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NORDIHYDROGUAIARETIC ACID: DUAL BEHAVIOR AS PRO- OR ANTIOXIDANT ON A EUKARYOTIC CELL MODEL (VERO CELLS).

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ABSTRACT

Since Nordihydroguaiaretic acid (NDGA), a potent natural antioxidant, can produce oxidation in several cell kinds, mainly by superoxide anion production ($O_2^{\bullet-}$), the current study was designed to assess its capacity to produce or not this reactive oxygen species on Vero cell line, with the aim to establish whether this lignan behaves as a pro- or antioxidant. The $O_2^{\bullet-}$ production was determined by the Nitro Blue Tetrazolium reduction test. Results show that NDGA has a dual-face behavior on this eukaryotic cell model depending on the biological environment and its concentration. The NDGA behaved as a pro-oxidant when it was tested single, by means of an increase in $O_2^{\bullet-}$ production that was directly concentration-dependent. Mixed with an antioxidant (ascorbic acid) or a moderate oxidant (glucose), the NDGA behaved as pro-oxidant at low concentrations and antioxidant at high concentrations.

KEYWORDS: Nordihydroguaiaretic acid, Vero cells, pro-oxidant, antioxidant, *Larrea divaricata*.

INTRODUCTION

Nordihydroguaiaretic acid (NDGA) is a natural phenolic compound, specifically defined as lignan, which has shown to have promising applications in the treatment of multiple diseases, including cardiovascular diseases, neurological disorders and cancers.[1] This lignan is the main metabolite of several species of *Larrea* Cav. (Zygophyllaceae), particularly in *Larrea tridentata* (Sessé & Moc. ex DC.) Coville, the most studied species, which is popularly known as "creosote-bush", "chaparral" or "greasewood" in United States and "gobernadora" or "hediondilla" in Mexico.[2] Our working group established that NDGA is also the main component in bioactive extracts of *Larrea divaricata* Cav.,[3] related specie with *L. tridentata*, but with habitat in arid regions of Argentina, in where it is popularly known as "jarilla", and used in folk medicine as anti-inflammatory and anti-rheumatic agent.[4]

Among the proposed biological properties for the NDGA, its antioxidant effect has been one of the most widely studied. Nevertheless, over the years, others biological activities have been studied and this compound has gained popularity and interest due to its antineoplastic, antiviral and anti-inflammatory characteristics;[2] even our research group has demonstrated that NDGA and the enriched-extracts in this lignan, obtained from *L. divaricata*, showed *in vitro* antiviral effect against Junin virus.[3, 5] Thus, the potential medical applications of NDGA have attracted much interest, and numerous investigations have been published in the last few years, which include studies on molecular mechanisms as well as the pharmacokinetics and toxicity.[2]

It has been widely demonstrated that the antioxidant effect exhibited by the NDGA is due to its ability to trap free radicals or reactive oxygen species (ROS);[6] even some of its beneficial effects (anticancer; preventive agent of several toxic effects such as renal, liver and lung toxicity; anti-ulcerogenic and anti-inflammatory activities) have been attributed to its antioxidant property, especially its ability to prevent oxidative stress.[1, 4, 6-9]

On the other hand, NDGA also has demonstrated pro-oxidant effects in various cell types, being this action responsible both for its toxicity and for antitumor activity.[10, 11] In addition, Sahu *et al.*[12] established that this polyphenol compound has the potential to act both as a pro- and antioxidant depending on its concentration and biological environment.

An interesting biological environment to study the NDGA behavior (pro- or antioxidant) that has not been previously used for this purpose, is a mammalian cellular eukaryotic line such as the Vero cells; which can be used in different studies, primarily to evaluate the *in vitro* antiviral effect of a compound, since they are susceptible to infection by different kind of viruses.

Therefore, the aim of this study was to assess whether the NDGA acts as $O_2^{\bullet -}$ generator (pro-oxidant) or as scavenger (antioxidant) of this reactive species (generated by an oxidizing agent) on Vero cell line; and how this behavior can be influenced by the NDGA concentration and the biological environment when it is present an antioxidant or an oxidizing agent.

