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Hydrophobicity and antioxidant activity acting together for the beneficial health properties of nordihydroguaiaretic acid

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ABSTRACT

Nordihydroguaiaretic acid (NDGA) and rosmarinic acid (RA), phenolic compounds found in various plants and functional foods, have known antioxidant and anti-inflammatory properties. In the present study, we comparatively investigated the importance of hydrophobicity and oxidability of NDGA and RA, regarding their antioxidant and pharmacological activities. Using a panel of cell-free antioxidant protocols, including electrochemical measurements, we demonstrated that the anti-radical capacity of RA and NDGA were similar. However, the relative capacity of NDGA as an inhibitor of NADPH oxidase (ex vivo assays) was significantly higher compared to RA. The inhibitory effect on NADPH oxidase was not related to simple scavengers of superoxide anions, as confirmed by oxygen consumption by the activated neutrophils. The higher hydrophobicity of NDGA was also a determinant for the higher efficacy of NGDA regarding inhibition of the release of hypochlorous acid by PMA-activated neutrophils and cytokines (TNF-α and IL-10) production by Staphylococcus aureus-stimulated peripheral blood mononuclear cells. In conclusion, although there have been extensive studies about the pharmacological proprieties of NDGA, our study showed, for the first time, the importance not only of its antioxidant activity, but also its hydrophobicity as a crucial factor for pharmacological action.

Keywords: nordihydroguaiaretic acid; rosmarinic acid; hydrophobicity; antioxidant activity; oxidative stress; NADPH oxidase.
1. Introduction

Polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom; these compounds have currently attracted much attention as active constituents in functional foods and food supplements, which promise to improve human health [1,2]. Polyphenols are known to possess antioxidant activity that inhibits oxidative damage and may consequently prevent inflammatory conditions [3,4], neurodegenerative diseases and ageing [5]. Certain phenolic compounds, such as nordihydroguaiaretic acid (NDGA) and rosmarinic acid (RA) present in many plants and functional foods have been studied due to their pharmacological properties.

NDGA (Figure 1) is a natural lignin and the main metabolite extracted from Creosote bush, *Larrea tridentate*, a plant that is abundant in the United States and Mexico [6]. NDGA was one of the earliest phenolic antioxidants permitted for the stabilisation of edible fats and oils [7]. NDGA was used as a food antioxidant in the USA in the 1940s; however, in 1970, the US Food and Drug Administration limited its use after some reports of toxicity [8]. However, its pharmacological form, also known as masoprocol, was approved in the United States and is currently used as a topical treatment for actinic keratosis under the name Actinex (Chemex Pharmaceuticals, Denver, CO) [9]. The number of potential beneficial health effects of NDGA is astonishing. It is a powerful scavenger of the most common reactive oxygen (ROS) and nitrogen (RNS) species, including peroxynitrite (ONOO⁻), hypochlorous acid (HOCl), singlet oxygen (¹O₂) and hydroxyl radical (HO•) [10]. NDGA is also an anti-inflammatory [11,12], neuroprotective [13], anti-viral [14], anti-hyperlipidaemic [15]
and anti-cancer agent [16]. A comprehensive review of NDGA and its pharmacological properties can be found in the works of Lü et al. [6] and Hernández-Damián et al. [8].

Rosmarinic acid (RA) (Figure 1) is a polyphenol found in some fern and hornwort species and higher plant species of the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae [17]. RA was detected also in rosemary, balm, sage, thyme, oregano, marjoram, savoury, peppermint, and basil [18,19]. RA has numerous potential pharmacological applications, including antioxidant [20,21], anti-nociceptive and anti-inflammatory effects [22,23] and chemopreventive properties [24].

As can be observed from their molecular structures, NDGA and RA are very similar polyphenols. Both compounds have two catechol moieties, which are responsible for their potent antioxidant capacities and, consequently, their biological effects. However, these molecules also have an important difference, which could directly affect their physicochemical properties. It is the carbon chain that connects the catechol moieties. In NDGA, it is a totally reduced aliphatic carbon chain, whereas in RA, an ester and carboxylic acid group confer a less hydrophobic character to this polyphenol. This structural difference requires attention because we have recently demonstrated that the simple esterification of phenolic acids was sufficient to significantly increase the inhibitory capacity of the NADPH oxidase compared to the acid precursors [25,26]. The improvement of beneficial pharmacological properties caused by the increase in the hydrophobicity of phenolic acids is not only related to NADPH oxidase inhibition. For instance, antibacterial activity [27], inhibitory effect upon tumour necrosis factor alpha (TNF-α)-induced activation of nuclear transcription factor kappa B (NF-kB) [28] and anti-mutagenicity activity [29] were also reported to be dependent on the alkyl chain length of gallates.
NADPH oxidase is a multi-enzymatic complex identified as a family of seven isoforms expressed in many cell types, including phagocytes, which contributes to the production of ROS [30,31]. Previous studies have shown that NADPH oxidase modulators may provide major opportunities in many diseases as novel therapeutics.

