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Original article

# Synthesis and effects of some novel tetrahydronaphthalene derivatives on proliferation and nitric oxide production in lipopolysaccharide activated Raw 264.7 macrophages

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ABSTRACT

In this study, novel *N*-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalene-2-yl)-carboxamide (**6**–**15**) and 5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**16**–**32**) derivatives were synthesized and their in vitro effects at 5 μM and 50 μM concentrations on proliferation and nitric oxide (NO•) production in lipopolysaccharide (LPS) activated RAW 264.7 macrophage cells were determined. Compounds **12**, **17**, **24** and **26** were found to decrease nitrite levels in a dose-dependent manner in LPS-activated cells. At the tested concentrations, these compounds did not exhibit cytotoxic effects. Interestingly, compound **27** which contains nitroxide free radical was the most active compound in this series showing 59.2% nitrite inhibition in LPS-activated macrophage cells.



## 1. Introduction

Free radicals and oxidative stress lead to uncontrolled reactions resulting in the oxidative damage of biological macromolecules including lipids, proteins and DNA. Protection of these molecules from oxidative stress is very important in preventing the progression of a number of diseases, such as cancer, diabetes, and inflammatory diseases. Antioxidant vitamins are very effective in inhibiting above-mentioned disorders which generally occur via free radical involvements. Retinoids, synthetic and naturally occurring derivatives of vitamin A (retinol) and its most active metabolite all-*trans*-retinoic acid (ATRA), have been known to expose diverse pharmacological effects such as mediation of cell growth and differentiation in both normal and neoplastic cells, and modulation of programmed cell death known as apoptosis [1]. These properties confer a significant therapeutic potential in the fields of oncology and dermatology [2,3] as well as in other free radical-induced diseases. Retinoid analogues have received considerable attention as agents that may be useful for both prevention and treatment of some cancers [4] due to their potent growth inhibiting activities on cancer cell lines in vitro and in vivo [1]. The use of retinoids in many biological models of carcinogenesis has suggested that their action may be related to their antioxidant properties [5]. Retinoids have been shown to scavenge several free radicals and inhibit microsomal lipid peroxidation [6,7]. However, beneficial usage of these compounds is restricted due to their undesirable side effects [8,9]. For optimal results in disease prevention, which would include reduced side effects and modulating the effectiveness of their beneficial applications, new retinoidal compounds should be then required for those aspects indicated above.

On the other hand, macrophages are the main immune system cells responsible for the defensive environment of vertebrate animals, acting against pathogens and other antigenic materials. It is reported that one of the mechanisms of macrophages involves the release of various inflammatory molecules including the nitric oxide (NO•) free radical [10,11]. Overproduction of the inflammatory mediators is involved in many diseases, such as rheumatoid arthritis, chronic hepatitis, and pulmonary fibrosis [12–14]. Inflammation that is mediated by tissue-resident macrophages is an essential component of many metabolic, endocrine and cardiovascular disorders. When activated, macrophages transcriptionally express inducible nitric oxide synthase (iNOS) enzymes which cause longterm and extensive NO• production [10,11]. Abnormal release of NO• may lead to inflammation and tissue injury; highly synthesized NO• reacts with DNA and causes cells

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to undergo apoptosis [15–17]. Therefore, pharmacological interventions that aim to control potentially harmful pro-inflammatory activity of macrophages present promising treatment options. RAW 264.7 macrophages undergo apoptosis due to high levels of NO• production by iNOS activation when treated with lipopolysaccharide (LPS) in a concentration- and time-dependent manner. The use of iNOS inhibitors and scavengers of NO• produced by these activated cells blocks cell injury and death [18,19] and brings benefit to the treatment of inflammatory diseases characterized with high NO• levels.

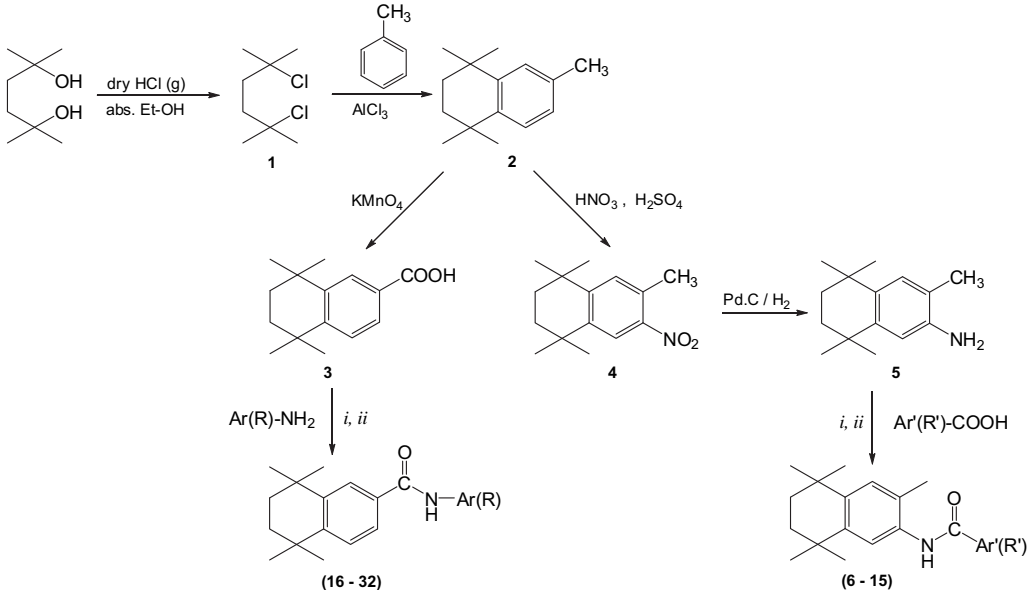
In the present study, we aim to reveal more active and less toxic novel retinoid analogues compared to ATRA. We have prepared a series of novel retinoid compounds consisting of a tetrahydronaphthalene ring system which is integrated with several moieties including alpha-lipoic acid, caffeic acid, ferulic acid, trolox and tempamine residues which have been reported to possess free radical scavenging activities [20,21]. We investigated the effects of these retinoid analogues in vitro on proliferation and NO• production in LPS-activated RAW 264.7 macrophages with comparison to known antioxidant compounds.

## 2. Chemistry

The synthetic procedures for the preparation of the compounds (**16**–**32**) are shown in Scheme 1. Commercially available 2,5-dimethyl-2,5-hexanediol and appropriate carboxylic acid or amine compounds served as starting materials. 2,5-Dichloro-2,5-dimethyl hexane (**1**) was prepared in 55% yield by passing dry hydrochloride gas over 2,5-dimethyl-2,5-hexanediol, as described by Boehm et al., 1994 [22]. Toluene was alkylated by **1** in dichloromethane catalyzed with aluminum chloride to produce 1,2,3,4-tetrahydro-1,1,4,4,6-pentamethylnaphthalene (**2**), in 68% yield [22]. Then, 5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalene carboxylic acid (**3**) was obtained by oxidation of **2** by $KMnO_4$ in alkali medium, in 57% yield [23]. A mixture of $HNO_3$ and $H_2SO_4$ was added to **2** at −10 °C to give 1,2,3,4-tetrahydro-1,1,4,4,6-pentamethyl-7-nitronaphthalene (**4**) in 35% yield. This nitro compound was hydrogenated by the usual method (35 psi $H_2$ and 10% Pd/C in EtOH, 61% yield) to produce 1,2,3,4-tetrahydro-1,1,4,4,6-pentamethyl-7-aminonaphthalene (**5**) [24]. The crude amine was used without purification. Trolox acetate was also synthesized for the preparation of **14**. A mixture of trolox (2 mmol), pyridine (5.1 ml), and acetic anhydride (4.3 ml) was stirred for 2 h at room temperature. The mixture was diluted with water (10 ml), and extracted with diethylether. The combined organic layers were washed with saturated copper sulfate solution and brine. It was dried with sodium sulfate and the solvent evaporated in vacuo to give acetyltrolox, which was used without further purification [25]. The acetylation process was confirmed by mass spectrum, 293 (M + 1) and 291 (M−1). The final products *N*-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalene-2-yl)-carboxamides (**6**–**15**), and 5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamides (**16**–**32**) were prepared by the amidification of compound **5** with appropriate acids, and compound **3** with several amines, respectively, by using different methods and various amidification agents which are stated in Experimental section.