MATERIALS AND METHODS

Samples and reagents: Standard NDGA was obtained from *L. divaricata* by Dr. C.E. Tonn (INTEQUI-CONICET, Argentina) and identified by its spectroscopic properties (MS and UV-V), in agreement with those found in the literature.[13] Ascorbic acid (AA) and glucose (Glu) were purchased in Merck.

The following reagents were used: Eagle's minimum essential medium (EMEM, Gibco), Fetal calf serum (FCS, Natocor), L-glutamine (Calbiochem), gentamicin (Klonal), dimethylsufoxide (DMSO, Tetrahedron), Neutral Red (NR, Gibco), Phosphate buffer saline (PBS), Nitro Blue Tetrazolium (NBT, Sigma).

Cells: African green monkey kidney cells (*Cercopithecus aethiops*, Vero 76 ATCC CRL-587) were used. They were grown and kept alive under humid atmosphere with 5 % CO_2 at 37 °C. EMEM supplemented with 10 % FCS, 1 % L-glutamine, and gentamicin (50 µg/mL) was used as growth medium (GM), whereas EMEM plus 2 % FCS containing the same formulation as described above and 1 % DMSO was used as maintenance medium (MM).

In vitro cytotoxicity Test

Samples for cytotoxicity assays: A stock solution of NDGA (10 mg/mL in DMSO) was used to achieve 15 consecutive dilutions with MM, in a range of 1-55 µg/mL. In the case of AA (100 mg/mL in MM), 15 dilutions were prepared between 5 and 2000 µg/mL in MM. From a Glu solution (2.5 M in PBS) 15 dilutions were performed between 500 and 10 mM in MM (this medium already has a low Glu concentration, 5 mM).

Cytopathic effect: By means of inverted optical microscopy, the action of NDGA and ascorbic acid on the morphology of Vero cells were observed.[14] Each dilution was inoculated in duplicate on a confluent cell monolayer ($2.5 \pm 0.6 \times 10^5$ cells/mL, 48 h incubation), grown in a 48 well-plaque. Cell controls (CC) that contain only MM were included (n = 2). The cells were incubated at 37 °C during 72 h, and the development of cellular alterations such as rounding, membrane retraction, cell detachment and the presence of granules in the cytoplasm was daily observed.[15]

Cell viability assay: Cellular viability (CV) *vs.* concentrations of each compound was measured by means of the uptake NR assay in a 96 well-plaque. The same 15 dilutions used before was inoculated in triplicate on a confluent monolayer of cells ($1.0 \pm 0.6 \times 10^5$ cells/mL), according to the methodology described by Borenfreund & Puerner.[14] The absorbance of the NR extracted after 48 h of incubation at 37 °C was measured at 540 nm on a microplate reader (BioTekELx800). The percentage of CV (CV%) was calculated by comparison with CC (100 % viability, without sample, n = 3). The concentration of the compound that reduces the viable cells to 50 % (CC_{50}) was determined by regression ($R^2 > 0.9$) from the plot of CV% vs. compound concentrations. Maximum Non-Cytotoxic Concentration (MNCC) was defined as the maximum concentration of sample that exhibits more than 90 % viable cells and exerts no cytotoxic effect detected by microscopic monitoring.[16] In addition, a subtoxic concentration (SubTC) was determined as the concentration that causes 10 - 20% cellular death[17] and produces slight morphologic changes observed by microscopy (less than 20% of swollen and rounded cells, with cytoplasmic inclusions, slight vacuolization, and the nuclear membrane remaining intact).

Determination of $O_2^{\bullet-}$ production: To evaluate the intracellular generation of $O_2^{\bullet-}$, the NBT bioassay adapted to a confluent monolayer of cells attached to a multiwell-plate was used.[18] In this test, the yellow-colored NBT is absorbed by cells and reduced to water-insoluble Blue Formazan by the action of $O_2^{\bullet-}$ intracellular, in the presence and absence (basal situation) of the tested compound.