For these reasons, here, we aimed to study the effect of the polyphenols NDGA and RA on NADPH oxidase inhibition, preventing oxidative damage, and to evaluate whether the difference in hydrophobicity between NDGA and RA could also be relevant regarding antioxidant and anti-inflammatory properties. Our hypothesis was confirmed, because, while the cell-free antioxidant properties were not affected, the relative capacity of NDGA as an inhibitor of NADPH oxidase and cytokine released by neutrophils was significantly higher compared to RA.
2. Results and Discussion

2.1. Structures and hydrophobicity

It is well-known that hydrophobicity may be crucial for the pharmacological action and toxicological effect of any substance present in drugs and functional foods. This is because hydrophobicity affects absorption, bioavailability and hydrophobic-receptor interactions. Regarding that, a simple inspection of the molecular structures of NDGA and RA reveals that the major chemical difference between these molecules must be related to their hydrophobicities. Figure 1 shows the molecular structures and relative hydrophobicities of NDGA and RA, which was calculated based on their hydrophobicity indexes (partitioning coefficient n-octanol/water). The log p values confirmed our expectation and show that NDGA is significantly more hydrophobic than RA. The higher hydrophobicity of NDGA can be explained by the totally reduced carbon chain that connects the catechol moieties in NDGA. On the other hand, the ester and carboxylic acid groups confer a more hydrophilic character to RA.
**Fig. 1.** Molecular structures of nordihydroguaiaretic acid (NDGA) and rosmarinic acid (RA) and their hydrophobicity indexes (log P, partition coefficients n-octanol/water). The functional groups responsible for the different hydrophobicities between these compounds are highlighted.

2.2. **Antioxidant capacity**

The presence of two catechol moieties in both NDGA and RA is an indication of their potential capacity as a reducing agent of ROS. Indeed, there are numerous demonstrations of the efficacy of these phytochemicals as scavengers of ROS in several experimental models [10,17]. However, they have never been studied together using the same analytical protocols. For this reason, and aiming to understand the importance of the hydrophobicity in their radical scavenging capacity, a panel of antioxidant assays was performed.
The first step was to study the redox properties of the compounds. For that, the oxidation peak potentials, measured as anodic peak potential (Epa), were investigated by cyclic voltammetry at pH 7.0. Figure 2 shows the well-defined cathodic and anodic waves peaking in the range 0.16 to 0.22 V. These low Epa values are consistent with related and well-established antioxidant phenolic acids such as protocatechuic acid (0.188 V) [32] and caffeic acid (0.183 V) [33]. The first anodic peak potential for NDGA (0.162 V) was only slightly lower compared to RA (0.173 V). This small difference could be explained by the presence of the α,β-unsaturated carbonyl moiety substituent in RA, but not in NDGA. This substituent has a small electron-withdrawing character, which could increase the oxidation peak potential of RA. Anyway, this difference seems to be insignificant regarding their radical scavenging capacities, as will be demonstrated below.

**Fig. 2.** Cyclic voltammograms of nordihydroguaiaretic acid (NDGA) and rosmarinic acid (RA) (0.1 mM) obtained in 0.2 M sodium phosphate buffer at pH 7.0. The scan rate was 5 mV.s⁻¹.

<table>
<thead>
<tr>
<th></th>
<th>Epa1</th>
<th>Epa2</th>
<th>Epc1</th>
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<tbody>
<tr>
<td>Rosmarinic acid (RA)</td>
<td>+0.17</td>
<td>+0.22</td>
<td>-0.2</td>
<td>-0.14</td>
</tr>
<tr>
<td>Nordihydroguaiaretic acid (NDGA)</td>
<td>+0.16</td>
<td>-</td>
<td>-0.11</td>
<td>-</td>
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*Epa1* = First anodic peak potential, *Epc1* = First cathodic peak potential
The next step was to measure whether the different hydrophobicities of the studied compounds could have an influence in their free radical scavenging capacity. Initially, they were tested as reducing agents of the stable free radical DPPH. This assay is based on the reduction of this free radical, which is monitored by a decrease in its absorbance. Figure 3 shows the concentration-response curves and the effective concentration (EC$_{50}$) values for both molecules. The EC$_{50}$ obtained were RA 9.1 µM (confidence interval 8.2 – 10.0 µM, p < 0.05) and NDGA 8.2 µM (confidence interval 7.6 – 9.6 µM, p < 0.05). As expected due to the similarities in their molecular structure and oxidation peak potential, both compounds were equally potent as reducing agents of the DPPH free radical. The difference observed in this set of experiments was not statistically significant. These results are in agreement with previous reports that showed a strong capacity of NDGA for scavenging free radicals, with EC$_{50}$=7.46 μM in the DPPH assay, and potency comparable to resveratrol and trolox [34]; some previous studies also indicated that RA is a potent antioxidant, with EC$_{50}$ values varying widely, from 1.51 µg/mL to 41.3 µg/mL [35,36]. Lapenna and collaborators (2003) [37] also studied the antioxidant activity of RA and found values of EC$_{50}$=30 µM using the DPPH assay.
Fig. 3. DPPH scavenging capacity of NDGA and RA. The results are mean and SEM of triplicate experiments. NGDA $r^2 = 0.9688$, RA $r^2 = 0.9756$.