## 3. Results and discussion

Regarding the toxic side effects, the usage of natural and synthetic retinoids is limited. Therefore, new retinoid derivatives are need to be synthesized that have increased beneficial properties and reduced adverse effects. Thus, in this study, we aimed to synthesize novel retinoid compounds which are expected to have certain antioxidative properties. To achieve this, two groups of novel twenty-seven retinoid derivatives expected to possess antioxidant activity with regard to nitric oxide radical inhibition were designed and synthesized with the substituents at 2nd and/or 3rd positions of the tetrahydronaphthalene ring. *N*-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydronaphthalene-2-yl)-carboxamide derivatives (**6**–**15**) were synthesized in five steps while the other seventeen compounds, 5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide derivatives (**16**–**32**), were synthesized in four steps (Scheme 1). The compounds (**6**–**15**) which have a methyl group at the 3rd position of the tetrahydronaphthalene moiety are linked to an amide group with appropriate substituents at the second position of the tetrahydronaphthalene ring. The amide bond formation was established with the carboxylic acid compounds. Some of these compounds




**Scheme 1.** Synthesis of novel retinoid compounds **6**–**32**. i: Coupling agent; *N*,*N*′-CDI (method I), EDCI and HOBT (method II). ii: a) $SOCl_2$, b) pyridine and triethylamine (method III); a) $SOCl_2$, b) $NaHCO_3$ (method IV); a) oxalyl chloride, b) DMAP and pyridine (method V).

show strong antioxidant properties, such as alpha-lipoic acid, caffeic acid, ferulic acid, and trolox [20]. A different substitution pattern exists in compounds **16**–**32** in which the second position of the tetrahydronaphthalene ring bears the substituents instead of the methyl group as seen in the first set of compounds. One of these linked amine compounds is a cyclic, stable nitroxide free radical tempamine which is known to possess antioxidant properties [21]. Thus, the first group of compounds indicate that the NH–CO moiety binds to tetrahydronaphthalene ring at 3rd position whereas the second substitution pattern occurred with the reverse amide formation (CO–NH) at the 2nd position of the ring.

Synthesized compounds were purified by column chromatography and recrystallization using appropriate solvent systems stated in Table 1. Expected chemical structures of the compounds have been proven by mass, IR and NMR spectral findings indicated in Experimental section and elemental analyses (Table 1).

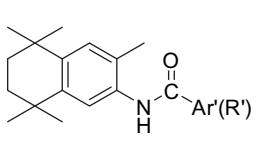
In order to determine whether compounds **9**, **10**, **11**, and **12** are *trans* or *cis* configured, a standard three-pulse NOESY NMR sequence [26] was applied in phase sensitive hypercomplex mode (States–Haberkorn method) [26] by using a relaxation delay of 1 s, an acquisition time of 0.16 s, 512 increments in t1 dimension, 1024 data points in t2 dimension, 8 transients to collect FIDs in t2 dimension, and a mixing time of 200 ms. All NOESY FIDs were acquired over 16 ppm spectral width and processed by Varian NMR v6.1C. The NOESY FIDs were zero-filled to 2K data points with a linear prediction function in both dimensions, apodized with a 90° sine-square and 6 Hz Gaussian functions in both dimensions and Fourier transformed in quadrature mode in the t2 dimension and in hypercomplex mode in the t1 dimension. NOESY spectra were displayed and analyzed either in phase sensitive or magnitude modes.

The proximity of the proton pairs $H_i$ and $H_j$ was estimated from 2D NOESY cross-peak intensities by the NMR ISPA (distance constraints via isolated spin pair approximation) method [27] which is expressed as follows,

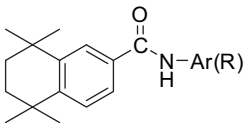
$$r_{ij} = r_{ref}\,(a_{ref}/a_{ij})^{1/6} \quad (1)$$

in which $r_{ij}$ is the distance between $H_i$ and $H_j$ to be estimated; $a_{ij}$ is the cross-peak intensity for $H_{ij}$, $r_{ref}$ and $a_{ref}$ are a known distance and a cross-peak intensity for a proton pair, respectively. The $r_{ref}$ was chosen to be the distance between either of the *trans* ethylene protons and one of the phenyl protons, 2.08 and 2.53 Å, whose distances were obtained from the crystal structure of cinnamic acid in complex with tyrosine ammonia-lyase [28]. Apparently, the distance between the ethylene protons ($r_{ij}$) exceeds 3 Å when the

**Table 1**
Isolation methods and some physico-chemical properties of compounds **6**–**32**.




**(6-15)**




**(16-32)**

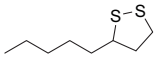
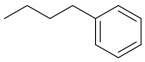
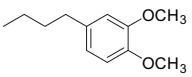
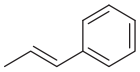
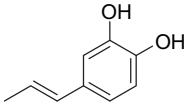
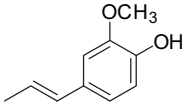
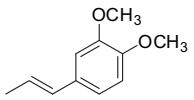
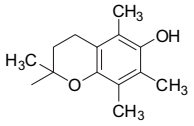
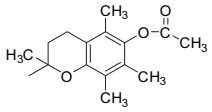
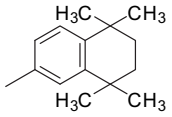
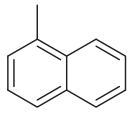
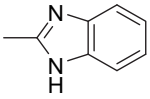
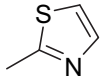
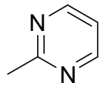
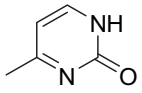
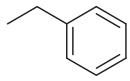
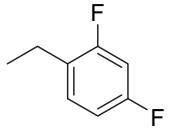
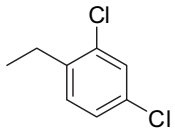
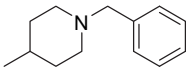
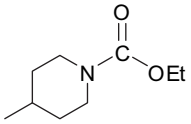
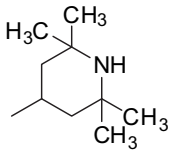
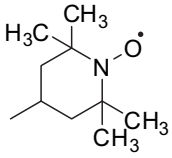
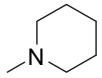
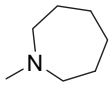
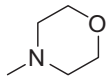
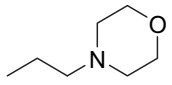
| No | –R′ (–Ar′) –R (–Ar) | Isolation | Yield (%) | M.p. (°C) | Elemental analyses (exp.) |
|---|---|---|---|---|---|
| **6** |  | *n*-Hexane:AcOEt (5:1), (4:1), cc | 85 | 87 | $C_{23}H_{35}NOS_2$: C: 67.93%; H: 8.55%; N: 3.55%; S: 15.70%. |
| **7** |  | *n*-Hexane:AcOEt (3:1), cc | 63 | 145–147 | $C_{25}H_{33}NO \cdot 0.1H_2O$: C: 82.12%; H: 9.11%; N: 3.99%. |
| **8** |  | *n*-Hexane:$CHCl_3$ (1:1), cc | 69 | 126–128 | $C_{27}H_{37}NO_3$: C: 76.29%; H: 8.77%; N: 3.41%. |
| **9** |  | Petroleum ether:AcOEt (3:1), (1:4), cc | 29 | 189–190 | $C_{24}H_{29}NO$: C: 83.00%; H: 8.16%; N: 3.91%. |
| **10** |  | *n*-Hexane:AcOEt (3:1), (1:1), cc | 27 | 198 | $C_{24}H_{29}NO_3 \cdot 0.05(CH_3)_2NCHO \cdot 0.75H_2O$: C: 73.15%; H: 7.85%; N: 3.55%. |
| **11** |  | *n*-Hexane:AcOEt (3:1), (1:1), cc | 48 | 214 | $C_{25}H_{31}NO_3$: C: 76.11%; H: 8.07%; N: 3.54%. |