Preformed Vero cell monolayers ($1.0 \pm 0.6 \times 10^5$ cells/mL, 48 h) in 24-well plate were washed 4 times with PBS (250 µL per well and per time) to remove any remaining GM. Dilutions of all compounds to assess were added, which were prepared in PBS from corresponding stock solutions. Thus, different concentrations of NDGA and AA, in a range covering the CC_{50}, were inoculated in duplicate. By contrast, the Glu was only tested at its SubTC in duplicate, which was determined by the *in vitro* cytotoxicity assay (Table 1). Then, NBT (0.1 mg/mL in PBS with 1% DMSO) was added. Wells with cells in PBS (1% DMSO) without sample were included as control cells (CC, n = 2) to determine the basal $O_2^{\bullet-}$ production. The multiwell-plate was incubated during 1h at 37 ºC under CO_2 atmosphere, following the methodology described by Choi *et al.*[19] The absorbance of intracellular Blue Formazan was measured on a microplate reader (BioTek ELx800) at 630 nm. The $O_2^{\bullet-}$ production in the presence of oxidant or antioxidant compounds was expressed as an increase or decrease in absorbance respectively, compared to the basal situation (CC). The increase in the percentage of $O_2^{\bullet-}$ production vs. concentrations of compound was plotted.

Data analysis: MNCC, SubTC and CC_{50} values were graphically obtained from the dose-response plots, which have a non-lineal regression analysis (Sigmoidal Origin, $R^2 > 0.9$). The values were expressed as (mean ± standard error) from three independent experiments. Thus, for each concentration, 6 replicates were carried out to determine cytopathic effect, 9 replicates to quantify cell viability, 6 replicates to evaluate the intracellular $O_2^{\bullet-}$ production. The *t*-test (Origin) was used to assess the degree of statistical difference of MNCC, CC_{50} and the SubTC values; differences between means were considered significant at $p < 0.05$.

Table 1: Cytotoxic concentration to 50% cells (CC_{50}), subtoxic concentration (SubTC) and Maximum Non-Cytotoxic Concentration (MNCC) for nordihydroguaiaretic acid (NDGA), ascorbic acid and glucose.

Compounds	CC_{50} (µg/mL)	SubTC (µg/mL)	MNCC (µg/mL)
NDGA	15.4 ± 0.4	9.9 ± 0.4	6.9 ± 0.3.
Ascorbic acid	852.2 ± 87.97	544.6 ± 50.1	306.7 ± 29.5
Glucose	nd	$(4.8 \pm 0.1) \times 10^4$	$(3.19 \pm 0.06) \times 10^4$

nd: not determined because it was not toxic to 50% of the cells.

RESULTS AND DISCUSSION

The CC_{50}, SubTC and a MNCC of NDGA (Table 1) were determined by extrapolation from the plot of CV% *vs.* NDGA concentrations (Fig. 1A). Thus, a concentration range where cellular viability is equal or higher than 90% was established from 1.0 µg/mL up to the MNCC (6.9 µg/mL).

Figure 2A shows the morphological changes of a Vero cell monolayer treated with NDGA at the CC_{50}. There are clearly signs of lack of cellular viability, since cells showed retraction with very little dye inside, compared to those that were not exposed to the compound (Fig. 2B), in where cellular lysosomes containing dye can be appreciated as a sign of viability.

The data obtained in the NBT assay are shown in Fig. 1B as the percentage increase in the production of $O_2^{\bullet-}$ respect to basal situation. After 1h incubation, NDGA increased the generation of $O_2^{\bullet-}$ in direct way to its concentration, and thus the oxidative stress over the cell monolayer was demonstrated. The maximum increment of $O_2^{\bullet-}$ (194.90 ± 0.02 %) was noted at the CC_{50}, whereas this production lowered at SubTC (103.00 ± 0.03 %) and at the MNCC (43.70 ± 0.04 %) (Data extrapolated from the plot, Fig. 1B).