In the sequence, NDGA and RA were compared by their efficacy as scavengers of peroxyl free radical (ROO•), generated by AAPH, an azo-compound that decomposes at 37°C in aqueous solutions to generate an alkyl radical (R•), which, in the presence of molecular oxygen, is converted to the corresponding ROO•. Here, the deleterious effect of ROO• was evaluated by the degradation of the conjugated triene present in eleostearic acid. The oxidation of the triene was monitored by the bleaching its absorption at 273 nm. The addition of the test compounds, by scavenging ROO•, delayed the bleaching and produced a concentration-dependent lag phase. The results depicted in Figure 4A show the kinetic profile of the absorbance decay of eleostearic
acid and the effect of the addition of NDGA and RA at increasing concentrations. Figure 4B shows the relationship between the area under the curve and concentrations of the studied compounds. The slope of the linear regression \( \frac{(\text{AUC}_{\text{test compound}} - \text{AUC}_{\text{control}})}{\text{concentration}} \) was used as an analytical parameter for assessment of the reactivity of the test compounds with ROO•. From the results depicted in Figure 4C and 4D, once again, it is possible to conclude that the antioxidant capacities of NGDA and RA were not significantly different. The slopes calculated from the linear regression curves were: NDGA 14320 ± 1165 and RA 13430 ± 664, for which the difference were not statistically significant. The protective effect of NDGA and RA against lipid peroxidation has been proved by other authors; according to Popov et al. (2013) [38], RA demonstrates both high antioxidant potential and protective effect upon the induced peroxidation of lipids, presenting higher activity than trolox, ascorbic acid and dihydroquercetin using different model systems. Lapenna et al. [37] showed strong antioxidant effects of NDGA on the peroxidation of linoleic acid induced by soybean 15-lipoxygenase, inhibiting the formation of conjugated diene hydroperoxides.
**Fig. 4.** Inhibitory effect of NDGA and RA on triene degradation by ROO•. A, C) Bleaching of triene (eleostearic acid) by ROO• and the protective effect provoked by the addition of NDGA and RA, respectively. B, D) Linear relationship between AUC\(_{t}\)-AUC\(_{c}\) and concentrations of NDGA and RA, respectively. The slopes calculated from the linear regression curves were: NDGA 14320 ± 1165 and RA 13430 ± 664. The results are means and SD of triplicate experiments.

Going a step further, the anti-radical efficacy of NDGA and RA was evaluated by their ability to impede the degradation or erythrocytes (haemolysis) provoked by ROO•. This assay has the same concept as the previous one, but is closer to a biological context, since it is based on the direct peroxidation of cell membranes. Figure 5 shows the kinetic profile of haemolysis and the protective effect of NDGA and RA. As can be observed, the haemolysis is time-dependent and reached its maximum at 6 h of incubation. The inhibition of haemolysis provoked by the addition of NDGA and RA...
was concentration and time-dependent. Hence, at 4 hours, both test compounds at both concentrations were able to inhibit haemolysis. At 6 hours, only RA at the higher concentration was an effective and statistically significant inhibitor. These results are consistent with the previous ones, although RA was slightly more effective.

**Fig. 5.** Inhibitory effect of (a) NDGA and (b) RA on erythrocyte haemolysis induced by ROO•. The reaction mixture (positive control) contained 10% (v/v) erythrocytes and 50 mM AAPH in PBS at 37°C. The results are means and SEM of duplicates of three separate experiments. *p < 0.05 relative to the control.
2.3. Inhibitory effect on the production of superoxide anion by activated leukocytes

The previous results showed that the higher hydrophobicity of NDGA did not significantly alter its antioxidant potency compared to RA. Hence, our next step was to evaluate whether the same tendency could be observed for enzymatic and cellular systems.

Initially, we investigated whether the generation of ROS by activated neutrophils could be affected by the presence of the studied compounds. For that, NDGA and RA were initially evaluated as inhibitors of lucigenin-dependent chemiluminescence elicited by activated neutrophils. In this reaction, $\text{O}_2^-$, which is produced by the activation of the NADPH oxidase multi-enzymatic complex, reduces lucigenin to form its cation radical, which reacts with a second $\text{O}_2^-$ leading to light emission [39]. The results depicted in Figure 6 show the kinetic profile of light emission produced by the $\text{O}_2^-$-mediated reduction of lucigenin and the effect of the previous incubation of the cells with NDGA and RA. As can be observed, in contrast to the previous cell-free antioxidant assays, NDGA was found to be significantly more potent than RA. In agreement with these findings, Lojek et al. [40] studied the antioxidative effects of NDGA on murine bone marrow phagocytes using lucigenin- and luminol-amplified chemiluminescence, verifying strong suppression in the opsonised zymosan particles’ stimulated response. In the same way, Verweij-van Vught et al. [41] detected the inhibition of PMN chemiluminescence by RA, measured in a luminol-enhanced system. Here, these results were confirmed; however, a comparison between RA and NDGA revealed that the latter is much more effective.
Fig. 6. Inhibitory effect of NDGA and RA on Lucigenin-dependent chemiluminescence elicited by PMA-activated neutrophils. The results are means and SEM of duplicates of three separate experiments. Different letters denote significant differences. One-way ANOVA and Turkey’s multiple comparison test, \( p < 0.05 \).

