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Nordihydroguaiaretic acid is a potent *in vitro* scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion and hypochlorous acid and prevents *in vivo* ozone-induced tyrosine nitration in lungs

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Abstract

The antioxidant nordihydroguaiaretic acid (NDGA) has recently become well known as a putative anticancer drug. In this paper, it was evaluated the *in vitro* peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$) and hypochlorous acid (HOCl) scavenging capacity of NDGA. It was found that NDGA scavenges: (a) $ONOO^-$ ($IC_{50} = 4 \pm 0.94\,\mu M$) as efficiently as uric acid; (b) $^1O_2$ ($IC_{50} = 151 \pm 20\,\mu M$) more efficiently than dimethyl thiourea, lipoic acid, *N*-acetyl-cysteine and glutathione; (c) $OH^{\cdot}$ ($IC_{50} = 0.15 \pm 0.02\,\mu M$) more efficiently than dimethyl thiourea, uric acid, trolox, dimethyl sulfoxide and mannitol, (d) $O_2^{\cdot-}$ ($IC_{50} = 15 \pm 1\,\mu M$) more efficiently than *N*-acetyl-cysteine, glutathione, tempol and deferoxamine and (e) HOCl ($IC_{50} = 622 \pm 42\,\mu M$) as efficiently as lipoic acid and *N*-acetyl-cysteine. NDGA was unable to scavenge H_2O_2. In an *in vivo* study in rats, NDGA was able to prevent ozone-induced tyrosine nitration in lungs. It is concluded that NDGA is a potent *in vitro* scavenger of $ONOO^-$, $^1O_2$, $OH^{\cdot}$, $O_2^{\cdot-}$ and HOCl and is able to prevent lung tyrosine nitration *in vivo*.

Keywords: *Nordihydroguaiaretic acid, scavenger, peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion*

Introduction

Larrea tridentata (Sessé & Mocino ex DC.) Coville, also known as Larrea, chaparral, creosote bush, greasewood, or *gobernadora* is a shrubby plant belonging to the family Zygophyllaceae, which dominates some areas of the desert southwest in the United States and Northern Mexico, as well as some areas of Argentina [1]. Chaparral tea has been used in the folk medicine for the treatment of more than 50 ailments including: infertility, tuberculosis, arthritis, diabetes, kidney and gallbladder stones, pain and inflammation, etc. (reviewed in [1]). Chaparral tea has

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been recognized as an antioxidant [1] and it has been shown that is able to scavenge superoxide anion $(O_2^{\cdot-})$ [2]. While consumption of low doses of *Larrea tridentata* appears to be nontoxic, high doses of this herbal have been associated with renal and hepatic diseases in humans [1]. The plant contains the inhibitor of lipoxygenase and antioxidant nordihydroguaiaretic acid (NDGA, Figure 1) at concentration between 5 and 10% by dry weight in the leaves. This compound has been used in commercially as a food additive to preserve fats and butter, although, it has been banned in some countries, including USA, since it was shown to induce cystic nephropathy in the rat [3]. In addition to be an antioxidant, it has been found that NDGA has health beneficial properties including: (a) Inhibition of the growth of a number of human cancer types both *in vitro* and *in vivo* [4–7], (b) cancer chemopreventive activity in models of carcinogenesis [8–11], (c) antiviral effects [12,13] and (d) degradation of pre-formed Alzheimer's β-amyloid fibrils *in vitro* [14] and protection of cultured rat hippocampal neurons against the toxicity of amyloid β-peptide [15], interrupting a neurodegenerative pathway relevant to the pathophysiology of Alzheimer's disease. In addition to NDGA, two new lignans isolated from the leaves of *Larrea tridentata*, (7S,8S,7′S,8′S)-3,3′,4′-trihydroxy-4-methoxy-7,7′-epoxylignan and meso-(rel 7S,8S,7′R,8′R)-3,4,3′,4′-tetrahydroxy-7,7′-epoxylignan) and another two epoylignans possessing a tetrahydrofuran moiety (4-*epi*-larreatricin and 3″-hydroxy-*epi*-larreatricin) showed strong antioxidant activity [16]. Although, NDGA is recognized as a potent antioxidant, to the best of our knowledge, it has not been studied the specific scavenging activity of NDGA. In the present paper, it was evaluated the *in vitro* peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), $O_2^{\cdot-}$ and hypochlorous acid (HOCl) scavenging capacity of NDGA by established spectrophotometric methods. In addition we evaluated if NDGA is able to prevent ozone-induced tyrosine nitration in lungs *in vivo*. Inhalation of toxic doses of ozone causes lung injury and inflammation in humans and experimental animals. It has been shown in a rodent model of ozone toxicity that macrophages recruited to the lung following exposure to this oxidant contribute to the pathogenesis of tissue injury [17–19]. It has been observed that ozone exposure induces overexpression of nitric oxide synthase II (NOS II) and enhanced 3-nitrotyrosine (3-NT) staining in lung macrophages [18]. Tyrosine nitration of proteins is regarded as an indication of the production of reactive nitrogen species *in vivo* and usually is measured by immunostaining of 3-NT. In addition, it has been shown that overexpression of superoxide dismutase or deficiency of NOS II prevents the ozone induced lung damage and 3-NT staining [17]. This strongly suggests that peroxynitrite ($ONOO^-$), is actively synthesized from $O_2^{\cdot-}$ and nitric oxide ($NO^{\cdot}$) and it is involved in tyrosine nitration in ozone exposed animals.

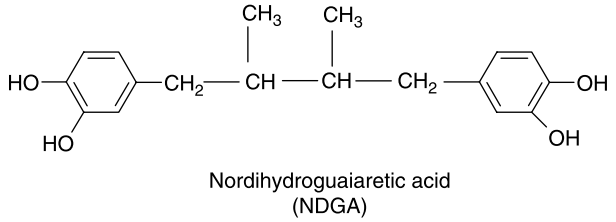

Figure 1. Structure of nordihidroguaiaretic acid.

