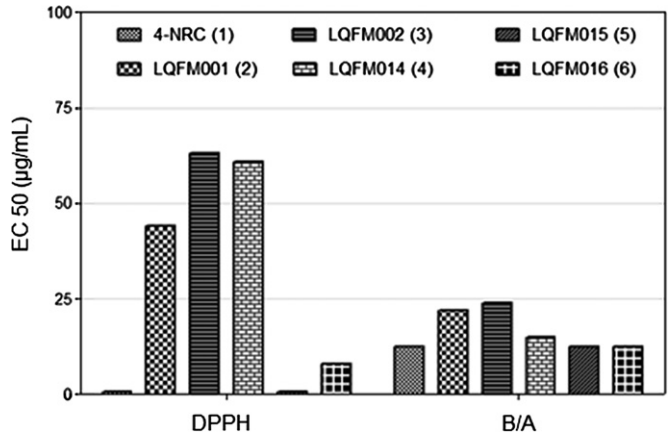


**Fig. 3.** Antioxidant activities of compounds (**1**–**6**) using DPPH (500–0.48 μg/mL) and β-carotene/linoleic acid (B/A) (200–12.5 μg/mL) assays. The figure shows the $EC_{50}$ values in relation to the test compounds for the two assays.

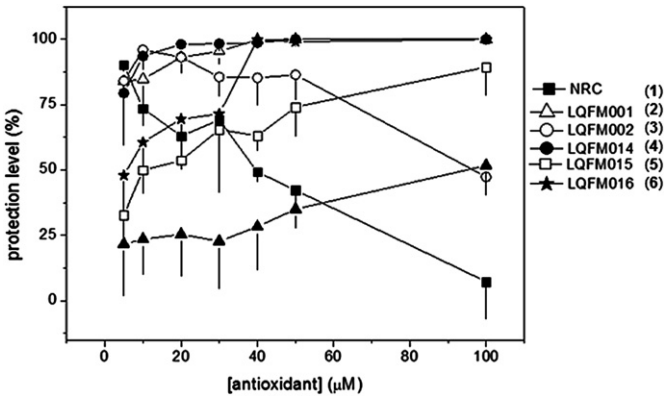


**Fig. 4.** Protective effect of compounds (**1**–**6**) (100–6.25 μM) on the erythrocyte membrane. Erythrocytes were incubated with compound test for 30 min and the oxidative stress was induced by $H_2O_2$ (300 μM). After for 3 h, the absorbances were measured at 540 nm. The data are presented as the mean ± S.D. of three experiments run in duplicate.

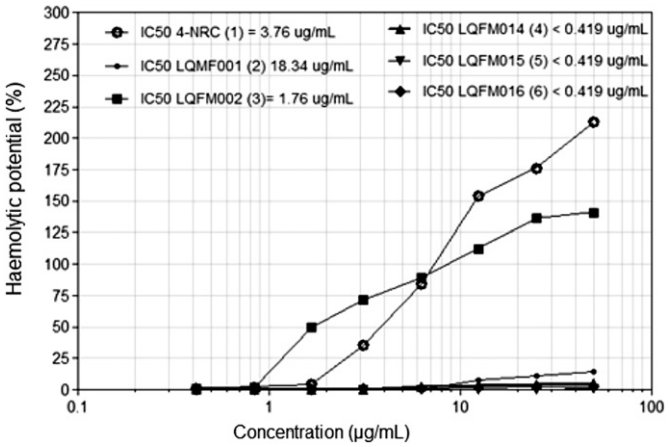


**Fig. 5.** Effects of the compounds (**1**–**6**) (50–0.419 μg/mL) on human erythrocytes. The erythrocytes (2% saline solution) were incubated with the test sample for 1 h. The absorbance was read at 450 nm. Variance was calculated by ANOVA assay and the Tukey test, and significance was set at $p < 0.05$.