Considering that the anti-radical properties of RA and NDGA were similar, the previous result is an indication that NDGA could be inhibiting the production and not just working as a scavenger of \( \text{O}_2^- \). For this reason, and because there are some controversies about the selectivity of the lucigenin assay for the detection of \( \text{O}_2^- \), we also studied the activation of NADPH oxidase in neutrophils using the sulphonated tetrazolium salt (WST-1), which is a specific chromogenic probe to \( \text{O}_2^- \). WST-1 is water soluble and membrane-impermeable. Hence, the formazan salt produced by its specific reaction with \( \text{O}_2^- \) can be detected in the extracellular medium using conventional absorbance measurements [42]. From the results depicted in Figure 7a, we
can conclude that the higher capacity of NDGA compared to RA was confirmed using the WST-1 assay.

The confirmation that NDGA was inhibiting the production of $O_2^\cdot$ in neutrophils, and not only acting as a scavenger of this ROS, was obtained using the xanthine/xanthine oxidase enzymatic system as a source of $O_2^\cdot$ and its production was measured by the WST-1 assay (Figure 7b). As can be observed, the scavenger effects were minimal and the difference between NDGA and RA was not statistically significant.

In short, by comparing the difference between the test compounds, in both neutrophil and xanthine/xanthine oxidase enzymatic studies, we can conclude that the NDGA seems to act as an inhibitor of the enzymatic activity of the NADPH oxidase complex in neutrophils and not only a scavenger of $O_2^\cdot$. It must be also emphasized that NDGA was more potent than apocynin, which is currently the most used NADPH oxidase inhibitor [43].

To confirm that NDGA was blocking the enzymatic activity of NADPH oxidase, we performed additional experiments where its inhibitory potency was studied via direct action on the oxygen uptake elicited by stimulated neutrophils. In agreement with the lucigenin and WST-1 assays, the NDGA but not RA was able to impede oxygen consumption by the cells, which is direct evidence of its inhibitory effect (Figure 7c). It is important to note that the concentration of the test compound used in this assay was higher than previous ones. This can be explained taking into account the concentration of neutrophils used for an effective measurement of oxygen consumption, which was 10-fold higher compared to WST-1 and lucigenin assays.
Fig. 7. NDGA, RA and apocynin (APO) as inhibitors of the production of superoxide anion by (a) stimulated neutrophil and (b) xanthine/xanthine oxidase. (c) The consumption of molecular oxygen by stimulated neutrophils. The results are mean and SEM of duplicates of three different experiments. Different letters denote significant differences. One-Way ANOVA and Tukey's multiple comparison test, $p < 0.05$. 
2.4. Inhibitory effect on the intracellular production of ROS

By inhibiting the generation of $\text{O}_2^-$, the production of $\text{H}_2\text{O}_2$ and subsequent downstream ROS should also be blocked in stimulated neutrophils. For this reason, NDGA and RA were also compared as inhibitors of the intracellular production of ROS through the dihydrorhodamine 123 flow cytometry assay [44]. In agreement with the previous cell-based assays, NDGA was a more effective inhibitor than RA regarding the production of intracellular ROS (Figure 8). However, two points must be emphasised in these results. First, the concentration of both drugs had to be increased compared to the previous experiments. This can be explained considering that the dihydrorhodamine 123 cytometry assays were performed with whole blood and not with isolated neutrophils. Hence, part of the test compounds can diffuse to the erythrocytes, which are in large numbers compared to neutrophils. Second, the difference between NDGA and RA was not as high compared to the lucigenin, WST-1 and oxygen uptake assays. This can also be justified considering that the dihydrorhodamine 123 assay is an analytical technique for measuring total ROS. In other words, although RA was not able to block $\text{O}_2^-$ production, it could act directly as a scavenger of $\text{H}_2\text{O}_2$ and others ROS.
Fig. 8. Flow cytometry assay for determination of intracellular ROS produced by stimulated neutrophils and the inhibitory effect of NDGA and RA. a) Percentage of inhibition was calculated in comparison to the control group. The results are mean and SEM of duplicates of three different experiments. Different letters denote significant differences. One-Way ANOVA and Tukey's multiple comparison test, p < 0.05. b) Representative example of spontaneous production of intracellular ROS by neutrophils. c) Representative example of PMA-stimulated production of ROS (control). d) After previous incubation with 200 μM NDGA. e) After previous incubation with 200 μM.
RA. The dot plot displays forward scatter (FSC) and cell granularity (side scatter, SSC). Left quadrant represented rhodamine-negative cells, while right quadrant represented rhodamine-positive cells.

2.5. Inhibition of the production of hypochlorous acid by activated leukocytes

Another consequence of the inhibition of O$_2^-$ production in neutrophils is the interruption of the extracellular release of HOCl. This microbicidal hypohalous acid is produced through MPO-mediated oxidation of chloride using H$_2$O$_2$ as a co-substrate [45]. For that, an analytical protocol based on trapping the HOCl produced with taurine, which is converted to taurine chloramine, was used. This stable oxidant was detected by the iodide-catalysed oxidation of TMB [46]. Corroborating the previous cell-based experiments, NDGA was significantly more effective than RA as inhibitor of the production of HOCl by activated neutrophils (Figure 9a).