Although the values of $O_2^{\bullet-}$ production at the MNCC and SubTC were elevated respect to CC, it can be inferred that this increase was not enough to cause significant damages on the cells during 1 h incubation, since no apparent morphological

alterations were observed by inverted optical microscopy (the cells were examined every 15 min during incubation). This agrees with results of a previous work of our research group,[18] in where we also observed that an increase in the $O_2^{\bullet-}$ production is not necessarily associated with the generation of cytopathic effect. Not forget that against an increase of ROS, the total antioxidant cell system is activated, in which enzymatic and non-enzymatic components are involved to counteract the effect of these reactive species.[20] However, it should be noted that NDGA began to be cytotoxic from concentrations greater than 10 µg/mL (near the SubTC), by means of a decrease in cellular viability, which would seem to be directly related with a rise in the $O_2^{\bullet-}$ production, probably because of an imbalance between the production of oxidant species and the triggering of antioxidant defenses.[20] This behavior is describing a pro-oxidant capacity of the NDGA, which has already been observed by other researchers but in other cell models;[10, 11, 21] even Sahua *et al*.[12] have shown that this pro-oxidant action produces an increase in oxidative stress, oxidative cell injury and cytotoxicity. Therefore, we decided to assess the behavior of a recognized antioxidant compound such as Ascorbic acid (AA), in order to compare its performance in this biological system with the effect exhibited by the NDGA, thus the AA was used as a positive control.

Comparison of cytotoxicity with $O_2^{\bullet-}$ production generated by the NDGA (Fig.1A and B) and AA (Fig. 3), shows that both compounds act as pro-oxidants in this biological system, in direct proportion to concentration; which in turn leads to an increase in cytotoxicity. Thus, this result confirms the findings found by other researchers about the double-faced character of AA, since it exhibits a pro-oxidant activity arising from its usual antioxidant property that generates reactive free radicals, which induce cytotoxic effects.[22-25] The pro-oxidant activity of AA is a dose-dependent effect and is a result of the Fenton mechanism.[24] Ascorbate is an excellent reducing agent (it donates electrons), so it is a powerful antioxidant; however, during this process it is easily auto-oxidized. There are enzymes (reductases) in the biological medium that reduce the ascorbate to recover its antioxidant effect.[23] However, the ascorbate can reduce the oxygen to generate ROS at a very low reaction rate,[26] but which can be accelerated by catalytic metals like Fe^{+3}.[23] Thus, in the presence of catalytic metals, ascorbate also has pro-oxidant effects, where the redox-active metal is reduced by ascorbate and then in turn reacts with oxygen, producing superoxide that subsequently dismutes to produce H_2O_2.[23] The pro-oxidant toxicity of NDGA may be related to its ability to undergo autoxidation, such as other phenolic compounds (flavonoids), to produce superoxide anions.[27] This is supported by the results of Bilinski & Krol,[28] who demonstrated that the ortho-quinone species generated by autoxidation of NDGA, produces lipid peroxidation, suggesting an increase in oxidative stress.

In addition, we have observed that it is necessary a greater concentration of AA (CC_{50}) than NDGA to produce a similar cytopathic effect (Table 1), hence the AA is less cytotoxic. However, the AA exhibited a higher production of $O_2^{\bullet-}$ than the NDGA at their CC_{50}. Therefore, it can be deduced that other mechanisms are involved in the cytopathic effect of NDGA besides oxidative stress. Based on this observation, we only compared the $O_2^{\bullet-}$ production between NDGA and AA without considering the cytopathic effect.