Materials and methods

Reagents

N-Acetyl-cysteine (NAC), glutathione (GSH), tempol, deferoxamine mesylate (DFO), pyruvate, dimethyl thiourea (DMTU), uric acid, dimethyl sulfoxide (DMSO), mannitol, lipoic acid, NDGA, histidine, ascorbic acid, deoxy D-ribose, tetramethoxy-propane, thiobarbituric acid, xylenol orange, butylated hydroxytoluene, iron (III) chloride ($FeCl_3$), *N*,*N*-dimethyl-4-nitrosoaniline, xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), potassium nitrite (KNO_2), dioxide manganese (MnO_2), diethylene-triamine-pentaacetic acid (DTPA) were from Sigma-Aldrich (St Louis, MO). Trolox was from Calbiochem (La Jolla, CA). 2′,7′-dichlorodihydrofluorescein diacetate (DCF) and anti-3-NT antibodies (Cat. No. 189542) were from Cayman Chemical (Ann Arbor, MI). Ferrous ammonium sulfate, H_2O_2 and sodium hypochlorite were from JT Baker (Xalostoc, Edo. Mexico, Mexico). All other chemicals were reagent grade. NDGA was dissolved in DMSO/Water (1:1 v/v) for the $ONOO^-$, $^1O_2$, $OH^{\cdot}$, $O_2^{\cdot-}$ scavenging assays and in ethanol:water (1:1 v/v) for the HOCl assay. We demonstrated that these mixtures were unable to interfere in the respective scavenging systems used in this work (data not shown).

In vitro *experiments*

$ONOO^-$ scavenging assay. The $ONOO^-$ used in our assay was synthesized as previously described by Beckman et al. [20] mixing an acidic solution of H_2O_2 with KNO_2. Concentrations of $ONOO^-$ were determined before each experiment at 302 nm using a molar extinction coefficient of $1670\,M^{-1}\,cm^{-1}$. $ONOO^-$ scavenging assay was performed spectrophotometrically at 500 nm according to Saito et al. [21] measuring fluorescein formation from DCF. The ability of NDGA (up to 50 μM) to scavenge $ONOO^-$ was compared with that of uric acid (up to 50 μM) and penicillamine (up to 200 μM).

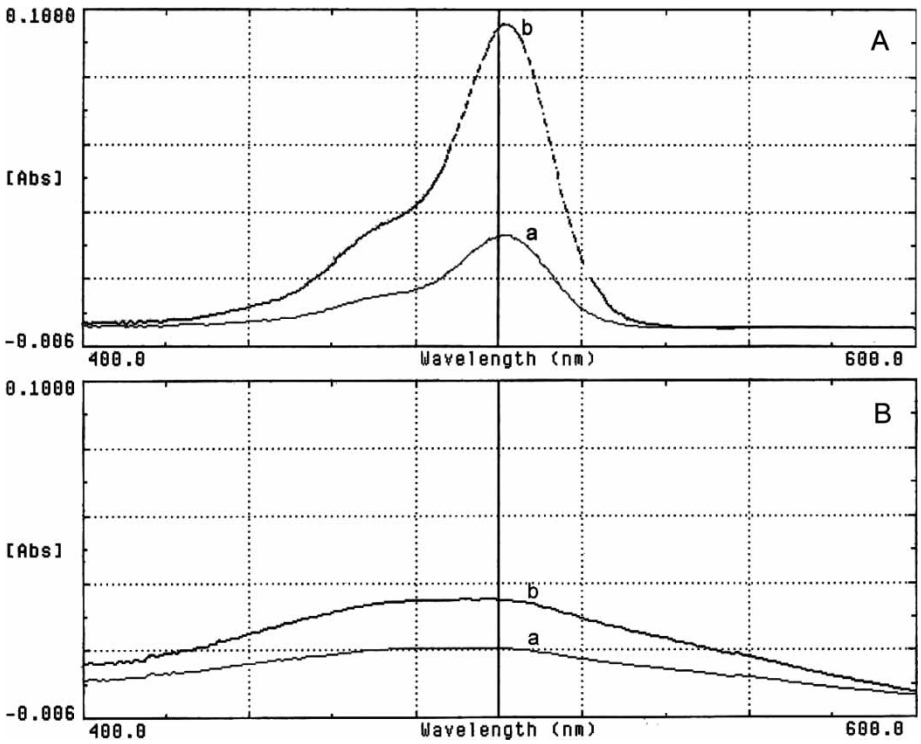

Figure 2. Spectrophotometric pattern of the assay system for $ONOO^-$. Line a was obtained at zero time and line b was obtained 1 min after starting the reaction in absence (Panel A) or presence (Panel B) of NDGA (50 μM, final concentration). In both panels, the continuous vertical line indicates 500 nm. It is clear that NDGA prevents the increase in optical density at 500 nm.

The spectrophotometric pattern of the assay system for $ONOO^-$ in absence or presence of NDGA is shown in Figure 2.

$^1O_2$ scavenging assay. The production of $^1O_2$ by sodium hypochlorite (NaOCl) and H_2O_2 was determined by using a spectrophotometric method [22] with minor modifications in which *N*,*N*-dimethyl-*p*-nitrosoaniline was bleached by $^1O_2$. The extent of $^1O_2$ production was determined by measuring the decrease in the absorbance of *N*,*N*-dimethyl-*p*-nitrosoaniline at 440 nm. The ability of NDGA to scavenge $^1O_2$ was compared with that of DMTU, lipoic acid, NAC and GSH. The spectrophotometric pattern of the assay system for $^1O_2$ in absence or presence of NDGA is shown in Figure 3.

$OH^\cdot$ scavenging assay. The ability of NDGA to scavenge $OH^\cdot$ was conducted in the Fe^{3+}–EDTA–H_2O_2–deoxyribose system [23]. The extent of deoxyribose degradation by the formed $OH^\cdot$ was measured directly in the aqueous phase by the thiobarbituric acid test at 532 nm [24]. The ability of NDGA to scavenge $OH^\cdot$ was compared with that of DMTU, uric acid, trolox, DMSO and mannitol. The spectrophotometric pattern of the assay system for $OH^\cdot$ in absence or presence of NDGA is shown in Figure 4.

$O_2^{\cdot-}$ scavenging assay. Xanthine–xanthine oxidase system was used to determine the $O_2^{\cdot-}$ scavenging activity of NDGA. $O_2^{\cdot-}$ production and xanthine oxidase activity were measured as NBT reduction (at 560 nm) and uric acid production (at 295 nm), respectively [25]. The ability of NDGA to scavenge $O_2^{\cdot-}$ was compared with that of GSH, NAC, tempol and DFO. The spectrophotometric pattern of the assay system for $O_2^{\cdot-}$ in absence or presence of NDGA is shown in Figure 5.