**Table 1** (*continued*)

| No | −R′ (−Ar′) −R (−Ar) | Isolation | Yield (%) | M.p. (°C) | Elemental analyses (exp.) |
|---|---|---|---|---|---|
| **12** |  | Petroleum ether:AcOEt (3:1), (1:4), cc | 37 | 175–179 | $C_{26}H_{33}NO_3$: C: 76.24%; H: 8.25%; N: 3.37%. |
| **13** |  | *n*-Hexane:AcOEt (5:1), (3:1), cc | 5 | 182–183 | $C_{29}H_{39}NO_3 \cdot 0.2H_2O$: C: 76.85%; H: 8.88%; N: 3.14%. |
| **14** |  | *n*-Hexane:AcOEt (5:1), (3:1), cc | 4 | 188–189 | $C_{31}H_{41}NO_4 \cdot 0.33H_2O$: C: 74.73%; H: 8.56%; N: 2.93%. |
| **15** |  | *n*-hexane:$CHCl_3$ (1:1), cc | 18 | 150–151 | $C_{30}H_{41}NO \cdot 0.5H_2O$: C: 81.83%; H: 9.60%; N: 3.17%. |
| **16** |  | *n*-Hexane:AcOEt (20:1), cc | 10 | 118 | $C_{25}H_{27}NO \cdot 1.75H_2O$: C: 77.28%; H: 7.63%; N: 3.49%. |
| **17** |  | *n*-hexane:AcOEt (3:1), cc | 32 | 236 | $C_{22}H_{25}N_3O \cdot 0.25\ (CH_3)_2NCHO$: C: 74.82%; H: 7.27%; N: 12.22%. |
| **18** |  | *n*-Hexane:AcOEt (3:1), cc | 48 | 144 | $C_{18}H_{22}N_2OS \cdot 0.1C_6H_{14} \cdot 0.1H_2O$: C: 68.56%; H: 7.43%; N: 8.45%; S: 9.65%. |
| **19** |  | *n*-Hexane:AcOEt (2:1), cc | 25 | 187 | $C_{19}H_{23}N_3O \cdot 0.25H_2O$: C: 72.82%; 7.32%; N: 13.47%. |
| **20** |  | *n*-hexane:AcOEt (3:1), cc | 21 | 320 | $C_{19}H_{23}N_3O_2 \cdot 0.25(CH_3)_2NCHO$: C: 69.19%; H: 6.95%; N: 12.96%. |
| **21** |  | *n*-hexane:AcOEt (3:1), cc | 75 | 143–144 | $C_{22}H_{27}NO$: C: 82.24%; H: 8.58%; N: 4.45%. |
| **22** |  | *n*-Hexane:AcOEt (3:1), cc | 70 | 139 | $C_{22}H_{25}F_2NO$: C: 73.83%; H: 7.17%; N: 4.06%. |
| **23** |  | *n*-hexane:AcOEt (3:1), cc | 38 | 160 | $C_{22}H_{25}Cl_2NO$: C: 67.40%; H: 6.27%; N: 3.69%. |

(*continued on next page*)

**Table 1** (*continued*)

| No | −R′ (−Ar′) −R (−Ar) | Isolation | Yield (%) | M.p. (°C) | Elemental analyses (exp.) |
|---|---|---|---|---|---|
| **24** |  | *n*-Hexane:AcOEt (3:1), cc | 54 | 165–166 | $C_{27}H_{36}N_2O \cdot 0.2H_2O$: C: 79.45%; H: 8.85%; N: 7.19%. |
| **25** |  | *n*-Hexane:AcOEt (3:1), (1:1), cc | 80 | 69–70 bubl. | $C_{23}H_{34}N_2O_3 \cdot 0.2H_2O$: C: 70.84%; H: 8.77%; N: 6.99%. |
| **26** |  | *n*-Hexane:AcOEt (20:1), cc | 46 | 213 | $C_{24}H_{38}N_2O \cdot 0.1H_2O$: C: 77.32%; H: 9.96%; N: 7.53%. |
| **27** |  | *n*-Hexane:AcOEt (20:1), cc | 29 | 210 | $C_{24}H_{37}N_2O_2\cdot$: C: 74.33%; H: 9.57%; N: 7.16%. |
| **28** |  | *n*-Hexane:AcOEt (3:1), cc | 32 | 199 | $C_{20}H_{30}N_2O \cdot 0.5H_2O$: C: 73.86%; H: 9.32%; N: 8.59%. |
| **29** |  | *n*-Hexane:AcOEt (3:1), cc | 27 | 202–203 | $C_{21}H_{32}N_2O$: C: 76.74%; H: 10.01%; N: 8.35%. |
| **30** |  | *n*-Hexane, recrystallization | 32 | 242–244 | $C_{19}H_{28}N_2O_2 \cdot 0.1H_2O$: C: 71.73%; H: 9.10%; N: 8.89%. |
| **31** |  | *n*-Hexane, recrystallization | 67 | 142–144 | $C_{21}H_{32}N_2O_2 \cdot 0.25H_2O$: C: 72.27%; H: 9.39%; N: 8.03%. |
| **32** |  | *n*-Hexane:AcOEt (3:1), cc | 92 | 157–158 | $C_{18}H_{25}NO \cdot 0.1H_2O$: C: 78.91%; H: 8.88%; N: 5.11%. |

given parameters $a_{ref}$, $a_{ij}$, and $r_{ref}$ are applied in Eq. (1), proving that the ethylene protons of the compounds **9**, **10**, **11**, and **12** are *trans* configured.

Determination of the feature of compound **27** using $^1$H NMR was difficult because of the paramagnetic nature of the nitroxide radical. It is reported that the reduction of similarly behaved nitroxide radicals by certain agents that transform the paramagnetic behavior to a diamagnetic feature could allow the more specifically determined features obtained from $^1$H NMR spectra [29–31]. Therefore, the conversion of paramagnetic nitroxide to diamagnetic hydroxylamine was achieved by the reduction of the compound with sodium dithionide in DMSO-$d_6$, which then yielded 5,5,8,8-tetramethyl-*N*-(1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide. A small amount of sodium dithionite in $D_2O$ was added to a solution of compound **27** in DMSO-$d_6$ in an NMR tube at 20 °C. The progress of the reduction was monitored visually by the disappearance of the light pink color of compound **27**, indicating the formation of *N*-hydroxylamine.

Stable free radical compound **27** was also proven by Electron Paramagnetic Resonance (EPR) spectra. EPR is a spectroscopic technique that directly detects chemical species with unpaired electron(s). Using persistent nitroxide spin probes, it has been widely utilized to investigate the organizational and dynamic properties of biomolecules for several decades [32–34]. Direct evaluations and single domain simulations were performed for the new TEMPO-labeled retinoid derivative **27** and tempamine (Fig. 1). The rotational correlation times ($\tau$), which is related to the mobility of the probe, and nitrogen hyperfine splitting constants ($a_N$), which is related to the polarity of the environment, of these spin probes were calculated by EPRSIM program [35] shown in Table 2. As

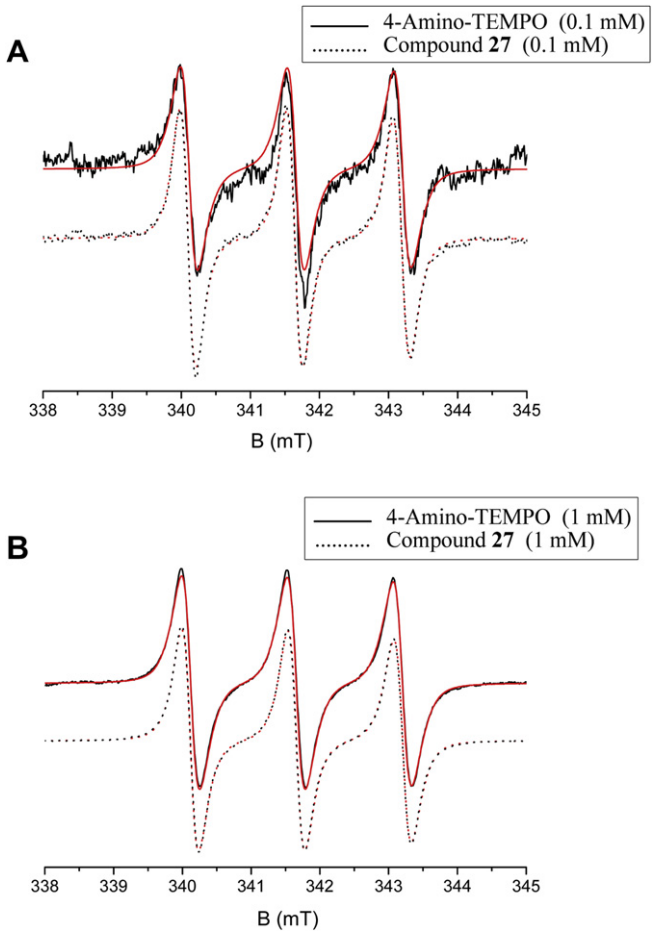



**Fig. 1.** Experimental (black) and simulated (red) EPR spectra of compound **27** and 4-amino-TEMPO (tempamine) in THF. A) 0.1 mM, B) 1 mM (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

a result of these calculations it was shown that there is no significant change on nitrogen hyperfine splitting constants of two spin probes. This shows that these two spin probes give information approximately from the same environment. The rotational correlation time of the new TEMPO-labeled retinoid derivative **27** is higher than the rotational correlation time of tempamine, indicating more restricted motion for this new probe.

As mentioned above, the ATRA was used as a reference for those synthesized compounds to make comparison for better understanding of the activities of the compounds. According to our findings, the nitrite inhibition pattern of ATRA is consistent with the similar results reported from various studies [36,37]. Although the activity of ATRA against NO• production was shown in different type of macrophage cells (J774A.1) with a dose-dependent manner [37], in this study, the RAW 264.7 macrophage system was utilized to achieve the inhibition of NO• production. The synthesized retinoid compounds were tested in vitro on LPS-activated RAW 264.7 macrophage cells for their nitric oxide radical inhibition activities. Moreover, the MTT test was used in order to investigate the effectiveness of the compounds on the viability of the cells. Consequently, our results showed that ATRA and the other standard compounds inhibit the nitrite production in LPS induced macrophages at concentrations of 5 μM and 50 μM (Table 3) without showing any effect on the cell viability. Biological activity findings of the targeted compounds **6**–**32** are summarized in Table 4.