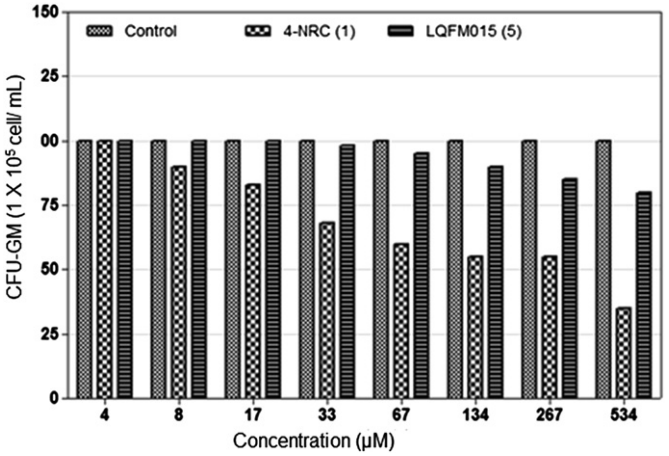


**Fig. 7.** Effects of 4-NRC (**1**) and **5** (LQFM015) (4.17–534 μM) on the growth and differentiation of bone marrow granulocyte/macrophagic progenitor cells (CFU-GM). The cells (1 × 105 cell/mL) were incubated with these compounds for seven days, and after that the number of CFU-GM was counted. *A ($p < 0.05$) in relation to the control (ANOVA).

quinones or semiquinones, which can generate Michael acceptor subunits. Michael acceptors are known to be reactive intermediates that deplete endogenous nucleophiles, *i.e.*, proteins, DNA and others, and can cause significant damage to cells (Rodriguez et al., 2004) [33]. Zapor (2004) evaluated the cytotoxic effects of five phenol derivatives (phenol, catechol, resorcinol, hydroquinone and phloroglucinol) in mouse 3T3 fibroblast cells and suggested that the cytotoxic activity of the phenolic compound most likely depends on the number of hydroxyl subunits as well as their position on the aromatic ring [34].

Considering that compounds (**1**) and (**5**) showed comparable antioxidant profiles to one another, beside biocompatibility, both were selected for the subsequent tests.

### 3.4.2. Clonal culture haematopoietic progenitor cell assay

Considering that antitumour agents are frequently haematotoxic, the myelosuppressive potentials of compounds (**1**) and (**5**) were studied and are shown in Fig. 7. Both compounds inhibited the growth and differentiation of granulocyte/macrophagic progenitor cells in a concentration-dependent manner when compared to control, non-exposed progenitor cells. Additionally, compound (**1**) ($IC_{50}$ 293 μM) was more toxic to granulocyte/macrophagic progenitor cells when compared to (**5**) ($IC_{50}$ 460.4 μM). However, when these $IC_{50}$ values were compared with the $IC_{50}$ values of classical antineoplastic compounds, such as 5-fluoruacil and cyclophosphamide, (**1**) and (**5**) showed lower haematotoxicity [35]. Usually, antineoplastic drugs and some phenols, catechols and hydroquinone compounds are able to cause myelosuppression with a direct reduction in circulating blood cells and bone marrow cellularity [36–38]. These toxic effects can cause leukopaenia, anaemia and thrombocytopaenia, which are the main limiting factors of the antitumoural treatments [39].

### 3.4.3. Comet assay for measuring DNA damage

DNA damage in lymphocyte cells was evaluated after exposure to compounds (**1**) or (**5**) at different concentrations (100, 50 and 25 μM). The results are illustrated in Table 2. DNA damage was observed only at higher concentrations of both compounds, which

