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p-Substituted Phenols as Inhibitors of Nitric Oxide Production by LPS-Activated Macrophages

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Abstract: Substances as resveratrol, quercetin, gallic acid, curcumin and their derivatives are among the most recognized natural antioxidants which are able to inhibit the production of Nitric Oxide (NO) by activated macrophages. Despite these antioxidants have very different molecular structures; they share the common and essential characteristic of to be phenolic compounds. For this reason, we studied the relative potency of commercial *p*-substituted phenols as inhibitors of the production of NO by LPS-stimulated macrophages. Our objective was to access the correlation between the reduction potential of the *p*-substituted phenols, which is directly influenced by the presence of electron-donating and electron-withdrawing substituents groups in the phenolic moiety, with the efficiency of inhibition. The production of NO by LPS-stimulated macrophages was measured by Griess's assay. The compounds were not cytotoxic in the concentration used as determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. We found that *p*-hydroxyphenol, the compound with lower reduction potential, was the best inhibitor of the production of NO. On the other hand, compounds like *p*-nitrophenol and *p*-acetylphenol, bearing electron-withdrawing substituents groups and, consequently, higher reduction potential, were ineffective as NO inhibitor. We also compared natural antioxidants as quercetin ($E° = 0.33$ V), apocynin ($E° = 0.66$ V) and melatonin ($E° = 0.95$ V) and, again, quercetin, the substance with lower reduction potential, was the more efficient inhibitor.

Key words: Nitric oxide, macrophage, *p*-substituted phenols, quercetin, melatonin, apocynin

INTRODUCTION

There is a very close relationship between the activation of transcription factors and the intracellular redox homeostasis (Baldwin, 2001). This is the case of the redox-sensitive transcription factor, Nuclear Factor-kappa B (NF-κB) which controls the expression of genes encoding the pro-inflammatory cytokines (e.g., IL-1, IL- 2, IL-6, TNF-α, etc.), chemokines (e.g., IL-8, MIP-1α, MCP1, RANTES, eotaxin, etc.), adhesion molecules (e.g., ICAM, VCAM, E-selectin), inducible enzymes (COX-2 and iNOS), growth factors, some of the acute phase proteins and immune receptors, all of which play critical roles in controlling most inflammatory processes (Baldwin, 2001; Haddad, 2002).

NF-κB is activated by an extraordinarily large number of conditions and agents, which usually generate a pro-oxidant state by disrupting the intracellular redox status. Hence, there are numerous evidence that antioxidants as *N*-acetylcysteine, dithiocarbamate, resveratrol, curcumin, flavonoids, etc, exert an inhibitory effect on NF-κB activation (Paterson *et al*., 2003; Csiszar *et al*., 2006; Sandur *et al*., 2007; Manna *et al*., 2007).

Nitric Oxide (NO) is synthesized from *L*-arginine by constitutive and inducible Nitric Oxide Synthase (cNOS and iNOS) in numerous mammalian cells and tissues. Constitutively expressed NO by neuronal NOS (nNOS) and endothelial NOS (eNOS) is a key regulator of homeostasis. In macrophage, NO production is induced by a variety of stimuli, such as oxidants, Lipopoly-Saccharide (LPS), bacteria and pro-inflammatory cytokines (MacMicking *et al*., 1997). NO can be directly cytotoxic but can also interact with superoxide anions and result in the formation of peroxynitrite, which is the most Reactive Nitrogen Species (RNS). Excess production of Reactive Oxygen Species (ROS), NO and RNS can damage DNA, lipids, proteins and carbohydrates, leading to impaired cellular functions and enhanced inflammatory reactions (Valko *et al*., 2007).