Bearing in mind that NDGA may have a dual biological behavior, as pro- or antioxidant, and this effect is directly dependent on its concentration and biological environment, we evaluated the behavior of NDGA under two experimental conditions at different concentrations: 1) along with another antioxidant compound (AA), and 2) together with an oxidizing compound as Glucose (Glu).[29]

To assess the effect of NDGA in the presence of AA, several experiments were performed, in where the $O_2^{\bullet-}$ production was only evaluated, since this is the first reactive oxygen species that occurs and therefrom other reactive species are generated (H_2O_2, $^{\bullet}OH$, y $^1O_2$), according to the Fenton reaction.[30] Each compound was tested at its SubTC (Table 1), which was estimated from cytotoxicity curves (Fig. 1A y 3A). This concentration was chosen because it does not produce a marked toxic effect on cells, and ensures a constant $O_2^{\bullet-}$ production (Fig. 1B y 3B).

Figure 4 shows the growth of $O_2^{\bullet-}$, expressed as increase in Abs of reduced-NBT by this ROS. It can be observed that the $O_2^{\bullet-}$ generated by the NDGA and AA (positive control) was higher than that produced by the mixture of both compounds (1:1), but this decline when both compounds are mixed was very slight; so the action of both compounds mixed in same proportion (1:1) was not enough to reach the basal production (cells alone).

Then, the $O_2^{\bullet-}$ produced by the combination of both compounds was studied, keeping constant the concentration of a compound and varying the concentration of the other (Fig. 5A y 5B). When NDGA was tested at a constant concentration (SubTC) combined with varying concentrations of AA (SubTC, 2xSubTC and 3xSubTC) (Fig 5A), it is observed that the mixture of both compounds behaves similarly to AA. A sum of the effects of both

compounds was not observed, but rather the effect of AA had primacy over the NDGA, since the $O_2^{\bullet-}$ production by this lignan was insignificant compared to AA (Fig 1B y 3B).

When the AA was tested at a constant concentration (SubTC) combined with varying concentrations of NDGA (1/2SubTC, SubTC, 1,5xSubTC and 2xSubTC) (Fig 5B), a slight increase in the $O_2^{\bullet-}$ production was observed with increasing concentrations of NDGA, reaching a peak production at 10 µg/mL (SubTC). Higher concentrations of NDGA in the mix produced a significant decrease in the $O_2^{\bullet-}$ generation, without achieving the total reduction of this radical. Therefore, the NDGA in the presence of a constant concentration of AA would have a reducing effect on the generation of $O_2^{\bullet-}$, which is concentration-dependent. It is difficult to give an explanation to the combined action of two antioxidants because each one has an affinity for one or more specific free radical, can act in different processes of the oxidative sequence and have more than one mechanism of action.[31]

In Figure 6, the $O_2^{\bullet-}$ production generated by different concentrations of NDGA in the presence and absence of an oxidant is assessed. For that, glucose (Glu) was used at its SubTC (Table 1), determined by the corresponding cytotoxicity curve (Data not shown), since this concentration ensures a constant production of $O_2^{\bullet-}$ and the cells remain viable. In the presence of a constant concentration of Glu (SubTC), NDGA showed a dual action. Concentrations lower than 10 µg/mL of NDGA (inflection point) slightly increased the $O_2^{\bullet-}$ production as compared with the single effect produced by Glu. In contrast, concentrations greater than 10 µg/mL of NDGA in the mix triggered a reduction in $O_2^{\bullet-}$ production, compared with the generation of $O_2^{\bullet-}$ caused individually by Glu and NDGA. It can be observed that a total inhibition of $O_2^{\bullet-}$ production is achieved when the concentration of NDGA in the mixture are greater than 15 µg/mL. Experiments, where Glu concentrations change in presence of a fixed concentration of NDGA, were not performed because it was not our interest to study the behavior of glucose.