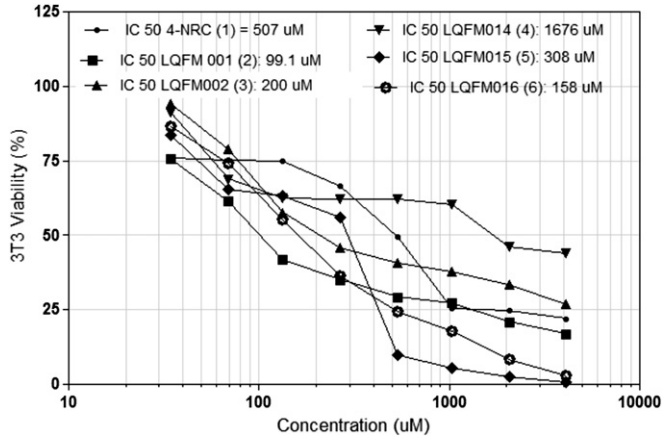


**Fig. 6.** Cytotoxic activity of compounds (**1**–**6**) on 3T3 cells (1 × $10^5$ cells) after a 48 h incubation. The curves show the cytotoxic effects of compounds (**1**–**6**) using the neutral red assay. Inhibition is expressed relative to control cell viability (100%), and each point represents the mean ± S.D. of two independent experiments run in triplicate.

**Table 2**
DNA damage in T lymphocytes exposed to three different concentrations (100, 50 and 25 μM) of compounds (**1**) and (**5**).

| Group | | Cells numbers | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Scores | | | | Scores total | WGD[a] (%) | GD[b] (%) |
| | | 0 | 1 | 2 | 3 | | | |
| Control | Negative | 45 | 5 | 0 | 0 | 5 | 90 | 10 |
| Exposed 1 | 4-NRC 25 μM | 43 | 7 | 0 | 0 | 7 | 86 | 14 |
| | 4-NRC 50 μM | 39 | 11 | 0 | 0 | 11 | 76 | 24 |
| | 4-NRC 100 μM | 37 | 13 | 0 | 0 | 13 | 74 | 26 |
| Exposed 2 | LQFM015 25 μM | 40 | 9 | 1 | 0 | 11 | 78 | 22 |
| | LQFM015 50 μM | 36 | 14 | 0 | 0 | 14 | 72 | 28 |
| | LQFM015 100 μM | 35 | 15 | 0 | 0 | 15 | 70 | 30 |

$n = 50$. Score 0: without genomic damage, score 1: low genomic damage, Score 2: moderate genomic damage and score 3: high genomic damage.
[a] WGD: cell numbers without genomic damage.
[b] GD: cells number with genomic damage.

were classified as having low genotoxicity because the cells were given the score of 1, which is the same classification attributed to the negative control. In this context, Valadares et al. (2007) showed a protective effect of compound (**1**) against the genotoxicity induced by cyclophosphamide using the micronucleus assay [40].

*3.4.4. Acute oral toxicity evaluation*

Evaluation of the acute oral toxicity of compounds (**1**) and (**5**), in agreement with OECD 423 (2001), showed low toxicity *in vivo* because death was not observed during the 14 days after exposure to the test compounds ($LD_{50} > 2000$ mg/kg). Although we did not observe death in the animals exposed to (**1**), agitation, reduced water and food consumption and diarrhoea were observed. Animals exposed to compound (**5**) initially exhibited agitation, followed by depression.

As reported by Scharage (2011), the Balb/c 3T3 method has advantages in toxicological studies because it can reduce the number of animals used for *in vivo* assays [41]. In this study, the screening performed with the 3T3 assay enabled the evaluation of the *in vivo* toxicity with a reduced number of animals. Barros et al. (2005) evaluated the *in vivo* acute toxicity of *P. umbellata* L. Miq. root extract (1, 2 and 5 g/kg), which is rich in 4-NRC, and did not observe the development of any clinical signs of toxicity either immediately or during the post-treatment period [10].

## 4. Conclusion

In conclusion, our results showed that the synthetic compound, LQFM015 (**5**), has an antioxidant profile similar to the 4-NRC (**1**) lead compound, (**5**) and that, in contrast with the lead compound, it appears to be highly biocompatible. The results of this study provide an experimental foundation that the molecular simplification strategy using 4-NRC (**1**) produced a new lead compound, which could be used further in pre-clinical antioxidant/chemopreventive properties investigations.

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