HOCl scavenging assay. The catalase assay involves a spectral analysis of the enzyme. A spectrum (200–500 nm) of catalase, catalase treated with HOCl and catalase containing varied mixtures of HOCl treated with increasing concentrations of NDGA or the reference compounds was obtained. The peak of catalase at 404 nm disappeared with HOCl. The HOCl scavenging capacity of NDGA or the reference compounds was evident by the inability of HOCl to eliminate/decrease the peak in a concentration-dependent way. Experiments were carried out essentially as described by Aruoma and

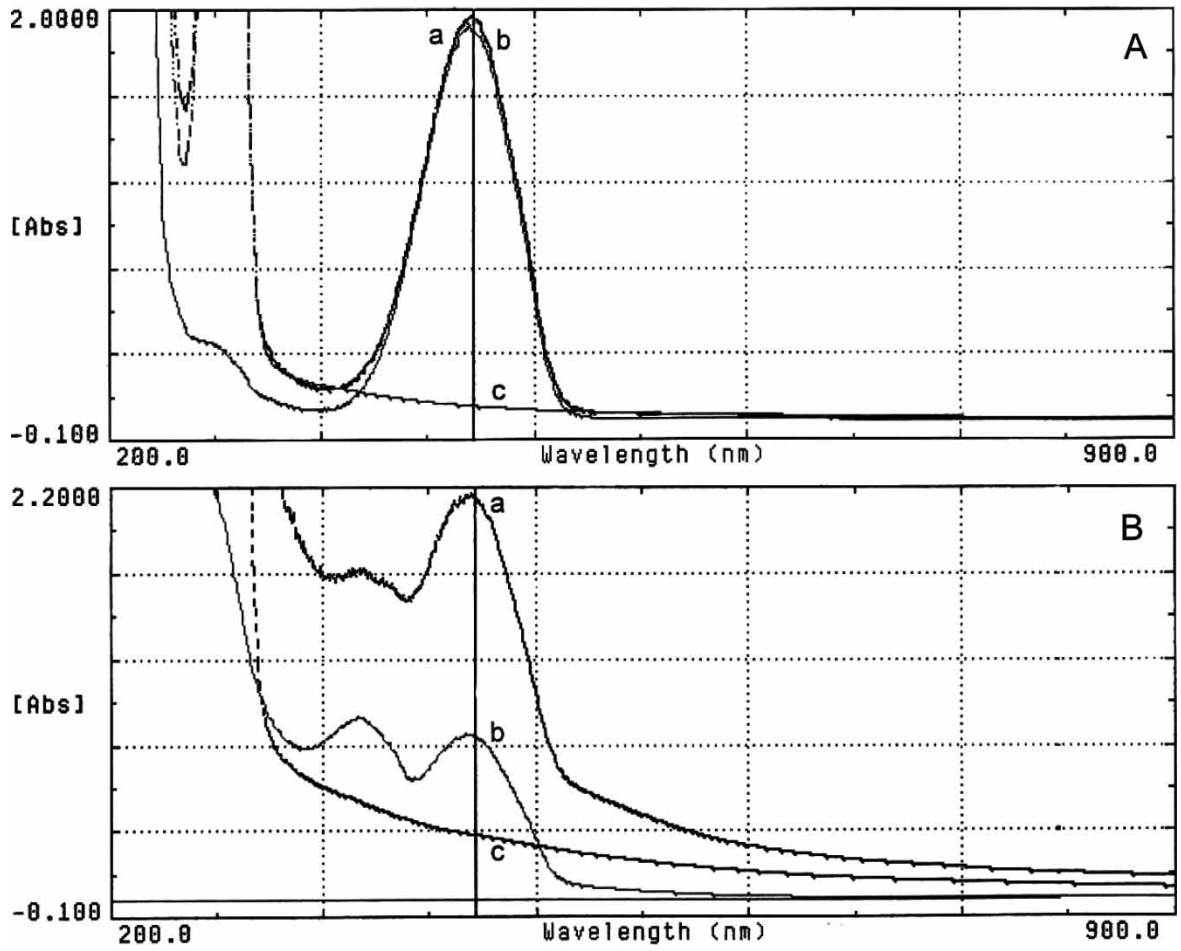

Figure 3. Spectrophotometric pattern of the assay system for $^1O_2$ at zero time (Panel A) and 40 min after the starting the reaction (Panel B). Line a was obtained in presence of NDGA (900 μM, final concentration). Line b was obtained in absence of NDGA. Line c was obtained with NDGA and without *N*,*N*-dimethyl-*p*-nitrosoaniline. In both panels, continuous vertical line indicates 440 nm. It is clear that NDGA decreases optical density at 440 nm.

Halliwell [26]. A solution of 49.8 μM bovine liver catalase (16.6 μM, final concentration) was mixed with 18 mM HOCl (6 mM, final concentration) with increasing concentrations of NDGA or the reference compounds. The spectrum was registered immediately. The spectrophotometric pattern of the assay system for HOCl in absence or presence of NDGA is shown in Figure 6. We were unable to measure the HOCl scavenging capacity of NDGA using the method of Ching et al. [27], based on the

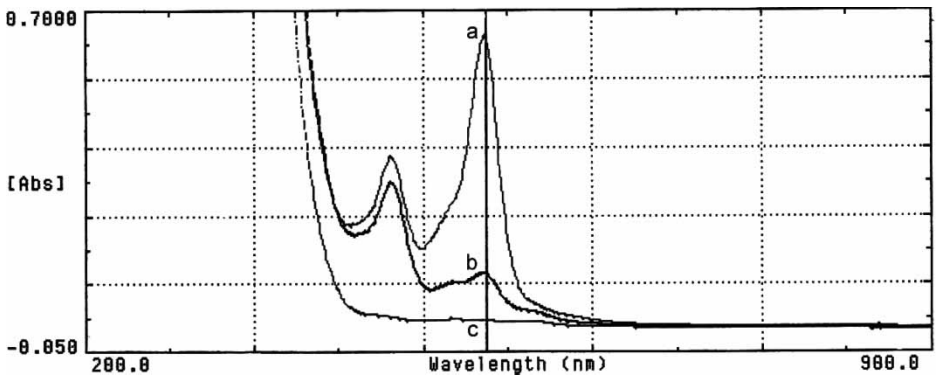

Figure 4. Spectrophotometric pattern of the assay system for $OH^{\cdot}$ measured as thiobarbituric acid reactive substances. Line a was obtained in absence of NDGA. Line b was obtained in presence of NDGA (10 μM, final concentration). Line c was obtained with NDGA and the assay system for $OH^{\cdot}$ generation but without thiobarbituric acid. The continuous vertical line indicates 532 nm. It is clear that NDGA decreases optical density at 532 nm.