Compounds **10**, **11**, **17**, **26**, **27**, **29**, and **31** have been found to provide 40% inhibition of nitrite dose-dependently, when compared with the antioxidant reference compounds ATRA, alpha-lipoic acid, ferulic acid, caffeic acid, trolox and tempamine (Table 4). However, three compounds (compounds **6**, **14** and **16**) in Table 4 show less inhibition at 50 μM than at 5 μM. Among these three compounds, compounds **14** and **16** very slightly affect NO• production. Since the activity was not potent we could not achieve a consistent inhibition of NO• production dose-dependently.

**Table 2**
Rotational correlation time and hyperfine splitting constant parameters of compound **27** and 4-amino-TEMPO in THF at 0.1 mM and 1 mM concentration calculated by EPRSIM program.

| Concentration | Sample | Rotational correlation time, $\tau_c$ (ns) | Hyperfine splitting constant, $a$ (mT) |
|---|---|---|---|
| 0.1 mM | 4-Amino-TEMPO | 0.016 | 1.543 |
| | **27** | 0.047 | 1.545 |
| 1 mM | 4-Amino-TEMPO | 0.025 | 1.540 |
| | **27** | 0.051 | 1.545 |

**Table 3**
The effects of the standards on the nitrite levels in RAW 264.7 macrophage cells.

| Compds | % Inhibition of nitrite levels | |
|---|---|---|
| | 5 μM | 50 μM |
| ATRA | 57.2 ± 0. 5 | 66.0 ± 1.1 |
| α-Lipoic acid | 25.2 ± 3.8 | 46.0 ± 1.2 |
| Caffeic acid | 23.2 ± 1.4 | 44.2 ± 0.2 |
| Ferulic acid | 24.8 ± 1.4 | 39.1 ± 8.9 |
| Trolox | 33.4 ± 1.9 | 39.3 ± 2.5 |
| Tempamine | 26.9 ± 5.1 | 45.6 ± 1.3 |

**Table 4**
The effects of the targeted compounds (**6**–**32**) on nitrite levels in RAW 264.7 macrophage cells (Nitrite levels in LPS treated group is represented as 100% and equal to 15.1 ± 0.1 μM where nitrite accumulation in untreated group is 2.9 ± 0.036 μM).

| Compds | % Inhibition of nitrite levels | |
|---|---|---|
| | 5 μM | 50 μM |
| **6** | 28.5 ± 0.6 | 22.8 ± 0.9 |
| **7** | NI[a] | NI |
| **8** | NI | NI |
| **9** | NI | NI |
| **10** | 23.3 ± 2.3 | 46.2 ± 1.4 |
| **11** | 17.8 ± 2.6 | 48.0 ± 2.6 |
| **12** | 24.1 ± 2.6 | 32.8 ± 1.9 |
| **13** | NI | 10.5 ± 1.5 |
| **14** | 15.8 ± 1.7 | 4.4 ± 0.8 |
| **15** | NI | NI |
| **16** | 10.5 ± 2.1 | 5.7 ± 0.5 |
| **17** | 15.6 ± 3.9 | 45.7 ± 0.7 |
| **18** | NI | 33.5 ± 1.9 |
| **19** | NI | 18.6 ± 2.6 |
| **20** | 11.5 ± 2.1 | 21.1 ± 1.7 |
| **21** | NI | 15.9 ± 4.9 |
| **22** | NI | 7.7 ± 1.3 |
| **23** | NI | 6.6 ± 1.5 |
| **24** | 21.0 ± 3.8 | 22.5 ± 3.4 |
| **25** | NI | 30.0 ± 1.1 |
| **26** | 19.1 ± 3.4 | 42.9 ± 8.7 |
| **27** | 18.2 ± 3.4 | 59.2 ± 0.3 |
| **28** | NI | 10.5 ± 1.0 |
| **29** | 25.7 ± 3.1 | 43.6 ± 1.6 |
| **30** | 5.8 ± 0.2 | 34.3 ± 2.4 |
| **31** | 14.3 ± 3.3 | 40.1 ± 3.7 |
| **32** | 20.5 ± 6.6 | 38.2 ± 0.7 |

[a] No inhibition.

Compound **6**, which bears a powerful antioxidant alpha-lipoic acid residue, showed decreased nitrite levels in the cells that were occurred in both concentrations (5 μM and 50 μM). The inhibitory effect of this compound was unexpectedly found to be lower at the 50 μM concentration than the one obtained from 5 μM (Table 4). Therefore, we could not achieve a dose-dependent response for this compound. At 5 μM concentration, a more potent effect was achieved, because of the probability that at higher concentrations, some antioxidants [38–40] may also act as pro-oxidants and this may lead to diminishment of their effects in the biological system.

Even though, the consequent toxic effects on the viability of the cells were elevated with increasing doses of the compounds **7**, **8**, and **9** (cinnamic acid derivative), observation on the nitrite inhibition was not obtained from these compounds. The latter has showed a completely different activity profile compared to those having the cinnamic acid residue (compounds **10**, **11**, and **12**) and also showed no inhibition in contrast to compounds **10**, **11**, and **12** which reduced the level of nitrite. The caffeic acid derivative (compound **10**) was found to possess more likely an identical inhibition pattern obtained from caffeic acid which is attributed as a potent antioxidant [41]. Approximately, a 3-fold increase in nitrite inhibition was observed with the increasing doses of compound **11**; the ferulic acid residue was utilized because of its potency to address antioxidant [42] and antiinflammatory [43] activities. The results obtained from compounds **10**, **11**, and **12** seemed to show no meaningful indications regarding the number of hydroxy groups within their molecular structures.

Nitrite levels in macrophage cells were reduced significantly by the implementation of compounds **10**, **11** and **27**, which consist of residues of known antioxidant structures, i.e. caffeic acid, ferulic acid, and tempamine. Compounds **6** and **14** are expected to show higher efficacy in decreasing the macrophage nitric oxide levels due to their constitution of antioxidant residues (alpha-lipoic acid and acetyltrolox). The results were surprisingly disappointing as they exerted slight decreases in the nitrite levels. Moreover, the trolox derivative (compound **13**) showed no inhibition at the 5 μM concentration whereas very little inhibition was observed at the 50 μM concentration. The finding that such antioxidant-residue-bearing compounds had no effect on the inhibition of nitrite formation in the macrophage cells is also surprising. Compound **15** (bis-retionid-bearing compound) did not exhibit inhibition; these results are similar to those obtained from compounds **7**, **8**, and **9**.

These results are thought to be associated with their lipophilic structures. Compound **15**, which contains two tetrahydronaphthalene rings, as explained above, showed no nitrite inhibition but some cell viability decreasing effect. The role of the tetrahydronaphthalene ring system most likely influenced the pattern of such biological activity, where in the retinoid moiety might be related to the features of nitrite inhibition and cell viability.

Cyclic stable nitroxide free radical 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) and its derivative tempamine possess anticancer and antioxidant properties due to its antitumor activities and protective effects against oxidative damage [21,44,45]. New TEMPO-labeled retinoid derivative **27** displayed 18.2% and 59.2% nitrite inhibition at 5 μM and 50 μM concentrations, respectively