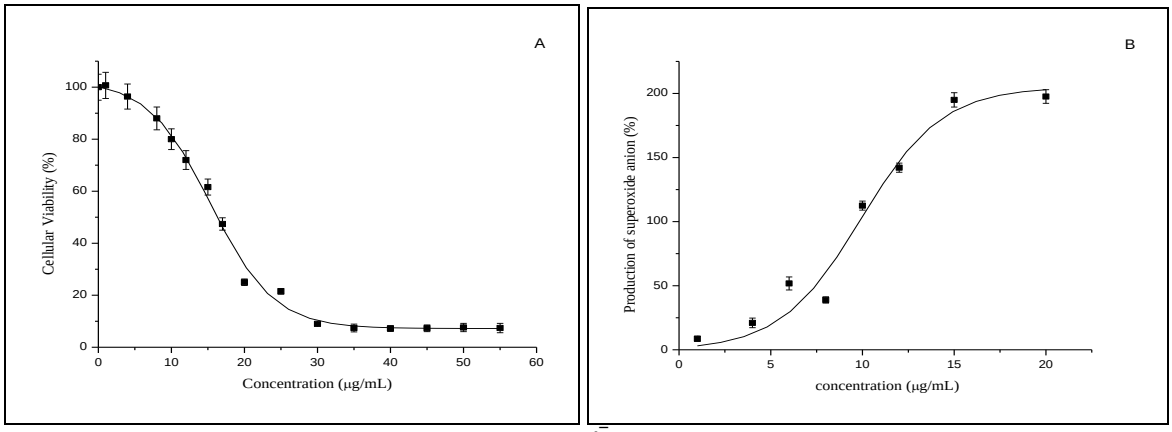


Figure 1: NDGA cytotoxicity (A) and Percentages of $O_2^{\bullet-}$ production *vs*. NDGA concentrations (B)
Error bars represent the standard deviation obtained from three independent experiments

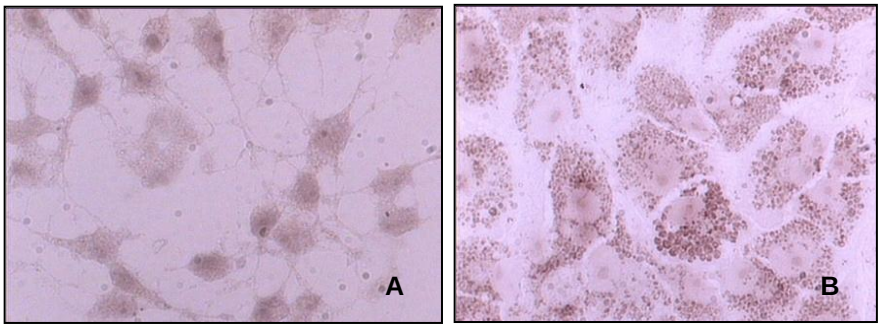


Figure 2: Morphological changes produced by NDGA at the CC_{50} on a Vero cells monolayer (A) compared to a monolayer without treatment (B). RN uptake assay. Photographs were captured with an inverted optical microscope (40X).