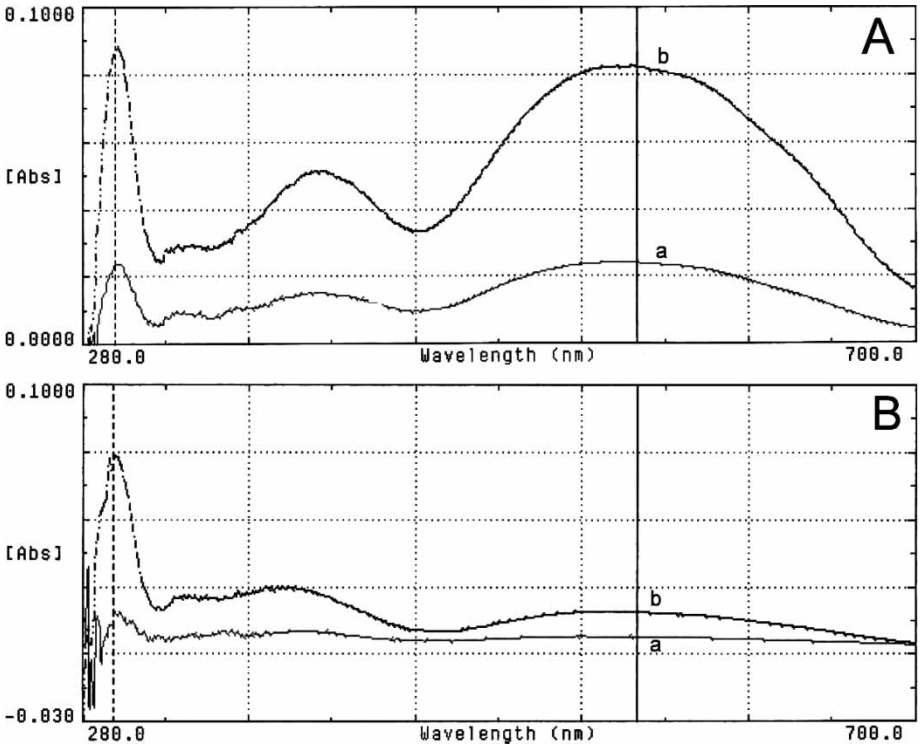

Figure 5. Spectrophotometric pattern of the assay system for $O_2^{\cdot-}$. Line a was obtained at zero time and line b was obtained 1 min after starting the reaction (see "Material and methods" section) in absence (Panel A) or presence (Panel B) of NDGA (100 μM, final concentration). In both panels, the continuous vertical line indicates 560 nm and the discontinuous vertical line indicates 295 nm. It is clear that NDGA prevents the increase in optical density at 560 nm (ΔOD/min = 0.058 in panel A and 0.0096 in panel B). In addition, it is clear that NDGA was without effect on the increase in optical density at 295 nm (ΔOD/min = 0.061 in panel A and 0.058 in panel B).

oxidation of thio-2-nitrobenzoic acid (TNB) or the elastase assay [28], because NDGA interfered with both assays.

H_2O_2 scavenging assay. A solution of 75 μM H_2O_2 was mixed with different concentrations of NDGA (1:1 v/v) and incubated for 4 h at room temperature. After this, H_2O_2 was measured by the method described by Long et al. [29] using the FOX reagent (xylenol orange, ammonium ferrous sulfate and H_2SO_4). The concentration of H_2O_2 was recorded at 560 nm. The ability of NDGA to scavenge H_2O_2 was compared with that of pyruvate and ascorbic acid.

In vivo *experiments*

Ozone model. Male Wistar rats weighing 200–230 g (10 weeks of age) were used. Rats were fed with Purina chow and water *ad libitum* and subjected to a 12:12

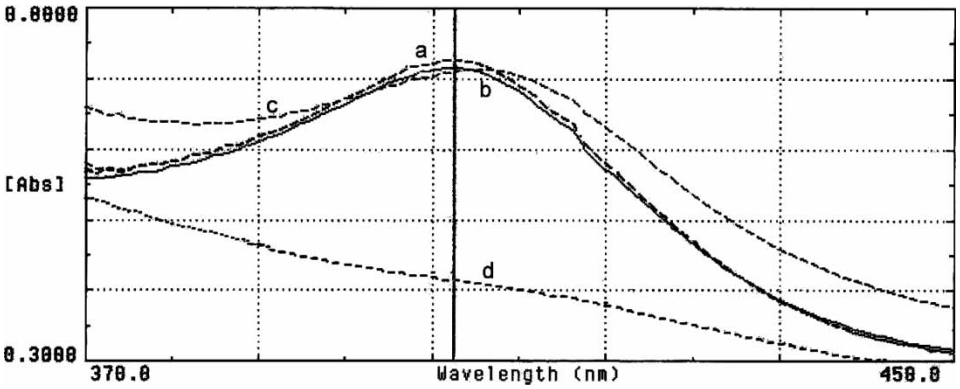

Figure 6. Spectrophotometric pattern of the assay system for HOCl. Line a was obtained in presence of catalase in phosphate buffer. Line b was obtained in presence of catalase and ethanol. Line c was obtained in presence of catalase, NDGA and HOCl. Line d was obtained in presence of catalase and HOCl. The continuous vertical line indicates 404 nm. It is clear that NDGA prevents the decrease in optical density at 404 nm. The concentrations in the assay were: [catalase] = 16.6 μM, [ethanol] = 72.3 mM, [HOCl] = 6 mM, [NDGA] = 1 mM.

light–dark cycle in a room equipped with filters. We studied four groups of rats: control ($n = 6$), NDGA ($n = 4$), ozone ($n = 6$) and ozone + NDGA ($n = 6$). The concentration of ozone was continuously registered with a monitor A21Z (IN USA, Inc., Needhman, MA) to guarantee that its concentration was less than 0.05 ppm in the control and NDGA groups. The rats from ozone and ozone + NDGA groups were exposed to ozone (0.25 ppm) 4 h/day by 7 days in an ozone test chamber Model OTC-1 (IN USA, Inc., Needhman, MA) [30]. Rats from NDGA and ozone + NDGA groups were placed Alzet osmotic pumps (Durect Corporation, Cupertino, CA, Model 2004) which released NDGA at a dose of 20 $mg^{-1}kg^{-1}day^{-1}$. The rats from the four groups were studied on day seven. The animals were anesthetized and perfused by intracardiac and intratracheal route with absolute ethanol and the lungs were obtained for the immunohistochemical analysis of 3-NT immunostaining as a marker of nitrosative stress.