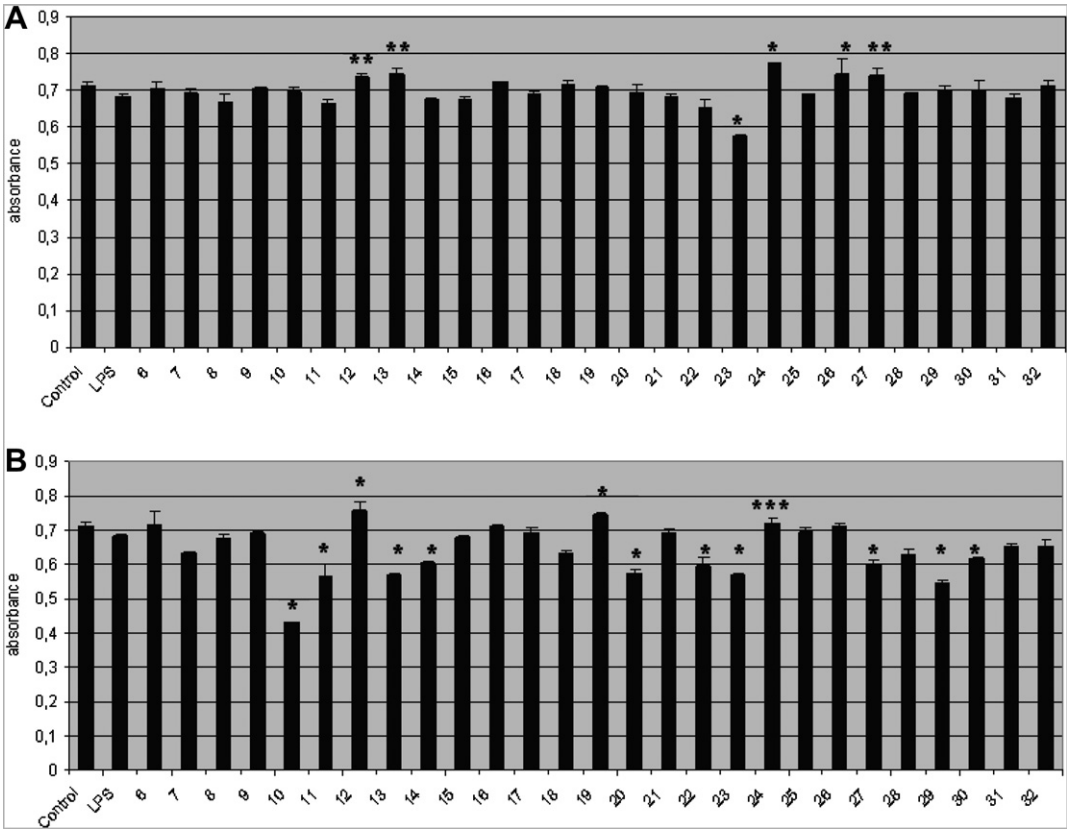



**Fig. 2.** (A) Effects of compounds **6**–**32** (5 μM) on cell viability. The bar chart represents MTT assay absorbance values of control, LPS and compounds **6**–**32** (5 μM) treated groups as mean ± S.D. Compounds **10**, **11**, **12**, **17**, **24**, **26**, **27**, **29** and **31** are not toxic to cells at 5 μM concentration which means that their inhibitory effect on nitrite production doesn't stem from cytotoxicity. (B) Effects of compounds **6**–**32** (50 μM) on cell viability. The bar chart represents absorbance values of control, LPS and compound **6**–**32** (50 μM) treated groups as mean ± S.D. Compounds **10**, **11**, **27** and **29** show cytotoxicity at 50 μM concentration which can lead to diminished nitrite values. (*): $p < 0.001$ vs. LPS treated. **$p < 0.01$ vs. LPS treated. ***$p < 0.05$ vs. LPS treated. (control: no stimulation. LPS: cells stimulated with LPS).

and therefore seemed to be the most active compound in this series with 59.2% nitrite inhibition. However it exhibited significant toxic effect at 50 μM which can be related with this high nitrite inhibition while tempamine did not show any cytotoxicity to RAW 264.7 macrophages at the concentrations tested.

The amount of nitrite might be related to decreased cell viability or the strength of compounds on the nitrite levels in the macrophage cells. The cinnamic acid derivative, compound **12**, and the benzimidazole containing compound, **17**, were found to decrease nitrite levels significantly in LPS-activated cells (Table 4) and were effective without showing cytotoxicity to the cells at tested concentrations (Fig. 2(A) and (B)). Of all of the novel synthesized compounds, compound **12** showed the greatest increase in cell viability at a dose of 50 μM. Compounds **24** and **26** were also found to decrease nitrite levels in a dose-dependent manner in LPS-activated cells and found to be effective without showing toxic effects on the viability of the cells. In this system, however, decreased cell viability was observed with increasing concentrations of compounds **10**, **11**, **13**, **18**, **20**, **22**, **27**, **28**, **29**, **30**, **31** and **32**. Therefore, the decline in nitrite levels in the presence of high concentrations of these retinoid compounds may be associated with the reduction in cell viability and due to cytotoxicity. Especially compound **10**, which has inhibitory effects on nitrite levels at both concentrations, exerted cytotoxicity at the 50 μM concentration (Fig. 2(A) and (B)).

## 4. Conclusion

Our results have shown that some of these novel retinoid analogues, especially the most active compound **27**, and non-toxic compounds **12**, **17**, **24** and **26**, have inhibitory effects on iNOS-related $NO^{\bullet}$ production and can therefore be used as promising treatment candidate compounds for diseases related with excess $NO^{\bullet}$ production. Indeed, there are still some undetermined mechanisms that exist. It is not clearly known if this inhibitory effect is achieved by direct scavenging of nitric oxide or inhibition of iNOS enzyme. Therefore we also tested direct in vitro $NO^{\bullet}$ scavenging activity of these novel compounds which may help us to explain the mechanisms of their effects on $NO^{\bullet}$ production in biological system [46]. Our results showed that majority of the compounds display poor $NO^{\bullet}$ scavenging activity. However compounds **10** and **11** have $NO^{\bullet}$ scavenging activity at both 5 μM and 50 μM concentrations (data not shown) which means that only these two compounds decrease nitrite levels by scavenging $NO^{\bullet}$ and/or effecting biological pathways. These findings suggest that most of these retinoid analogues show their effects biologically such as inhibition of target protein (iNOS) rather than scavenging $NO^{\bullet}$ in the media.

Further investigation is needed to determine mechanisms underlying this effect and the most effective concentrations.

## 5. Experimental section

### 5.1. General synthetic

All starting materials and reagents were high-grade commercial products purchased from Aldrich, Merck or Fluka. The structures of all synthesized compounds were assigned on the basis of $^1$H NMR, Mass, and IR spectral analyses. Analytical thin-layer chromatographies (TLC) were run on silica gel 60 $F_{254}$ plates (Merck, Germany). Column chromatographies were accomplished on silica gel 60 (40–63 μm particle size) (Merck, Germany). Melting points were determined with an electrothermal 9100 melting point apparatus and are uncorrected. $^1$H NMR (400 MHz) spectra were recorded with a Varian Mercury-400 spectrometer (Varian Inc., Palo Alto, CA, USA), in $CDCl_3$, DMSO-$d_6$ or acetone-$d_6$, δ scale (ppm) from internal standard TMS. Mass spectra were recorded on a Waters ZQ micromass LC-MS spectrometer (Waters Corporation, Milford, MA, USA) by the method of ESI and IR spectra were recorded on a Jasco FTIR-420 spectrophotometer (JASCO Research Ltd., Victoria, British Columbia, Canada). Elemental analyses were performed on LECO CHNS-932 instrument and were within ±0.4% of the theoretical values. $^1$H NMR, Mass, FTIR and elemental analyses were performed at The Central Instrumentation Laboratory of the Pharmacy Faculty of Ankara University, Ankara, Turkey. The EPR spectra of compound **27** and tempamine were performed on Bruker EMX-131 X-Band EPR spectrometer, at Nuclear Magnetic Resonance Laboratory of Physics Engineering Department, Faculty of Engineering, Hacettepe University, Beytepe, Ankara, Turkey.

#### 5.1.1. General methods for the preparation of the N-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalene-2-yl)-carboxamide derivatives (**6**–**15**), and 5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide derivatives (**16**–**32**)

*5.1.1.1. Method I.* *N*,*N*′-Carbonyldiimidazole (*N*,*N*′-CDI) was used as an amidification agent. *N*,*N*′-CDI (1.3 mmol) was added to a solution containing compound **3** (or the appropriate acid) (1.2 mmol) in DMF (or THF) (10 ml) at 0 °C. The mixture was stirred at room temperature for 3.5 h. An appropriate amine (or the compound **5**) (1.2 mmol) was added to the reaction mixture in an ice-cooled bath, and then stirred overnight at room temperature. The reaction mixture was evaporated, extracted with ethyl acetate, and washed with water. The organic layer was dried over $Na_2SO_4$, and the solvent was evaporated in vacuo. The residue was purified by silica gel column chromatography using the solvents given in Table 1 [47,48]. Compounds **6**, **7**, **8**, **13**, **14**, **17**, **18**, **20**, **21**, **22**, **23**, **24**, **25**, **26**, **27**, **28**, **29**, **30**, **31** and **32** were prepared according to the method I.

*5.1.1.2. Method II.* *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide HCl (EDCI) and 1-hydroxybenzotriazole hydrate (HOBT) were used as amidification agents. EDCI (1.095 mmol) and HOBT (1.095 mmol) were added to a solution of caffeic acid (or ferulic acid) (0.91 mmol) in dry $CH_2Cl_2$ (15 ml) at 0 °C and the mixture was stirred at room temperature for 15 min under $N_2$. After the addition of compound **5** (1.095 mmol), the mixture was stirred overnight at the same temperature. Cold 1% HCl aq. was added to the reaction mixture and the whole was extracted with $CHCl_3$. The organic layer was washed with saturated $NaHCO_3$ aq. and then brine, dried over $Na_2SO_4$ and evaporated [49,50]. The residue was purified by silica gel column chromatography using *n*-hexane:AcOEt (3:1 and 1:1) as eluent to afford amides. Compounds **10** and **11** were prepared according to the method II.