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NO production by iNOS is regulated at the transcriptional level by NF-κB. Consequently, the efficacy of antioxidant against the production of NO via NF-κB signaling pathways is well established. Substances as resveratrol, quercetin, gallic acid, curcumin, etc and their derivatives are among the most recognized natural antioxidants able to inhibit the production of NO in macrophages (Ma *et al*., 2005; De Stefano *et al*., 2007; Kim *et al*., 2007; Gafner *et al*., 2004). Despite these antioxidants have very different molecular structures; they share the common and essential characteristic of to be phenolic compounds. Hence, we decide to study the relative potency of *p*-substituted phenols as model of inhibitors of the production of NO by LPS-stimulated macrophages. Our objective was to access the importance of the presence of electron-donating and electron-withdrawing substituents groups in the phenolic moiety and their relevance in the efficiency of inhibition.

MATERIALS AND METHODS

Chemicals: Quercetin, melatonin, apocynin, *p*-nitrophenol, *p*-methoxyphenol, *p*-aminophenol, *p*-hydroxyphenol (hydroquinone), 4-hydroxyacetophenone, catechol, resorcinol, RPMI 1640 medium, streptomycin, 2-mercaptoethanol, *L*-glutamine, penicillin, sulfanilamide and sodium dodecyl sulphate were purchased from Sigma-Aldrich (St. Louis, MO, USA); Fetal Bovine Serum (FBS) from Cult lab (Campinas, SP, Brazil); 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazoliumbromide) (MTT) from USB Corporation (Cleveland, USA); phosphoric acid from Mallinckrodt (Mexico); naphthylethylenediamine and isopropanol from Merck (Germany); sodium thioglycollate from Difco Laboratories (EUA); crystal violet from Vetec (RJ, Brazil).

Animals: Seventy-six male Swiss mice weighing 18-25 g were supplied by the Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Araraquara, SP, Brazil. They were maintained in polycarbonate boxes at 23±2 °C, 56±2% humidity, 10-18 cycles h^{-1} and a 12 h light/dark cycle, with water and food available *ad libitum*.

Peritoneal macrophage: Thioglycollate-elicited Peritoneal Exudate Cells (PEC) were harvested from swiss male mice using 5.0 mL of sterile PBS, pH 7.4. The cells were washed twice by centrifugation at 200 rpm for 5 min at 4°C and re-suspended in appropriate medium for each test.

Cytotoxicity evaluation: For determination of the cytotoxic effect of the phenolic compounds, the macrophages were plated at a concentration of 5×10^6 per well in RPMI 1640, supplemented with 10% FBS, 100U of penicillin,

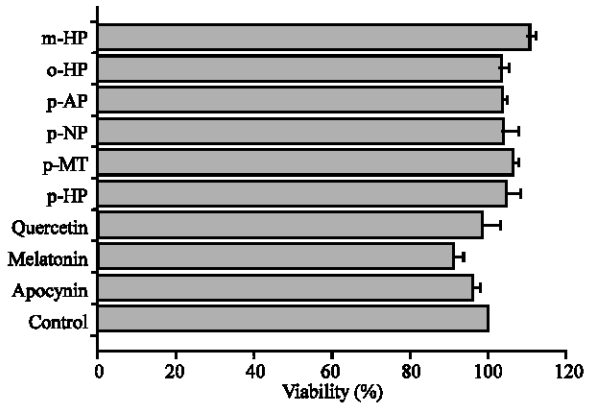


Fig. 1: Evaluation of the cytotoxic effect of substituted phenols, melatonin, quercetin and apocynin on macrophage. Adherent cells were incubated for 24h in the presence or absence (positive control) of 0.05 mM *p*-Nitrophenol (p-NP), *p*-Acetylphenol (p-AP), *p*-Methoxyphenol (p-MP), *p*-Hydroxyphenol (p-HP), *o*-Hydroxyphenol (o-HP), *m*-Hydroxyphenol (m-HP), quercetin, apocynin and melatonin. Cell in the culture medium (control) correspond to 100%. The cell viability was determined by the MTT method (Materials and methods). The results are mean and SEM of 4 replicates