Preparation of tissue samples for immunohistochemistry. Ethanol-perfused rat lungs were included in paraffin. Serial cuts of 3 μm of thickness were mounted on poli-L-lisina coated slides (Sigma, St Louis, MO). Sections were initially deparaffinized by washing in xylene and decreasing ethanol concentrations, then boiled in Declere (Cell Marque, Hot Springs, AR) to unmask antigen sites. Following cooling with distillated water, slides were washed in phosphate buffer saline (PBS). Endogenous peroxidase activity was blocked by exposing slides to 0.6% H_2O_2 in PBS for 30 min. After washing in PBS nonspecific binding was avoided by incubation with 5% blocking solution (5% normal goat serum in PBS) for 20 min. Sections were incubated overnight (16 h) with primary anti-3-NT antibody (1:100). Following removal of the antibodies and repetitive rinsing with PBS, slides were incubated with a biotinylated goat anti-IgG secondary antibodies (1:500, Jackson ImmunoReseach, West Grove, PA). Immunocytochemical identification of positive cells was performed by the use of an avidin-biotinylated peroxidase complex (ABC-kit Vectastain, Vector Laboratories, Burlingame, CA) and diaminobenzidine substrate (Vector Laboratories, Burlingame, CA). After intensive washing in PBS, slides were counterstained with hematoxylin. Sections were dehydrated in graded alcohols, treated with xylene and subsequently mounted. All specimens were examined by light microscopy (Axiovert 200 M, Carl Zeiss, Germany), photographs were taken with a digital camera (Axiocam HRC, Carl Zeiss, Germany). The number of positive cells was determined with a computerized image analyzer KS-300 3.0 (Carl Zeiss, Germany). For the negative control normal goat sera was used instead of the primary antibodies.

Statistical analysis

Data are expressed as mean ± SEM. The data were compared against the tube without NDGA or the reference compounds using one way analysis of variance (ANOVA) followed by Dunnett test. Scavenging capacity was expressed as 50% of inhibitory concentration (IC_{50}) value, which denotes the concentration of NDGA or the reference compounds (μM or mM) required to give a 50% reduction in scavenging capacity relative to the tube without NDGA or reference compound. The lower the IC_{50} value the higher is the scavenging capacity of the compound. The IC_{50} values were compared by ANOVA followed by Dunnett test or by Kruskall–Wallis analysis of variance followed by Dunn test, as appropriate. The immunohistochemical data (positive 3-NT macrophages) were analyzed by ANOVA followed by Tukey multigroup comparison test. $p < 0.05$ was considered significant.

Results

In vitro *studies*

NDGA scavenges $ONOO^-$ in a concentration-dependent way (Figure 7). The IC_{50} of NDGA for $ONOO^-$ was of 4.0 ± 0.94 μM (Table I). NDGA was as efficient as uric acid but more efficient than penicillamine to scavenge $ONOO^-$ (Table I). The efficiency to scavenge $ONOO^-$ was the following: NDGA = uric acid > penicillamine.

NDGA scavenges $^1O_2$ in a concentration-dependent way (Figure 8). The IC_{50} of NDGA for $^1O_2$ was of 151 ± 20 μM (Table I). NDGA was more efficient than DMTU, lipoic acid, NAC and GSH to scavenge $^1O_2$ (Table I). The efficiency to scavenge $^1O_2$ was the following: NDGA > DMTU > lipoic acid > NAC = GSH.

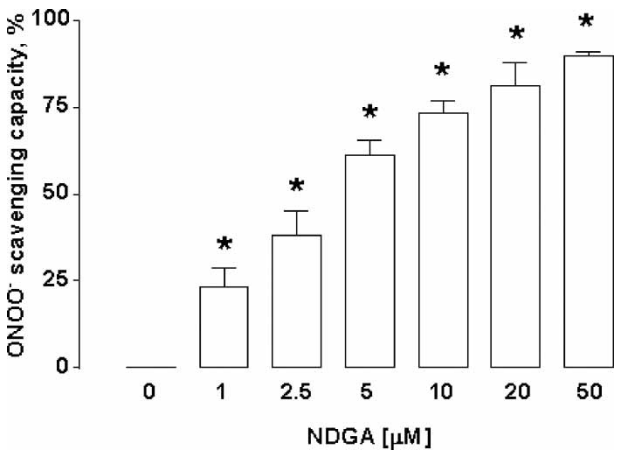

Figure 7. NDGA scavenges $ONOO^-$ in a concentration-dependent way. Data are mean ± SEM, $n = 6$ assays. $*p < 0.001$ vs 0 μM.

Table I. Scavanging ability of NDGA and different reference compounds. Date are expressed as IC_{50} (μM).

	$ONOO^-$	$^1O_2$	$OH^\cdot$	$O_2^{\cdot-}$	HOCl
NDGA	4.0 ± 0.9 (6)	151 ± 19 (6)	0.15 ± 0.02 (5)	15 ± 1 (7)	622 ± 42 (10)
Uric acid	13.1 ± 1.7 (6)		134 ± 12* (5)		
penicillamine	31.4 ± 3.2* (6)				
Lipoic acid		4,067 ± 174* (6)			858 ± 85 (5)
NAC		6,844 ± 585* (6)		3,600 ± 480* (5)	996 ± 76 (3)
GSH		7,023 ± 323* (6)		5,400 ± 970* (8)	
DMTU		1,689 ± 289* (5)	106 ± 10* (5)		
Trolox			174 ± 16* (6)		
DMSO			247 ± 14* (6)		5,103 ± 293* (8)
Mannitol			3,304 ± 197* (6)		
Tempol				7,100 ± 780* (5)	
DFO				8,200 ± 350* (11)	

Data are mean ± SEM. Number of data are in parenthesis. NDGA: nordihydroguaiaretic acid, NAC: *N*-acetyl–cysteine, GSH: glutathione, DFO: deferoxamine, DMTU: dimethyl thiourea, DMSO: dimethyl sulfoxide, $ONOO^-$: peroxynitrite anion, $^1O_2$: singlet oxygen, $OH^\cdot$: hydroxyl radicals, $O_2^{\cdot-}$: superoxide anion, HOCl: hypochlorous acid.
*$p < 0.001$ vs NDGA.