Obviously, as HOCl is a strong oxidant, its direct reaction with NDGA cannot be excluded. In the same way, we also could not exclude that NDGA had some effect upon the chlorinating activity of MPO. Hence, to discriminate these effects, we measured the capacity of the test compounds as inhibitors of the chlorinating activity of purified MPO. From the results depicted in Figure 9b, it can be concluded that both NDGA and RA were unable to inhibit the formation of HOCl in this cell-free system. A comparison can be made with 5-fluorotryptamine, an indole derivative that has been well-established as a potent inhibitor of MPO activity [47].

In short, inhibition of the production of HOCl without affecting MPO enzymatic activity is an additional confirmation that NDGA, but not RA, was acting as an inhibitor of NADPH oxidase in neutrophils. In other words, by blocking the activation of
NADPH oxidase, the entire downstream cascade of ROS produced by neutrophils was affected. Finally, it is important to note that despite the high HOCl scavenger capacity of NDGA and RA, demonstrated by several studies [8,10], our results prove that inhibition of the production of HOCl and not scavengers occurs, because the HOCl produced by MPO or by the cells is captured in the form of taurine chloramine, which does not react with RA and NDGA at the concentrations used here (controls not shown).
Fig. 9. NDGA, RA and 5-fluorotryptamine (FT) as inhibitors of the production of hypochlorous acid by (a) stimulated PMN and (b) purified MPO. The results are the mean and SEM of duplicates of three different experiments. Different letters denote significant differences. One-Way ANOVA and Tukey’s multiple comparison test, $p < 0.05$. 
2.6. Hydrophobicity of NDGA and proposal for its efficacy as inhibitor of NADPH oxidase

There are several classes of substances that have been used as potential inhibitors of the NADPH oxidase complex. Among the natural-based antioxidants, phenolic acids [25,26], flavonols [48] and catechols [49] are examples of compounds for which there are many demonstrations of their efficacies. Although widely used, the mechanism by which these compounds inhibit the activation of the NADPH oxidase complex has not been completely established. However, there is no doubt that the presence of redox active moieties is essential for their actions. For instance, the oxidation of phenolic moieties leading to quinone intermediate has been proposed as a pathway for inactivation of essential thiols and prevention of the NADPH oxidase assembly [50]. Apocynin, a methoxy-substituted catechol, has its mechanism of action based on its oxidation and the consequent generation of a dimeric product, which impedes the assembly of the NADPH oxidase complex [43]. In the same direction, oligophenols derived from apocynin oxidation inhibit critical interactions that are involved in the assembly and activation of human vascular NADPH oxidase [51]. Obviously, NDGA and RA, which have two catechol moieties, could also follow similar interactions with the cytosolic and membrane components of the NADPH oxidase complex. However, the findings obtained here show that the hydrophobicity of these phenolic compounds is also an important factor for their inhibitory potency. As we have demonstrated, although RA and NDGA have practically the same oxidation potential and antioxidant capacity, they were totally different as inhibitors of the enzymatic activity of NADPH oxidase. From these findings, we propose that the higher hydrophobicity of NDGA could facilitate their access to the membrane-bound protein, and consequently, its action upon redox-reaction involved in the inhibitory pathway. In
addition, the diffusion of NDGA through the cell-membrane could also facilitate its interaction and inhibition of the NADPH oxidase complex.

2.7. Inhibition of the production of Cytokines

Until now, we have demonstrated that the higher hydrophobicity of NDGA compared to RA was decisive for its action as an inhibitor of the enzymatic activity of NADPH oxidase and the consequent production of H$_2$O$_2$ and HOCl. On the other hand, the cell-free antioxidant capacity was not significantly affected. Hence, aiming to confirm this differentiated behaviour, our next step was to measure whether other cell-based processes could also be sensitive to the higher hydrophobicity of NDGA.

In this regard, the capacity of NDGA and RA as inhibitors of the production of the pro-inflammatory tumour necrosis factor-α (TNF-α) and the anti-inflammatory interleukin-10 (IL-10) was evaluated. This choice was based on the well-known relationship between intracellular oxidative stress and the production of cytokines [38]. This is the case for TNF-α, the major pro-inflammatory cytokine involved in the inflammatory response. As has already been well-established, the release of TNF-α through the activation of redox-sensitive transcription factors such as nuclear factor-kappa B (NFkB) can be initiated by activation of the NADPH oxidase complex [52-54]. TNF-α is also able to prime the NADPH oxidase in neutrophils through the partial phosphorylation of p47PHOX [55]. Moreover, many substances able to inhibit NADPH oxidase substance are also able to reduce the production of cytokines. Hence, our hypothesis was that by inhibiting NADPH oxidase, NDGA could also affect the production of TNF-α. From the results depicted in Figure 10, it can be seem that our expectation was confirmed because NDGA was, indeed, a more effective inhibitor. In
these experiments, *Staphylococcus aureus* was used for the activation of PBMC and the production of cytokines measured by conventional ELISA assay.