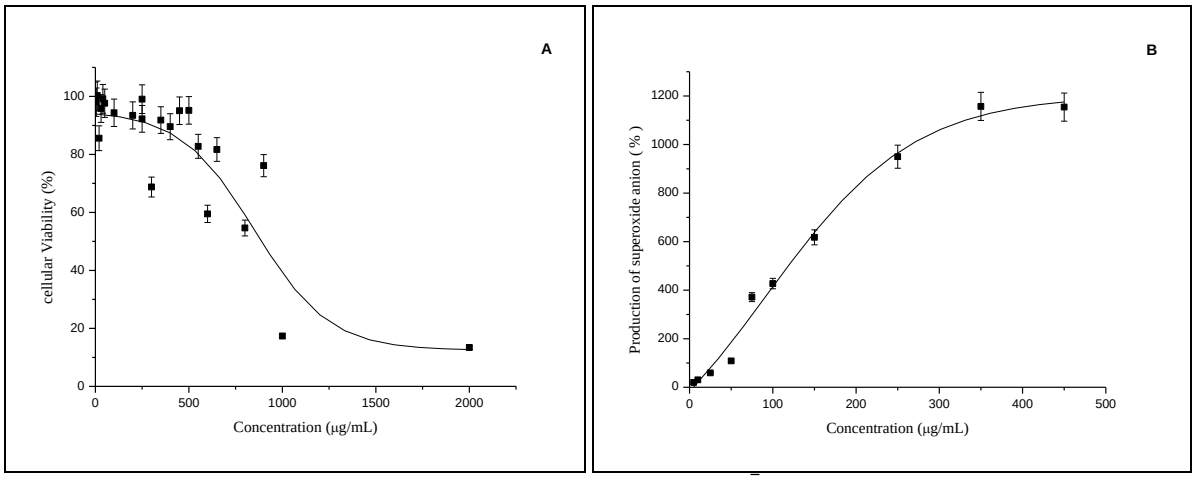


Figure 3: Ascorbic acid (AA) cytotoxicity (A) and Percentages of $O_2^{\bullet -}$ production vs. AA concentrations (B)
Error bars represent the standard deviation obtained from three independent experiments

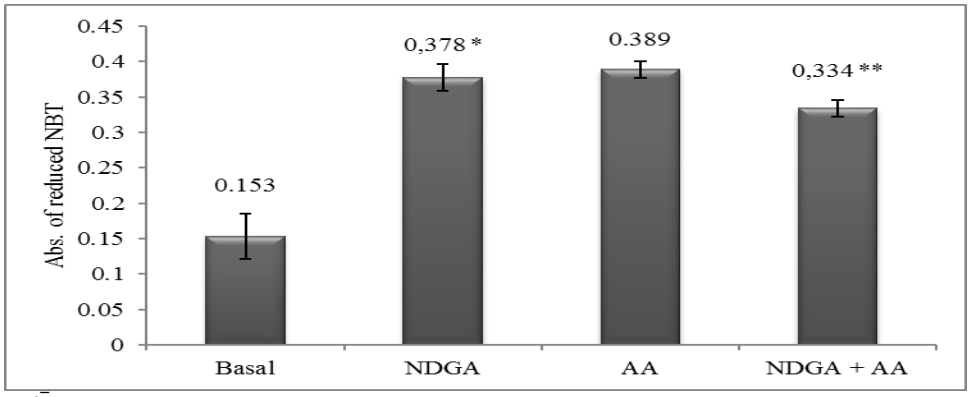
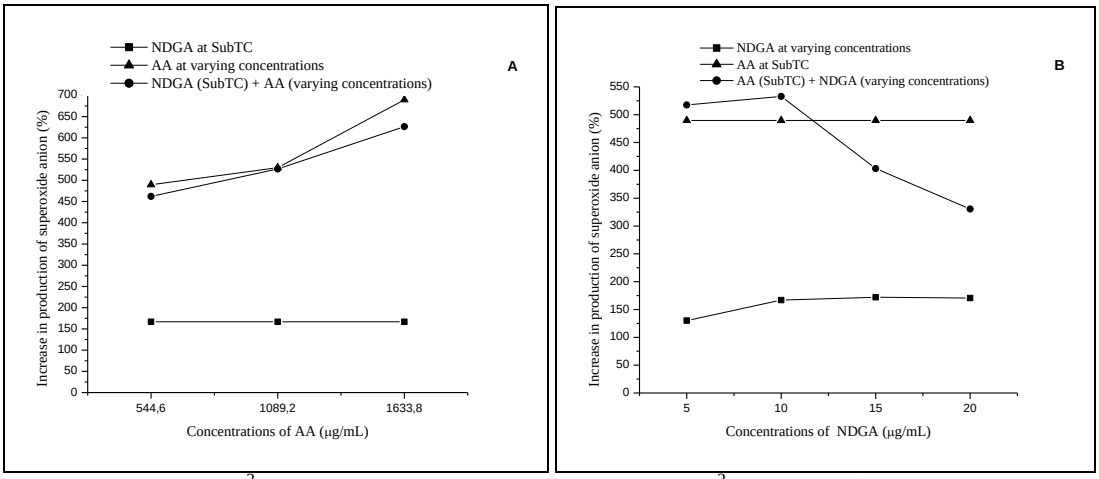


Figure 4: $O_2^{\bullet -}$ production by NDGA, ascorbic acid (AA) and a mixture of both compounds (1:1) at its subtoxic concentrations: NDGA (9.9 ± 0.4 µg/mL) and AA (544.6 ± 50.1µg/mL).
Error bars represent the standard deviation obtained from three independent experiments
* p>0,01, NDGA vs AA (α = 0,05). **p<0,01, NDGA + AA vs NDGA; NDGA + AA vs. AA (α = 0,05).