*5.1.1.3. Method III.* A mixture of compound **3** (or the appropriate acid) (2 mmol), $SOCl_2$ (2 ml), and benzene (10 ml) was heated at reflux for 4 h. The resulting solution was rotary evaporated to yield acid chloride. An appropriate amine (1 mmol) was suspended in pyridine (2 ml) and triethylamine (0.5 ml). After stirring for 20 min at room temperature, the resulting mixture was filtered. The filtrate was cooled to −5 °C, and at this temperature $CH_2Cl_2$ (5 ml) with acyl chloride (1.2 mmol) was added. After being stirred and refluxed for 4 h, the reaction mixture was filtered to remove white precipitation, and the filtrate was extracted with ethyl acetate, and washed with water. The organic extracts were combined, dried over $Na_2SO_4$, and the solvent was evaporated. The residue was purified by silica gel column chromatography [51,52]. Compounds **9**, **12**, and **16** were prepared according to the method III. Cinnamoyl chloride was commercially available for the synthesis of compound **9**.

*5.1.1.4. Method IV.* The procedure used by Ayhan-Kılcıgil and Altanlar [53] was modified. Compound **3** (0.32 mmol) was refluxed in benzene (5 ml) with $SOCl_2$ (5 ml) for 4 h at 80 °C. Then, solvent and excess of $SOCl_2$ were evaporated completely and the residue was dissolved in ether (10 ml). This mixture was added to an ice-cold mixture of **5** (0.32 mmol), $NaHCO_3$ (0.64 mmol), ether (5 ml) and water (5 ml) over a 1 h period. The mixture was stirred overnight at room temperature. Water and organic layers were separated. The organic layer was washed with 1 N HCl and then water, and the solvent was evaporated. The residue was purified by column chromatography using chloroform:*n*-hexane (1:1) as eluent. Compound **15** was prepared by using method IV.

*5.1.1.5. Method V.* A solution of **3** (0.45 mmol) and oxalyl chloride (0.25 ml) in $CH_2Cl_2$ (2 ml) was stirred for 1 h. After removal of the solvent, a solution of the appropriate amine derivative (0.74 mmol) and 4-dimethylaminopyridine (DMAP) (10 mg) in pyridine (2 ml) was added to the residue, and the mixture was stirred overnight at the room temperature. The reaction mixture was poured into 2 N HCl (20 ml), and extracted with $CH_2Cl_2$. The organic layer was dried over $Na_2SO_4$, and concentrated [51]. The residue was purified by column chromatography using *n*-hexane:AcOEt (2:1) as eluent. Compound **19** was prepared by using method V.

Isolation methods and some physical data of the synthesized compounds **6**–**32** are summarized in Table 1.

*5.1.2. 5-(1,2-Dithiolan-3-yl)-N-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)pentanamide (**6**)*

$ESI^+$-MS (*m*/*z*, %): 406 (M + H, 100). IR (KBr, $cm^{-1}$): 3279 (NH), 1652 (CO). $^1H$ NMR δ ppm ($CDCl_3$): 7.70 (s, 1H), 7.09 (s, 1H), 6.88 (br s, 1H, −NH), 3.59 (m, 1H), 3.15 (m, 2H), 2.47 (m, 1H), 2.39 (t, 2H), 2.20 (s, 4H), 1.92 (m, 1H), 1.75 (m, 4H), 1.66 (s, 3H), 1.54 (m, 2H), 1.25 (d, 12H).

*5.1.3. N-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-4-phenylbutanamide (**7**)*

$ESI^+$-MS (*m*/*z*, %): 364 (M + H, 100). IR (KBr, $cm^{-1}$): 3215 (NH), 1658 (CO). $^1H$ NMR δ ppm ($CDCl_3$): 7.69 (s, 1H), 7.32–7.20 (m, 5H), 7.09 (s, 1H), 6.81 (br s, 1H, −NH), 2.73 (t, 2H), 2.37 (t, 2H), 2.19 (s, 3H), 2.09 (m, 2H), 1.66 (s, 4H), 1.26 (d, 12H).

*5.1.4. 4-(3,4-Dimethoxyphenyl)-N-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)butanamide (**8**)*

$ESI^+$-MS (*m*/*z*, %): 446 (M + Na, 100), 424 (M + H, 83). IR (KBr, $cm^{-1}$): 3384 (NH), 1692 (CO). $^1H$ NMR δ ppm ($CDCl_3$): 7.71 (s, 1H), 7.09 (s, 1H), 6.83 (br s, 1H, −NH), 6.80 (d, 1H), 6.75 (d, 1H), 6.74 (s, 1H), 3.87 (d, 6H), 2.68 (t, 2H), 2.38 (t, 2H), 2.19 (s, 3H), 2.07 (m, 2H), 1.66 (s, 4H), 1.26 (d, 12H).

*5.1.5. E-N-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cinnamamide (**9**)*

$ESI^+$-MS (*m*/*z*, %): 411 (M + Na + $CH_3CN$, 100), 370 (M + Na, 91), 348 (M + H, 27). IR (KBr, $cm^{-1}$): 3244 (NH), 1658 (CO). $^1H$ NMR δ ppm ($CDCl_3$): 7.93 (br s, 1H, −NH), 7.77 (d, 1H, $J$ = 15.2 Hz), 7.55 (m, 2H), 7.38 (m, 3H), 7.13 (s, 1H), 7.08 (s, 1H), 6.59 (d, 1H, $J$ = 15.2 Hz), 2.27 (s, 3H), 1.68 (s, 4H), 1.28 (d, 12H).

*5.1.6. E-3-(3,4-Dihydroxyphenyl)-N-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)acrylamide (**10**)*

$ESI^+$-MS (*m*/*z*, %): 402 (M + Na, 100), 380 (M + H, 35.5). IR (KBr, $cm^{-1}$): 3522 (NH), 3235 (OH), 1652 (CO). $^1H$ NMR δ ppm (acetone-$d_6$): 8.36 (br s, 1H, −NH), 8.02 (s, $(CH_3)_2NCHO$), 7.84 (d, 1H), 7.52 (d, 1H, $J$ = 15.2 Hz), 7.16 (s, 1H), 7.11 (s, 1H), 6.99 (d, 1H, $J_o$ = 8.4 Hz), 6.85 (d, 1H, $J_o$ = 7.6 Hz), 6.75 (d, 1H, $J$ = 15.2 Hz), 2.78, 2.94 (s, s, $(CH_3)_2NCHO$), 2.89 (br s, 2H, Ar–3′-OH, Ar–4′-OH), 2.25 (s, 3H), 1.68 (s, 4H), 1.26 (d, 12H).

*5.1.7. E-3-(4-Hydroxy-3-methoxyphenyl)-N-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)acrylamide (**11**)*

$ESI^+$-MS (*m*/*z*, %): 416 (M + Na, 100), 394 (M + H, 15). IR (KBr, $cm^{-1}$): 3306 (NH), 3050 (OH), 1659 (CO). $^1H$ NMR δ ppm (acetone-$d_6$): 8.47 (br s, 1H, −NH), 7.84 (d, 1H), 7.58 (d, 1H, $J$ = 15.6 Hz), 7.22 (s, 1H), 7.16 (s, 1H), 7.11 (d, 1H, $J_o$ = 8.4 Hz), 6.86 (d, 1H, $J_o$ = 8 Hz), 6.82 (d, 1H, $J$ = 15.6 Hz), 3.89 (s, 3H), 2.25 (s, 3H), 1.68 (s, 4H), 1.26 (s, 12H).

*5.1.8. E-3-(3,4-Dimethoxyphenyl)-N-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)acrylamide (**12**)*

$ESI^+$-MS (*m*/*z*, %): 408 (M + H, 100). IR (KBr, $cm^{-1}$): 3356 (NH), 1661 (CO). $^1H$ NMR δ ppm ($CDCl_3$): 7.93 (br s, 1H, −NH), 7.72 (d, 1H, $J$ = 15.2 Hz), 7.13–7.01 (4H), 6.88 (d, 1H, $J_o$ = 8.4 Hz), 6.46 (d, 1H, $J$ = 15.6 Hz), 3.93 (d, 6H), 2.27 (s, 3H), 1.68 (s, 4H), 1.28 (d, 12H).

*5.1.9. 6-Hydroxy-2,5,7,8-tetramethyl-N-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)chroman-2-carboxamide (**13**)*

$ESI^+$-MS (*m*/*z*, %): 450 (M + H, 100). IR (KBr, $cm^{-1}$): 3480 (NH), 3414 (OH), 1669 (CO). $^1H$ NMR δ ppm ($CDCl_3$): 8.16 (br s, 1H, −NH), 8.04 (s, 1H), 7.02 (s, 1H), 4.30 (s, 1H, −OH), 2.65 (m, 2H), 2.50 (m, 1H), 2.24 (s, 3H), 2.18 (s, 3H), 2.08 (s, 3H), 1.97 (s, 3H), 1,92 (m, 1H), 1.64 (d, 4H), 1.57 (s, 3H), 1.28 (s, 6H), 1.22 (d, 6H).