Similarly, the production of IL-10 was also affected by the test compounds (Figure 10b). In agreement with the fact that antioxidants can inhibit the production of this anti-inflammatory cytokine in stimulated PBMC [56], both NDGA and RA were able to reduce IL-10 release; however, and probably due to its higher hydrophobicity, NDGA was more effective.

Again, it is important to note that the inhibition of cytokine production by NDGA and RA has been proven by other authors: Brennan & O’Neill [57] observed this activity of NDGA by inhibiting NFκB in TNF-α activated Jurkat T lymphoma cells and interleukin 1-activated EL4.NOB-1 thymoma cells; and Swarup et al. [58] showed significant decreases in pro-inflammatory cytokine levels (IL-12, TNF-α, gamma interferon (IFN-γ), MCP-1, and IL-6) in JEV-infected animals treated with RA compared to levels in infected mice without treatment. Hence, our main contribution here is to show that NDGA can be significantly more effective due to its higher hydrophobicity.
Fig. 10. Production of (a) TNF-α and (b) IL-10 by stimulated PBMC and the inhibitory effect of NDGA and RA. The results are mean and SEM of duplicates of six different experiments. Different letters denote significant differences. One-Way ANOVA and Tukey’s multiple comparison test, p < 0.05.
3. Conclusions

NDGA presented higher inhibitory capacity of NADPH oxidase compared to RA in all cell-based models studied in this work, but not in the cell-free assays. These results can be attributed to the difference in the hydrophobicity between these polyphenols. Hydrophobicity is an important property for drugs and compounds present in functional food, mainly because it affects the absorption, bioavailability and hydrophobic substance-receptor interactions of the substance. Although other extensive studies have focused on the pharmacological proprieties of NDGA, such as potent scavenger of ROS, inhibitor of lipoygenase, and cytoprotector in non-tumour cells, among others, our study showed, for the first time, the importance not only of its antioxidant activity, but also its hydrophobicity as a crucial factor for its pharmacological action.

4. Materials and Methods

4.1. Chemicals

Nordihydroguaiaretic acid, rosmarinic acid, Brij®35, tung oil, 5-fluorotryptamine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3,3’,5,5’-tetramethylbenzidine (TMB), taurine, apocynin, 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH), dimethyl sulphoxide (DMSO), lucigenin, phorbol 12-myristate 13-acetate (PMA), dihydrorhodamine 123, Histopaque®-1077 and Histopaque®-1119 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Myeloperoxidase (MPO) (EC 1.11.1.7) was purchased from Planta Natural Products (Vienna, Austria).
and its concentration was determined from its absorption at 430 nm ($\varepsilon_{430} = 89,000 \text{ M}^{-1} \text{ cm}^{-1}$). Hydrogen peroxide was prepared by diluting a 30% stock solution and calculating its concentration from its absorption at 240 nm ($\varepsilon_{240} = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$). Stock solutions of the test compounds were prepared in DMSO for cellular studies or in ethanol for electrochemical and DPPH scavenging assays. For the antioxidant assays, the DMSO stock solutions (50 mM) were diluted in 10 mM phosphate-buffered saline pH 7.4 (PBS) generating working solutions with lower concentrations (5 and 0.5 mM). PMA stock solutions were prepared in DMSO at a concentration of 50.0 µM and diluted to 0.5 µM in PBS at the time of use. TMB solution was prepared by dissolving 14 mM TMB and 100 μM potassium iodide in 50% dimethylformamide and 50% acetic acid (800 mM) (v/v). All reagents used for buffers were of analytical grade. Ultrapure Milli-Q water from Millipore (Belford, MA, USA) was used for the preparation of buffers and solutions.

### 4.2. Hydrophobicity index

The molecular hydrophobicities of NDGA and RA were calculated based on their log P values (partitioning coefficient in n-octanol/water) based on Crippen’s fragmentation method and performed using ChemDraw software (ChemDraw Ultra 7.0.1, CambridgeSoft) [59].

### 4.3. Cyclic Voltammetry

Voltammetric curves were recorded at room temperature using a 3-electrode setup cell. The working electrode was a glassy carbon disk electrode (GC electrode, 3 mm diameter). The counter electrode was a platinum plate and the reference an
Ag/AgCl saturated KCl at 3 M electrode. The working electrode surface was carefully polished with 0.5 μm alumina slurries before each experiment and thoroughly rinsed with distilled water. The experiments were performed using an Autolab PGSTAT 30 potentiostat/galvanostat (Eco-Chemie, Utrecht, Netherlands). A solution of sodium phosphate buffer 0.2 M (pH = 7) was used as a supporting electrolyte. The solutions were purged with nitrogen for 5 min before recording the voltammograms. The ethanolic solutions (5 mM) of the test compounds were diluted in the electrochemical cell at final concentrations of 0.1 mM using the supporting electrolyte solution. The cyclic voltammograms were recorded at a potential scan rate of 5 mV s\(^{-1}\) [60].