NDGA scavenges $OH^\cdot$ in a concentration-dependent way (Figure 9). The IC_{50} of NDGA for $OH^\cdot$ was of 0.15 ± 0.02 μM (Table I). NDGA was more efficient than DMTU, uric acid, trolox, DMSO and mannitol to scavenge $OH^\cdot$ (Table I). The efficiency to scavenge $OH^\cdot$ was the following NDGA > DMTU > uric acid > trolox > DMSO > mannitol.

NDGA scavenges $O_2^{\cdot-}$ in a concentration-dependent way (Figure 10, closed bars). The IC_{50} of NDGA for $O_2^{\cdot-}$ was of 15 ± 1 μM (Table I). NDGA was more efficient than NAC, GSH, tempol and DFO to scavenge $O_2^{\cdot-}$ (Table I). The efficiency to scavenge $O_2^{\cdot-}$ was the following: NDGA > > NAC > GSH > tempol = DFO. Uric acid production in the system was constant indicating that xanthine oxidase was unaffected by NDGA (Figures 5 and 10, open bars).

NDGA scavenges HOCl in a concentration-dependent way (Figure 11). The IC_{50} of NDGA for HOCl was of 622 ± 42 μM (Table I). NDGA was as efficient as lipoic acid and NAC but more efficient than DMSO to scavenge HOCl (Table I). The efficiency to scavenge HOCl was the following: NDGA = lipoic acid = NAC > DMSO.

NDGA was unable to scavenge H_2O_2 (data not shown) as compared to pyruvate (IC_{50} = 0.032 ± 0.001 mM) and ascorbic acid (IC_{50} = 115 ± 3.9 mM).

In vivo *studies*

Figure 12 shows the representative images of the immunohistochemical localization of 3-NT in the lung from the rats of the four groups studied and the Figure 13 shows the quantitative data of 3-NT immunoreactive alveolar macrophages. Since alveolar macrophages were readily identified in histologic sections (Figure 12), macrophage quantitation for 3-NT immunoreactivity was performed by counting positive alveolar macrophages in tissue sections. The 3-NT immunostained macrophages decreased in NDGA-treated rats and increased in ozone-exposed

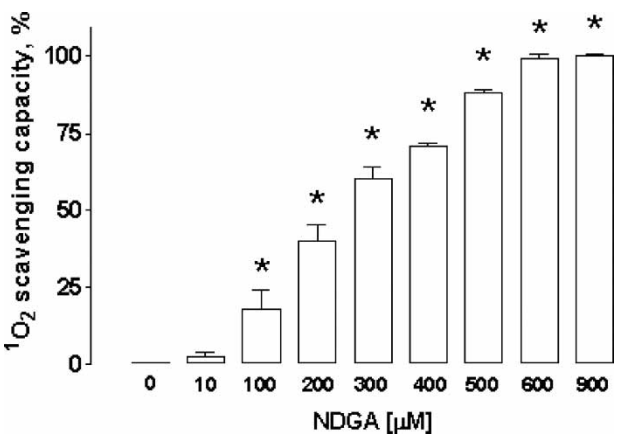

Figure 8. NDGA scavenges $^1O_2$ in a concentration-dependent way. Data are mean ± SEM, $n = 6$ assays. *$p < 0.001$ vs 0 μM.

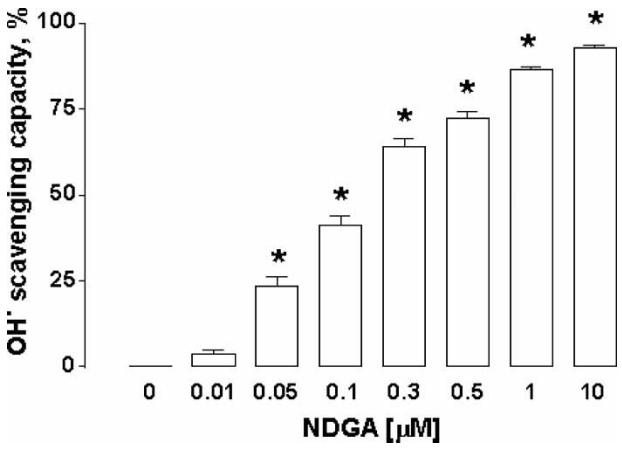

Figure 9. NDGA scavenges $OH^\cdot$ in a concentration-dependent way. Data are mean ± SEM, $n = 5$ assays. *$p < 0.001$ vs 0 μM.

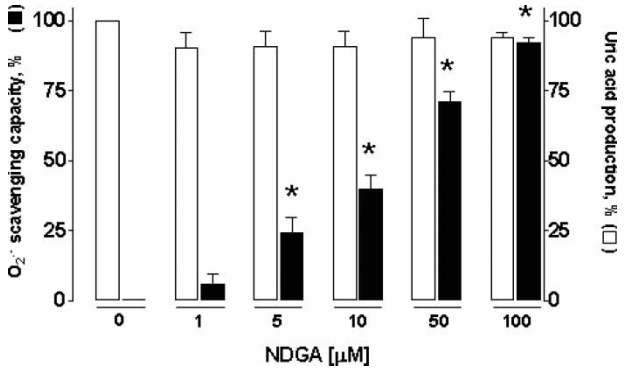

Figure 10. NDGA scavenges $O_2^{\cdot-}$ in a concentration-dependent way. Data are mean ± SEM, $n = 7$ assays. $^{\star}p < 0.001$ vs 0 μM.

rats (Figures 12 and 13). In addition, the immunostained macrophages decreased significantly in ozone + NDGA treated rats compared to ozone-treated rats (Figures 12 and 13).

Discussion

NDGA is a polyphenol-bearing *o*-dihydroxy (catechol) structure; it possesses four phenolic hydroxyl groups (Figure 1). NDGA is a recognized antioxidant with several health beneficial effects [1,4–15].