R^2: 0,99915 (A) R^2: 0,99836 (B)

Figure 5: $O_2^{\bullet -}$ production by a mixing of NDGA with another antioxidant (Ascorbic acid, AA), keeping constant the concentration of one compound (SubTC) and varying the concentration of the other. A) NDGA at constant concentration with varying concentrations of AA. B) AA at constant concentration with varying concentrations of NDGA.

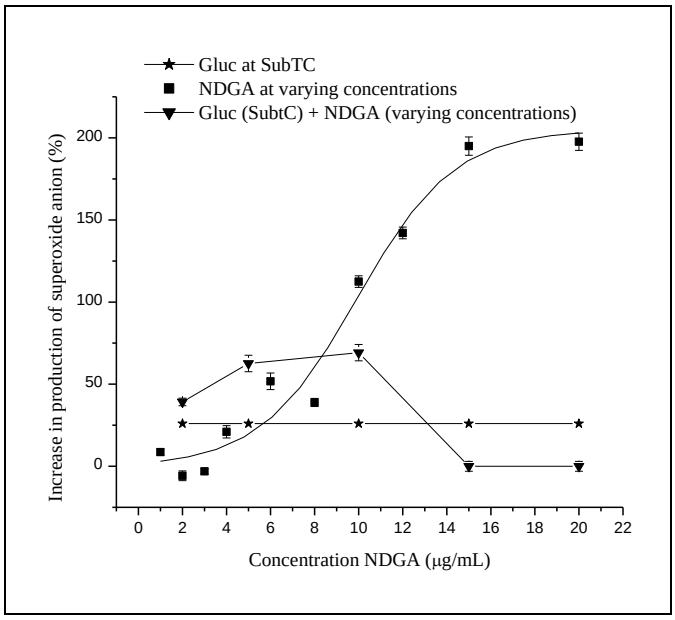


Figure 6: $O_2^{\bullet-}$ production by NDGA and mixed with a compound able to produce oxidative stress (Glucose, Gluc).

Error bars represent the standard deviation obtained from three independent experiments

CONCLUSIONS

Our results show that NDGA has a dual-face behavior (pro- or antioxidant) on Vero cells. When the NDGA was tested single on the cellular line, it increased the $O_2^{\bullet-}$ production (pro-oxidant) in a directly concentration-dependent manner, which also caused an increment in the cytotoxic effect, probably due to a stimulation of the oxidative stress. This same behavior was observed for the AA, another antioxidant used as positive control in this study. This dual-face effect of NDGA and AA would be related to its polyphenol nature, since those having a phenol ring, like these compounds, turn out pro-oxidants.[27] Moreover, it should be noted that in this cellular model (Vero cells), the AA (recognized antioxidant) behaves as a strong pro-oxidant, since its $O_2^{\bullet-}$ production was greater than that produced by the NDGA (Figs. 3B vs 1B). Therefore, our results are further evidence that the NDGA and AA can act as prooxidants and thus generate oxidative stress.

On the other hand, in presence of an antioxidant (AA) or a moderate oxidant (Glu), NDGA exhibited a similar behavior that was pro- or antioxidant depending on the concentration. That is, low concentrations of NDGA (≤ 9.9 μg/mL) increased slightly the $O_2^{\bullet-}$ production (pro-oxidant). However, concentrations exceeding 9.9 μg/mL decreased the generation of this ROS (antioxidant), achieving the basal situation only when in the biological environment was present a moderate oxidant such as Glu.

Thus, the NDGA is able to behave as a pro- or anti-oxidant, according to the environment in which it is located and also this behavior would be concentration-dependent.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

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