*5.1.10. 2,5,7,8-Tetramethyl-2-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-ylcarbamoyl)chroman-6-yl acetate (**14**)*

$ESI^+$-MS (*m*/*z*, %): 514 (M + Na, 100), 492 (M + H, 22). IR (KBr, $cm^{-1}$): 3428 (NH), 1746 (CO), 1683 (CO). $^1H$ NMR δ ppm ($CDCl_3$): 8.15 (br s, 1H, −NH), 8.04 (s, 1H), 7.03 (s, 1H), 2.65 (br s, 2H), 2.50 (br s, 1H), 2.34 (s, 3H), 2.23 (s, 3H), 2.05 (s, 3H), 1.97 (s, 7H), 1.65 (s, 7H), 1.29 (s, 6H), 1.22 (d, 6H).

*5.1.11. 5,5,8,8-Tetramethyl-N-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**15**)*

$ESI^+$-MS (*m*/*z*, %): 432 (M + H, 100). IR (KBr, $cm^{-1}$): 3289 (NH), 1645 (CO). $^1H$ NMR δ ppm ($CDCl_3$): 7.97 (br s, 1H, −NH), 7.91 (s, 1H), 7.58 (s, 1H), 7.54 (dd, 1H, $J_o$ = 9.6 Hz), 7.40 (d, 1H, $J_o$ = 8 Hz), 7.13 (s, 1H), 2.28 (s, 3H), 1.72 (s, 4H), 1.68 (s, 4H), 1.32 (d, 12H), 1.26 (d, 12H).

*5.1.12. 5,5,8,8-Tetramethyl-N-(naphthalen-1-yl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**16**)*

$ESI^+$-MS (*m*/*z*, %): 358 (M + H, 100). IR (KBr, $cm^{-1}$): 3269 (NH), 1643 (CO). $^1H$ NMR δ ppm ($CDCl_3$): 8.25 (br s, 1H, −NH), 7.99 (d, 2H, $J_o$ = 10.8), 7.88 (dd, 2H, $J_o$ = 9.2 Hz, $J_m$ = 2.4 Hz), 7.72 (d, 1H, $J_o$ = 8 Hz), 7.68 (dd, 1H, $J_o$ = 9.6 Hz, $J_m$ = 1.4 Hz), 7.47–7.51 (m, 2H), 7.43 (d, 2H, $J_o$ = 8 Hz), 1.73 (s, 4H), 1.33 (d, 12H).

*5.1.13. N-(1H-Benzo[d]imidazol-2-yl)-5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**17**)*

$ESI^+$-MS (*m*/*z*, %): 348 (M + H, 100). IR (KBr, $cm^{-1}$): 3350 (NH), 1667 (CO). $^1H$ NMR δ ppm (acetone-$d_6$): 8.16 (d, 1H, $J_m$ = 2 Hz), 7.91 (dd, 1H, $J_o$ = 8.4 Hz, $J_m$ = 2 Hz), 7.50 (d, 1H, $J_o$ = 8 Hz), 7.23 (br s, 2H), 7.09–7.07 (m, 2H), 2.78, 2.94 (s, s, $(CH_3)_2NCHO$), 1.71 (m, 4H), 1.32 (s, 6H), 1.24 (s, 6H).

*5.1.14. 5,5,8,8-Tetramethyl-N-(thiazol-2-yl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**18**)*

$ESI^+$-MS (*m*/*z* %): 315 (M + H, 100). IR (KBr, $cm^{-1}$): 3147 (NH), 1667 (CO). $^1H$ NMR δ ppm ($CDCl_3$): 10.88 (br s, 1H, −NH), 7.95 (d, 1H, $J_m$ = 2 Hz), 7.69 (dd, 1H, $J_o$ = 8 Hz, $J_m$ = 2.4 Hz), 7.44 (d, 1H,

$J_o = 8$ Hz), 7.26 (d, 1H, $J_o = 3.6$ Hz), 6.98 (d, 1H, $J_o = 3.6$ Hz), 1.72 (s, 4H), 1.32 (d, 12H).

*5.1.15. 5,5,8,8-Tetramethyl-N-(pyrimidin-2-yl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**19**)*

$ESI^+$-MS (*m*/*z*, %): 310 (M + H, 100). IR (KBr, $cm^{-1}$): 3211 (NH), 1670 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 8.67 (d, 2H, $J_o = 4.8$ Hz), 8.62 (br s, 1H, −NH), 7.89 (s, 1H), 7.66 (d, 1H, $J_o = 8.4$ Hz), 7.42 (d, 1H, $J_o = 8.8$ Hz), 7.06 (t, 1H), 1.72 (s, 4H), 1.32 (d, 12H).

*5.1.16. 5,5,8,8-Tetramethyl-N-(2-oxo-1,2-dihydropyrimidin-4-yl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**20**)*

$ESI^+$-MS (*m*/*z*, %): 326 (M + H, 100). IR (KBr, $cm^{-1}$): 3216 (NH), 3145 (NH), 1710 (CO), 1698 (CO). $^1H$ NMR $\delta$ ppm (DMSO-$d_6$): 8.01 (d, 1H, $J_m = 1.2$ Hz), 7.93 (s, $(CH_3)_2NCHO$), 7.84 (d, 1H, $J_o = 6.8$ Hz), 7.72 (dd, 1H, $J_o = 8$ Hz, $J_m = 2$ Hz), 7.43 (d, 1H, $J_o = 8$ Hz), 7.22 (d, 1H, $J_o = 5.2$ Hz), 2.70, 2.88 (s, s, $(CH_3)_2NCHO$), 1.64 (s, 4H), 1.27 (s, 6H), 1.23 (s, 6H).

*5.1.17. N-Benzyl-5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**21**)*

$ESI^+$-MS (*m*/*z*, %): 322 (M + H, 100). IR (KBr, $cm^{-1}$): 3295 (NH), 1625 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.81 (d, 1H, $J_m = 1.6$ Hz), 7.46 (dd, 1H, $J_o = 8.4$ Hz, $J_m = 2$ Hz), 7.36–7.28 (5H), 7.34 (d, 1H, $J_o = 8.4$ Hz), 6.36 (br t, 1H, −NH), 4.65 (d, 2H), 1.69 (s, 4H), 1.31 (s, 6H), 1.28 (s, 6H).

*5.1.18. N-(2,4-Difluorobenzyl)-5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**22**)*

$ESI^+$-MS (*m*/*z*, %): 358 (M + H, 100). IR (KBr, $cm^{-1}$): 3294 (NH), 1626 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.79 (d, 1H, $J_m = 2$ Hz), 7.45 (dd, 1H, $J_o = 8$ Hz, $J_m = 2$ Hz), 7.73–7.38 (m, 1H), 7.34 (d, 1H, $J_o = 8.4$ Hz), 6.86–6.78 (m, 2H), 6.50 (br s, 1H, −NH), 4.63 (d, 2H), 1.69 (s, 4H), 1.28 (d, 12H).

*5.1.19. N-(2,4-Dichlorobenzyl)-5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**23**)*

$ESI^+$-MS (*m*/*z*, %): 394 (M + H + 4, 18), 392(M + H + 2, 57), 390 (M + H, 100). IR (KBr, $cm^{-1}$): 3286 (NH), 1629 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.80 (d, 1H, $J_m = 1.6$ Hz), 7.45 (dd, 1H, $J_o = 8$ Hz, $J_m = 2$ Hz), 7.41 (d, 1H, $J_o = 8.4$ Hz), 7.39 (d, 1H, $J_m = 2$ Hz), 7.35 (d, 1H, $J_o = 8.4$ Hz), 7.22 (dd, 1H, $J_o = 8.4$ Hz, $J_m = 2$ Hz), 6.55 (br t, 1H, −NH), 4.68 (d, 2H), 1.69 (s, 4H), 1.29 (d, 12H).

*5.1.20. N-(1-Benzylpiperidin-4-yl)-5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**24**)*

$ESI^+$-MS (*m*/*z*, %): 405 (M + H, 100). IR (KBr, $cm^{-1}$): 3284 (NH), 1626 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.75 (d, 1H, $J_m = 1.6$ Hz), 7.40 (dd, 1H, $J_o = 8$ Hz, $J_m = 2$ Hz), 7.34 (d, 1H, $J_o = 7.6$ Hz), 7.32–7.25 (5H), 5.91 (d, 1H, −NH), 4.01 (m, 1H), 3.51 (s, 2H), 2.85 (m, 2H), 2.17 (t, 2H), 2.01 (d, 2H), 1.69 (s, 4H), 1.57–1.53 (m, 2H), 1.29 (d, 12H).