4.4. DPPH scavenging assay

The test compounds were incubated for 30 min with 100 μM DPPH in ethyl alcohol in the dark. The scavenging activity was evaluated spectrophotometrically at 517 nm using the absorbance of unreacted DPPH radical as a control and calculated as: 
\[
\frac{\text{(Absorbance of control } - \text{ absorbance of sample})}{\text{absorbance of control}} \times 100
\]

4.5. Triene degradation assay

An emulsion was prepared by mixing 2.5 mg of tung oil (not stripped of tocopherols) in 25 mL of PBS containing 17 μM of the tensoactive Brij®35. The solution was vigorously vortexed to produce a homogeneous emulsion. The assays were performed as follows: the tung oil suspension (50 μL) was incubated with freshly prepared 1 mM AAPH (source of peroxyl radical) in PBS at 37°C in the absence
(control) or presence of the test compounds in the wells of a microplate for 3 hours. The final reaction volume was 200 µL. The microplate was read at 5 min intervals with shaking for 5 seconds before the measurements were taken. The absorbances were measured at 273 nm using a Synergy 2 Multi-Mode microplate reader (BioTek, Winooski, VT, USA). The degradation of the eleostearic acid (conjugated triene) produced the absorbance versus time curve for which the area under the curve (AUC) was calculated. Curves of \((AUC_{\text{test compound}} - AUC_{\text{control}})\) against the concentration of each test compound were plotted and their slopes were used as analytical parameters [26,62].

4.6. Preparation of erythrocyte suspension

Human erythrocytes from healthy donors (age range 20-30 years) were obtained from peripheral blood, centrifuged at 770g for 10 min and washed 3 times with PBS at pH 7.4. The supernatant and buffy coat were removed by aspiration after each wash. The cells were resuspended to 20% (v/v) in PBS. The blood samples were taken from healthy volunteers. Experiments were performed in accordance with regulations of the Research Ethics Committee (21496413.8.0000.5426), Faculty of Pharmaceutical Sciences, Unesp, São Paulo, Brazil.

4.7. Haemolysis Assays

Equal amounts of erythrocytes suspension and 100 mM AAPH in PBS were gently homogenised while being incubated for 6 h at 37°C (blood tube rotator). Aliquots (75 µL) were removed at regular intervals, diluted 1:20 with PBS, and centrifuged at
4000 rpm for 10 min. The degree of haemolysis was measured in the supernatant by its absorbance at 540 nm. Reference values (100% haemolysis) were determined with the same aliquot of erythrocytes but diluted in 1500 μL of distilled water instead of PBS to provoke the total lysis of erythrocytes. By diluting the 100% haemolysis sample in PBS, a calibration curve of percent haemolysis against absorbance was constructed for conversion of the absorbance measurement to degrees of haemolysis. The blank was PBS and the measurements were made in a UV-1240 spectrophotometer (Shimadzu, Japan). Ten μL aliquots of the test compounds dissolved in ethanol were added at the beginning of the reaction. The same volume of ethanol (10 μL) was added to the negative (without AAPH) and positive (with AAPH) controls [63,64].

4.8. Isolation of human leukocytes

Blood samples were obtained from healthy volunteers (age range 20-30 years) using heparin as an anticoagulant. Polymorphonuclear neutrophil (PMN) and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Histopaque®-1077/1119 gradient at 700 x g for 30 min at room temperature [65]. After isolation, the cells were resuspended in PBS supplemented with 1 mM calcium chloride, 0.5 mM magnesium chloride, and 1 mg/mL glucose (supplemented PBS). Experiments were performed in accordance with regulations of the Research Ethics Committee (21496413.8.0000.5426), Faculty of Pharmaceutical Sciences, Unesp, São Paulo, Brazil.
4.9. Superoxide anion production by activated leukocytes (Lucigenin-dependent chemiluminescence assay)

PMN and PBMC (1 x 10^6 cells/mL) were pre-incubated at 37°C in supplemented PBS with the test compounds for 10 min. Next, lucigenin (10 µM) and PMA (100 nM) were added and the light emission was measured for 30 min at 37°C using a plate luminometer (Centro Microplate Luminometer LB960, Berthold Technologies, Oak Ridge, TN, USA). The final assay volume was 250 µL. The integrated light emission was used as an analytical parameter to measure the O_2^- produced by the stimulated cells. The inhibitory potency was calculated using the light emission generated by the control, in which activated cells were incubated in the absence of the test compounds as a reference [39]. It is worth noting that the inhibitory effects were not the result of a cytotoxic effect on leukocytes, as confirmed by the trypan blue exclusion assay. At the higher concentration used for leukocyte studies, the viability of the cells was >98% (results not shown).