In the present paper, we have shown that NDGA is a potent *in vitro* scavenger of $ONOO^-$, $^1O_2$, $OH^{\cdot}$, $O_2^{\cdot-}$ and HOCl. These specific scavenging properties of NDGA contribute to explain their recognized antioxidant properties. In addition, our data show that the antioxidant properties of NDGA may not be explained by its ability to scavenge H_2O_2 because this compound was unable to scavenge H_2O_2, at least in our assay. The ability of NDGA to scavenge the above mentioned reactive species was compared with reference compounds with the purpose to know the relative efficiency of NDGA to scavenge these species. To our knowledge, this is the first time that the IC_{50} values of NDGA for $ONOO^-$, $^1O_2$, $OH^{\cdot}$, $O_2^{\cdot-}$ and HOCl are described and the scavenging ability of NDGA for the above mentioned reactive species is compared with reference compounds to establish its scavenging efficiency. Based on these comparisons, we have concluded that NDGA is more efficient $^1O_2$, $OH^{\cdot}$ and $O_2^{\cdot-}$ scavenger than the reference compounds studied. The IC_{50} of NDGA for $ONOO^-$ (4.0 ± 0.9 μM) was lower but not significantly different from that of uric acid (13.1 ± 1.7 μM) (see Table I). The IC_{50} of NDGA for HOCl (622 ± 42 μM) was lower but not significantly different from those of lipoic acid (858 ± 85 μM) and NAC (996 ± 76 μM) (see Table I). NDGA is about: (a) 8-fold more efficient than penicillamine to scavenge $ONOO^-$, (b) 11-fold more efficient than DMTU to scavenge $^1O_2$, (c) 700-fold more efficient than DMTU to scavenge $OH^{\cdot}$, (d) 240-fold more efficient than NAC to scavenge $O_2^{\cdot-}$ and (e) about 8-fold more efficient than DMSO to scavenge HOCl. All four reducing equivalents from the two catechol groups in NDGA may be involved in the reactive oxygen and nitrogen species scavenging properties described in the paper. It has been suggested that hydrogen atoms of the four phenolic hydroxyl groups react with reactive oxygen species [31]. Abou–Gazar et al. [16] suggested that the number and position of the hydroxy and methoxy substituents of the phenyl moieties may contribute to the antioxidant effect in an epoxylignan. Also, it has been suggested that the resulting phenolate anion structure of NDGA chelates ferrous ions to prevent the Fenton reaction from occurring to generate hydroxyl radicals.

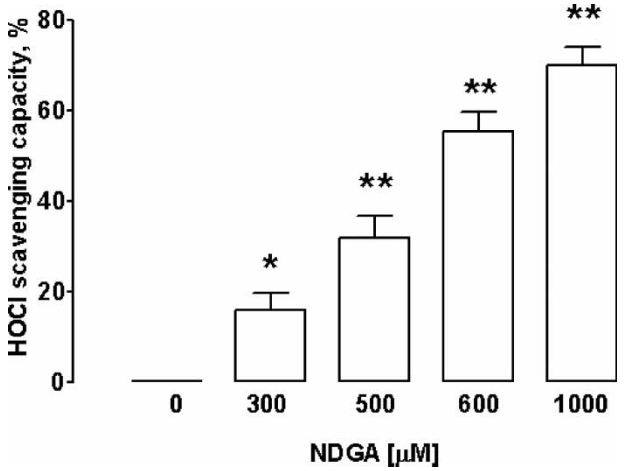

Figure 11. NDGA scavenges HOCl in a concentration-dependent way. Data are mean ± SEM, $n = 10$ assays. $^{\star}p < 0.05$, $^{\star\star}p < 0.001$ vs 0 μM.

The hydrophobicity of the antioxidants plays a role in the efficacy of inhibition. The oil/water partition coefficients of the antioxidants stress their different hydrophobicity. It has been shown that the oxidation of linoleic acid was more inhibited by lipid soluble than by hydrophilic antioxidants. Pryor et al. [32] found that the autoxidation of linoleic acid in micelles is inhibited by water soluble ascorbate and to a much greater extent, by lipid soluble ascorbyl palmitate. Doba et al. [33] reported that ascorbate is an effective inhibitor of peroxidations initiated in the aqueous phase, but it is a very poor inhibitor of peroxidation initiated in the lipid phase. Carlotti et al. [34] found that the oil/water partition coefficient of NDGA is 1.36 and the inhibition of lipoperoxidation of linoleic acid in oil/water emulsions by five antioxidants (butylated hydroxytoluene, butylated hydroxyanisole, NDGA, quercetin and rutin) was lower in the hydrophilic antioxidants quercetin and rutin supporting the fact that the hydrophobicity of the antioxidants plays a role in the efficacy of inhibition. In our study, it was observed that NDGA, which is a hydrophobic compound, has *in vivo* and *in vitro* antioxidant properties.

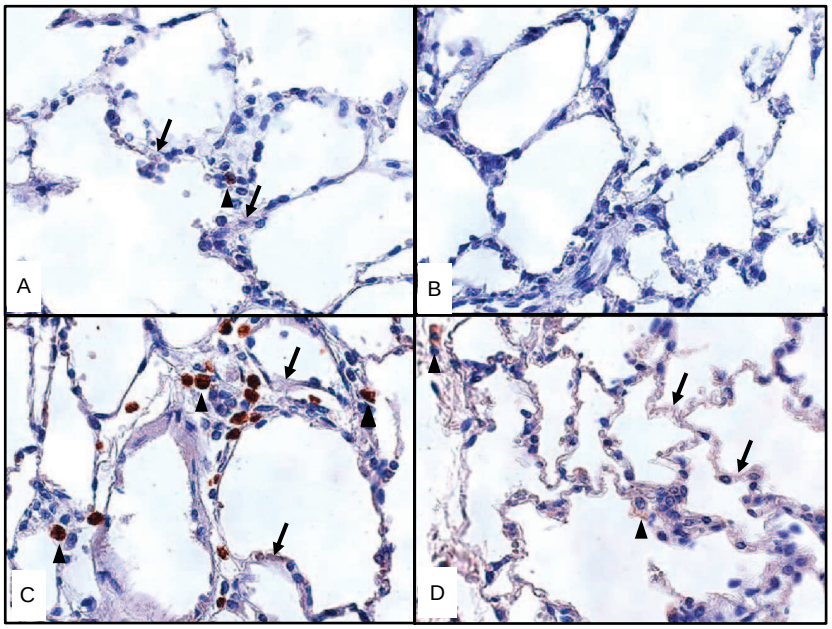

Figure 12. Immunohistochemical localization of 3-NT in the lungs of the rats from the four groups studied on week one. Representative images of the staining for 3-NT in lung sections from (A) control rats (air-exposed), (B) NDGA, (C) ozone-exposed and (D) ozone + NDGA-exposed rats. The alveolar epithelium (arrows) and positive alveolar macrophages (arrowheads) show positive immunostaining for 3-NT. (A) It is observed one positive macrophage in the photography and weak zones of immunoreactivity in the pulmonary alveoli. (B) Immunoreactivity for 3-NT was absent in both interstitial cells and alveolar epithelium. (C) Many positive alveolar macrophages and pulmonary parenchyma from an ozone-exposed rat, shown positive immunoreactivity for 3-NT. (D) Positive immunostaining for 3-NT is weak and diffuse in alveolar epithelium of ozone + NDGA-exposed rats, similar to ozone-exposed group. Nevertheless, few positive alveolar macrophages are observed and their intensity of 3-NT immunoreactivity is pale compared with the ozone-exposed group. Pictures are representative ($n \geq 6$ slides), panel represent $\times 400$ magnification.