*5.1.21. Ethyl 4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamido)piperidine-1-carboxylate (**25**)*

$ESI^+$-MS (*m*/*z*, %): 409 (M + Na, 100), 387 (M + H, 96). IR (KBr, $cm^{-1}$): 3305 (NH), 1699 (CO), 1634 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.75 (d, 1H, $J_m = 1.6$ Hz), 7.42 (dd, 1H, $J_o = 8.4$ Hz, $J_m = 2$ Hz), 7.34 (d, 1H, $J_o = 8.4$ Hz), 5.97 (d, 1H, −NH), 4.11–4.19 (m, 5H), 2.95 (t, 2H), 2.04 (d, 2H), 1.69 (s, 4H), 1.43 (m, 2H), 1.29 (d, 12H), 1.28 (t, 3H).

*5.1.22. 5,5,8,8-Tetramethyl-N-(2,2,6,6-tetramethylpiperidin-4-yl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**26**)*

$ESI^+$-MS (*m*/*z*, %): 371 (M + H, 100). IR (KBr, $cm^{-1}$): 3290 (NH), 1627 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.78 (s, 1H), 7.42 (d, 1H, $J_o = 8$ Hz), 7.34 (d, 1H, $J_o = 8.2$ Hz), 5.82 (d, 1H), 4.45 (m, 1H), 2.03 (d, 1H), 2.00 (d, 1H), 1.70 (s, 4H), 1.29 (d, 12H), 1.25 (d, 12H), 1.08 (t, 2H).

*5.1.23. 5,5,8,8-Tetramethyl-N-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-yl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**27**)*

$ESI^+$-MS (*m*/*z*, %): 386 (M + H, 100). IR (KBr, $cm^{-1}$): 3283 (NH), 1628 (CO). $^1H$ NMR $\delta$ ppm (DMSO-$d_6$ + $Na_2S_2O_4$ + $D_2O$): 8.44 (d, 1H, −NH), 7.78 (s, 1H), 7.57 (d, 1H, $J_o = 8$ Hz), 7.41 (d, 1H, $J_o = 8$ Hz), 4.36 (m, 1H), 1.96 (d, 2H), 1.66 (s, 4H), 1.60 (d, 2H), 1.43 (d, 12H), 1.25 (d, 12H).

*5.1.24. 5,5,8,8-Tetramethyl-N-(piperidin-1-yl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**28**)*

$ESI^+$-MS (*m*/*z*, %): 315 (M + H, 100). IR (KBr, $cm^{-1}$): 3191 (NH), 1641 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.70 (s, 1H), 7.41 (d, 1H, $J_o = 8.4$ Hz), 7.32 (d, 1H, $J_o = 8.4$ Hz), 6.73 (br s, 1H, −NH), 2.87 (t, 4H), 1.75 (m, 4H), 1.68 (s, 4H), 1.44 (m, 2H), 1.28 (d, 12H).

*5.1.25. N-(Azepan-1-yl)-5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**29**)*

$ESI^+$-MS (*m*/*z*, %): 329 (M + H, 100). IR (KBr, $cm^{-1}$): 3214 (NH), 1636 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.72 (d, 1H, $J_m = 2$ Hz), 7.40 (dd, 1H, $J_o = 8$ Hz, $J_m = 2$ Hz), 7.33 (d, 1H, $J_o = 8.4$ Hz), 3.19 (t, 4H), 1.74 (m, 4H), 1.68 (s, 4H), 1.65 (m, 4H), 1.29 (d, 12H).

*5.1.26. 5,5,8,8-Tetramethyl-N-morpholino-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**30**)*

$ESI^+$-MS (*m*/*z*, %): 339 (M + Na, 100), 317 (M + H, 71). IR (KBr, $cm^{-1}$): 3189 (NH), 1639 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.72 (s, 1H), 7.42 (d, 1H, $J_o = 8.4$ Hz), 7.35 (d, 1H, $J_o = 8.4$ Hz), 6.76 (br s, 1H, −NH), 3.87 (t, 4H), 2.97 (t, 4H), 1.70 (s, 4H), 1.29 (d, 12H).

*5.1.27. 5,5,8,8-Tetramethyl-N-(2-morpholinoethyl)-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**31**)*

$ESI^+$-MS (*m*/*z*, %): 345 (M + H, 100). IR (KBr, $cm^{-1}$): 3289 (NH), 1628 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.78 (d, 1H, $J_m = 2$ Hz), 7.45 (dd, 1H, $J_o = 8.4$ Hz, $J_m = 1.8$ Hz), 7.36 (d, 1H, $J_o = 8$ Hz), 6.73 (br t, 1H, −NH), 3.73 (t, 4H), 3.54 (q, 2H), 2.60 (t, 2H), 2.51 (t, 4H), 1.70 (s, 4H), 1.30 (d, 12H).

*5.1.28. N-Cyclopropyl-5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamide (**32**)*

$ESI^+$-MS (*m*/*z*, %): 272 (M + H, 100). IR (KBr, $cm^{-1}$): 3272 (NH), 1631 (CO). $^1H$ NMR $\delta$ ppm ($CDCl_3$): 7.73 (d, 1H, $J_m = 2$ Hz), 7.41 (dd, 1H, $J_o = 8.4$ Hz, $J_m = 2$ Hz), 7.31 (d, 1H, $J_o = 8$ Hz), 6.28 (br s, 1H, −NH), 2.87 (m, 1H), 1.68 (s, 4H), 1.28 (d, 12H), 0.84 (m, 2H), 0.61 (m, 2H).

### *5.2. General biological assays*

#### *5.2.1. Reagents for biological activity*

RAW 264.7 cell line was a gift from Dr. Georg Bauer. (Department of Virology, University of Freiburg, Freiburg, Germany). RPMI 1640 medium, L-glutamine, penicillin/streptomycin and fetal bovine serum were from PAA Laboratories; LPS, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and BSA (bovine serum albumin) were from Sigma; plastic flasks and 96-well plates were from Orange Sci.

Compounds **6**–**32** were dissolved in dimethyl sulfoxide to make stock solutions, respectively, and kept at −30 °C. The final concentration of the vehicle in the solution never exceeded 0.1% and had no effects on $NO^{\bullet}$ production and cell viability.

#### *5.2.2. Cell culture*

RAW 264.7 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine and

penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% $CO_2$ in air.

*5.2.3. Assay for nitrite quantitation*

Cells ($4 \times 10^4$) were seeded to 96-well plates and co-incubated with different concentrations of compounds (**6**–**32**) for 1 h. Following this co-incubation, cells were incubated in the absence or presence of LPS (1 μg/ml) for 20 h. Nitrite levels were determined spectrophotometrically with Griess assay. For this purpose, supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. Using $NaNO_2$ to generate a standard curve, nitrite production was measured by an absorbance reading at 550 nm [54].

Nitrite is the major NO• byproduct upon reaction with oxygen. Therefore, the easiest method for NO• measurement is the standard technique for measuring inorganic nitrite. This assay is based on the observation that the adduct of nitroxides and sulfanilic acid interacts with *N*-(1-naphthyl)ethylenediamine, generating a product that can be measured spectrophotometrically [54].

*5.2.4. Assay for cytotoxic activity*

Proliferation of cells was estimated by MTT test. Non-toxic concentrations of compounds were determined according to MTT test and concentrations of 5 μM and 50 μM were chosen to test the effects of different retinoid analogues on nitric oxide production. Cell respiration as an indicator of cell viability was determined on the basis of mitochondrial dependent reduction of MTT to formazan. After removal of the supernatants from the plate for nitrite determination, the cells were incubated at 37 °C with 0.5 mg/mL MTT for 1 h. The medium was aspirated, and the insoluble formazan product was dissolved in dimethyl sulfoxide for at least 2 h in the dark. The extent of MTT reduction was quantified by measuring the absorbance at 550 nm [55].

*5.3. Statistic analysis*

Each experiment was performed in triplicate. The results were expressed as means ± SD. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Student–Newman–Keuls multiple-comparison for differences between multiple groups. Differences were considered significant when the $p$ values were $<0.001$, $<0.01$ and $<0.05$.

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## Abbreviations

*μM:* micromolar
*mM:* milimolar
*cc:* column chromatography
*NI:* no inhibition
*SD:* standard deviation
$\tau_c$: rotational correlation time
*a:* hyperfine splitting constant
*EPR:* Electron Paramagnetic Resonance
*TEMPO:* 2,2,6,6-tetramethylpiperidine-*N*-oxyl
*ATRA:* all-*trans*-retinoic acid
*NO˙:* nitric oxide
*iNOS:* nitric oxide synthase
*LPS:* lipopolysaccharide
*MTT:* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
*BSA:* bovine serum albumin