4.10. Superoxide anion production by activated neutrophils (WST-1 assay)

PMN (1.0 x 10^6 cells/mL) were pre-incubated at 37°C in supplemented PBS with the test compounds for 10 min. Next, WST-1 (500 µM) and PMA (100 nM) were added and the extracellular release of O_2^- was measured by the reduction of WST-1 monitoring the absorbance increase at 450 nm for 30 min at 37°C; this was performed using Synergy 2 Multi-Mode microplate reader (BioTek, Winooski, VT, USA). The inhibitory potency was calculated using the absorbance of the control, in which the PMA-activated cells were incubated in the absence of the test compounds as a reference [42].
4.11. Superoxide anion production by xanthine/xanthine oxidase (WST-1 assay)

The test compounds were incubated at 37°C in PBS supplemented with 500 µM WST-1 and 100 µM xanthine. The reactions were initiated by the addition of 0.05 unit/mL xanthine oxidase and the reduction of WST-1 was assessed by monitoring the absorbance increase at 450 nm for 15 min at 37°C using Synergy 2 Multi-Mode microplate reader (BioTek, Winooski, VT, USA).

4.12. Oxygen uptake elicited by activated neutrophils

Neutrophils (1 × 10^7 cells/ml) were incubated at 37°C in supplemented PBS in the presence or absence of the test compounds. The reaction was triggered by adding 0.5 mM PMA. The oxygen consumption was measured in a Clark-type oxygen electrode (Yellow spring instruments 5300A, Cincinnati, OH, USA). The buffer solution was initially saturated with air by stirring the opened cell for 5 min. The rate of oxygen consumption was used as an analytical parameter [66].

4.13. Dihydrorhodamine 123 flow cytometry assay

Blood samples were obtained from healthy volunteers using heparin as the anticoagulant. Leucocytes were counted and the samples diluted with PBS to reach 3x10^5 cell/mL. Then, the test compounds were added and the sample incubated for 30 min at 37°C. Next, the stimulus (PMA, 2 µM) was added and incubated for an additional 20 min followed by the addition of dihydrorhodamine 123 (20 µg/mL). At this stage, the reaction volume was completed to 100 µL with PBS. After a further 7
min incubation at 37°C, 150 µL of Staining Buffer (Becton Dickinson, San Jose, CA) was added and the solution was centrifuged at 630xg for 7 min. The supernatant was discarded and the erythrocytes lysed by adding 230 µL BD FACSTM Lysing Solution and incubated for 20 min at room temperature in the absence of light. In the sequence, the cells were washed twice using the 150 µL Staining Buffer and finally resuspended to 300 µL of Staining Buffer. Fluorescence of gated neutrophils was detected at FL1 counting 2,000 events/gate in an FACS LRS II Fortessa flow cytometer (BD, Biosciences, NJ, USA). Data were analysed using the Flowjo Flow Cytometry Analysis Software (Treestar Inc., Ashlan, OR, USA), and results were recorded as mean fluorescence intensity (MFI). Negative (absence of PMA) and positive (absence of the test compound) controls were used for calculation of the inhibitory potency of the test compounds [44].

4.14. Production of hypochlorous acid by activated neutrophils and by H₂O₂/MPO

The neutrophils (1.0 × 10⁶ cells/mL) were pre-incubated at 37°C for 10 min in supplemented PBS containing 10 mM taurine and test compounds. The cells were stimulated by the addition of PMA (100 nM) and incubated for an additional 30 min at 37°C. The reactions were stopped by the addition of catalase (20 µg/mL) and centrifuged at 6000 rpm. Then, 200 µL was transferred into a 96-well plate and the accumulated taurine chloramine was measured by adding 50 µL of TMB solution. The oxidised TMB was detected spectrophotometrically at 655 nm using Synergy 2 Multi-Mode microplate reader (BioTek, Winooski, VT, USA). The amount of HOCl produced was calculated using a standard curve which was generated using pure HOCl and submitted to the same analytical protocol. Negative (absence of PMA) and positive
(absence of the test compounds) controls were used for calculation of the inhibitory potency of the test compounds.

For the cell-free experiments, the reactions were performed in a 96-well plate containing PBS, taurine (10 mM), pure MPO (50 nM), H₂O₂ (50 μM) and various concentrations of the test compounds. The reactions were triggered by the addition of H₂O₂ and incubated at 37°C. After 30 min, the reactions were stopped by the addition of catalase (20 μg/mL) and the accumulated taurine chloramine was measured as described above. The chlorination inhibitory potency was calculated using the control, in which the MPO/H₂O₂ was incubated in the absence of the test compounds as a reference [46].

4.15. Cytokines production by activated peripheral blood mononuclear cells

Peripheral blood mononuclear cells (1x10⁶ cells/mL per well) were cultured in RPMI-1640 (Gibco®, Life Technologies, Foster City, CA, USA) medium added with foetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) at 37°C in an atmosphere of 5% CO₂, overnight. Cells were stimulated with Staphylococcus aureus (10 to 1 microorganism per cell) after 5 hours of incubation with the test compounds. After 18 hours of incubation had elapsed, the supernatants were stored at -80°C. TNF-α and IL-10 were quantified by Enzyme-linked Immunosorbent Assay (ELISA) using BD OptEIA™ Human TNF ELISA Set (Cat. Nº 555212) and BD OptEIA™ Human IL-10 ELISA Set (Cat. Nº 555157), respectively, according to the manufacturer's instructions.
4.16. Statistical analysis

Comparisons were performed using one-way ANOVA multiple comparisons among means, with the Turkey's post hoc test. Results were considered statistically significant when $p < 0.05$. The results were expressed as mean and SEM.

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