These scavenging properties of NDGA described in this work may explain some of the health beneficial effects of this compound since the excessive production of $ONOO^-$, $^1O_2$, $OH^{\cdot}$, $O_2^{\cdot-}$ and HOCl is involved in several pathologies [35].

In our *in vivo* studies it was shown for the first time $ONOO^-$, the reaction product of $NO^{\cdot}$ and $O_2^{\cdot-}$, is a strong oxidant and nitrating agent; its formation may be beneficial in inflammatory states in terms of an oxidative destruction of invading microorganisms. On the other hand, $ONOO^-$ may oxidize and covalently modify all major types of host biomolecules, including DNA, protein and lipids. $ONOO^-$-induced modification of proteins has been connected with the development of several diseases [36–38]. $^1O_2$, an electronically excited species of oxygen, has been reported to be generated in myeloperoxidase- and eosinophil peroxidase-catalyzed reactions and by certain activated cells including neutrophils, eosinophils and macrophages. It has been implicated in the toxic properties of the above mentioned cells. Direct exposure of a number of compounds to UV light has been shown to generate $^1O_2$. As a result of the exposure of humans to UV and visible light, $^1O_2$ has been suggested to play a key role in the development of a number of human pathologies including aging, some skin cancers, sunburn and cataract. $^1O_2$ can significantly and quite often adversely alter several biological molecules including lipids, DNA, cholesterol, amino acids and proteins (reviewed in [39]). $OH^{\cdot}$, which is formed via the Fenton reaction in living systems, is the most reactive oxygen radical. The half-life of $OH^{\cdot}$ in cells, has been estimated to be around 10 s. $OH^{\cdot}$ is generally considered to be a harmful byproduct of oxidative metabolism, causing molecular

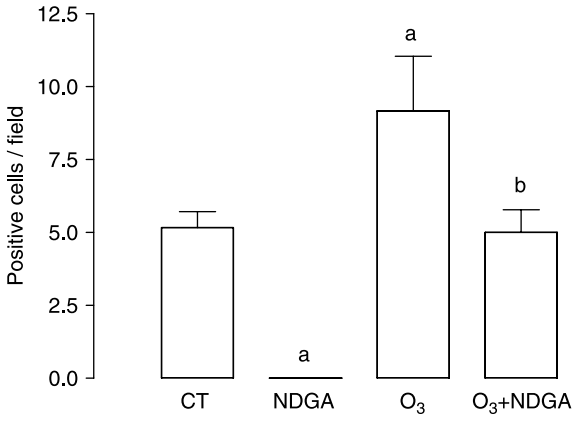

Figure 13. Quantitation of 3-NT immunostained macrophages in the lungs from the four groups of rats on week one. Values represent mean ± SEM ($n = 4$). [a]$p < 0.05$ vs control rats, [b]$p < 0.05$ vs ozone. O_3 = ozone.

damage in living systems. These radicals have been postulated to play a role in several pathological conditions, such as carcinogenesis, cardiovascular disease, Parkinson's disease, rheumatoid arthritis, brain ischemia, etc. (reviewed in [40]). Living organisms produce large quantities of $O_2^{\cdot-}$, a relatively benign radical. Although, it readily inactivates iron–sulfur proteins, it does not react with most biological molecules. However, the iron released from iron–sulfur proteins can give rise to secondary products such as $OH^{\cdot}$ which together with $ONOO^-$ are thought to be major contributors to $O_2^{\cdot-}$ toxicity. $O_2^{\cdot-}$ reacts with $NO^{\cdot}$ to generate $ONOO^-$. This reaction is extremely quick and $ONOO^-$ is capable of undergoing a wide range of oxidative processes. These include direct oxidations and secondary reactions due to nitrogen dioxide, hydroxyl and carbonate radicals (reviewed in Ref. [41]). HOCl, or its anion hypochlorite (^-OCl), is produced *in vivo* by activated phagocytes in myeloperoxidase–hydrogen peroxide–chloride ion system and it is responsible for the bacterial cell killing action of neutrophils and macrophages. Hypochlorite is a strong oxidant and reacts quickly with thiols and amines, thus modifying proteins and enzymes. Thus, hypochlorite may exert both beneficial and toxic effects *in vivo* [42] that NDGA was able to prevent ozone-induced tyrosine nitration in lungs in rats. These *in vivo* data are consistent with the ability of NDGA to scavenge $ONOO^-$ in our *in vitro* system. It has been considered that tyrosine nitration is secondary to the excessive production of reactive nitrogen species, including $ONOO^-$. Our *in vivo* results are also consistent with previous studies *in vivo* showing the antioxidant ability of NDGA. Ansar et al. [10] found that NDGA prevents renal and hepatic toxicity as well as oxidative damage induced by ferric–nitrilotriacetate in mice. NDGA prevented the increase in renal and hepatic microsomal lipid peroxidation, H_2O_2 generation and the decrease in GSH content and in the activity of antioxidant enzymes [10]. Anjaneyulu and Chopra [43] found that NDGA prevents oxidative stress, the development of renal damage and the decrease in glutathione and antioxidant enzymes in rats with streptozotocin-induced diabetes [43]. It has also been shown that NDGA is a potent antioxidant in cell culture; it was able to: (a) prevent the formation of reactive oxygen species induced by *tert*-butyl hydroperoxide as well as the DNA damage induced by γ-irradiation or the Fenton reaction [44], (b) prevent the oxidative-induced cytotoxicity in Madin–Darby bovine kidney cells [45] and (c) inhibit the oxidative DNA damage induced by H_2O_2 in Jurkat T-lymphocytes [46].

In conclusion, our studies clearly show that NDGA (a) is a potent *in vitro* scavenger of $ONOO^-$, $^1O_2$, $OH^{\cdot}$, $O_2^{\cdot-}$ and HOCl and (b) is able to prevent lung tyrosine nitration *in vivo*.

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