100 μg mL^{-1} of streptomycin, 2 mM of *L*-glutamine and 5×10^{-2} M 2-mercaptoethanol; this mixture was named complete RPMI-1640 (RPMI 1640-C). Then, samples of 100 μL of peritoneal cells suspension (5×10^6 cell mL^{-1}) in RMPI-1640-C medium were added to each well of a 96-well tissue culture plate and 100 μL of different concentrations of tested samples. Cells were incubated for 24 h at 37°C in a humidified atmosphere containing 7.5% of CO_2. After incubation, the medium was poured off and macrophages were incubated with 100 μL of solution of MTT (5 mg mL^{-1}) in each well. The plates were incubated for 3 h at 37°C and 7.5% CO_2. The formazan formed was dissolved with 100 μL de isopropanol and the optical density was measured using a microplate reader (Multiskan Ascent, Labsystems Research Tech. Div, Helsinki, Finland) equipped with a 540 nm filter and 620 nm reference filter. The optical density of dissolved formazan in the control (untreated cells) was taken as 100% of viability (Mossman, 1983). The phenolic compounds and antioxidants were not cytotoxic in the concentrations used in the NO assay (Fig. 1).

Nitric oxide production: The NO was determined by measuring Nitrite (NO_2^-), a stable degradation product of nitric oxide, in the culture supernatants (Green *et al*., 1982). PEC (adherent cells) at 5×10^6 cells mL^{-1} was

incubated with various concentrations of the tested samples in the presence of LPS (1 mg mL^{-1}) for 24 h at 37°C in a 7.5% CO_2 atmosphere. Cell-free supernatant (50 μL) was mixed with 50 μL of Griess reagent (sulfanilamide 0.1%, phosphoric acid 3%, naphthylethylenediamine 0.1%) and incubated at room temperature for 10 min. Cells incubated with LPS were used as a positive control. After incubation, the absorbance of the wells was determined by using a microplate reader (Multiskan, Ascent) equipped with a 540 nm filter. Nitrite concentration was determined using dilutions of sodium nitrite in culture medium as standards.

Statistical analysis: All values are given as the mean± S.E.M. Statistical differences among values were calculated Kruskal-Wallis One Way Analysis of Variance on Ranks followed by multiple comparisons versus Control Group (Dunn's Method). Differences were considered statistically significant if the p-value was less than 0.05.

RESULTS AND DISCUSSION

It is well established the role of polyphenols as effective inhibitor of the generation of NO by stimulated macrophages. Indeed, among the natural antioxidant substances, call attention compounds as resveratrol, quercetin, rutin, curcumin and their derivatives for which there are innumerous reports regarding their inhibitory properties (Ma *et al*., 2005; De Stefano *et al*., 2007; Kim *et al*., 2007; Gafner *et al*., 2004). The majority of these effects can be explained or linked to the inhibitory effect that antioxidants develop upon the activation of the Nuclear Factor-kappa B (NF-κB) (Cho *et al*., 2003; Comalada *et al*., 2006). This transcription factor is sensitive to the redox status of the cells, hence, irrespectively to its molecular structure; any antioxidant will be able to, in lower or higher degree, to reduce the NF-κB activation and consequently the expression of iNOS, pro-inflammatory cytokines, etc (Paterson *et al*., 2003; Manna *et al*., 2007; Ma *et al*., 2005; De Stefano *et al*., 2007; Kim *et al*., 2007; Gafner *et al*., 2004).

The antioxidant potency of a molecule is related to its reduction potential, an electrochemical property directly influenced by the presence of hydroxyl substituents in the aromatic moiety. Indeed, it is quite common to see reports where the relative potency of polyphenols acting as antioxidant and/or anti-inflammatory substances is decreased when hydroxylations at specific positions are not present or are substituted by alkyl or aryl groups (Comalada *et al*., 2006; Wang *et al*., 2006; Cho *et al*., 2002). Since, these substitutions in the aromatic ring have direct effect on the redox potential of these substances, we decided to compare ordinary *p*-substituted phenols as inhibitors of the production of NO by activated macrophage.

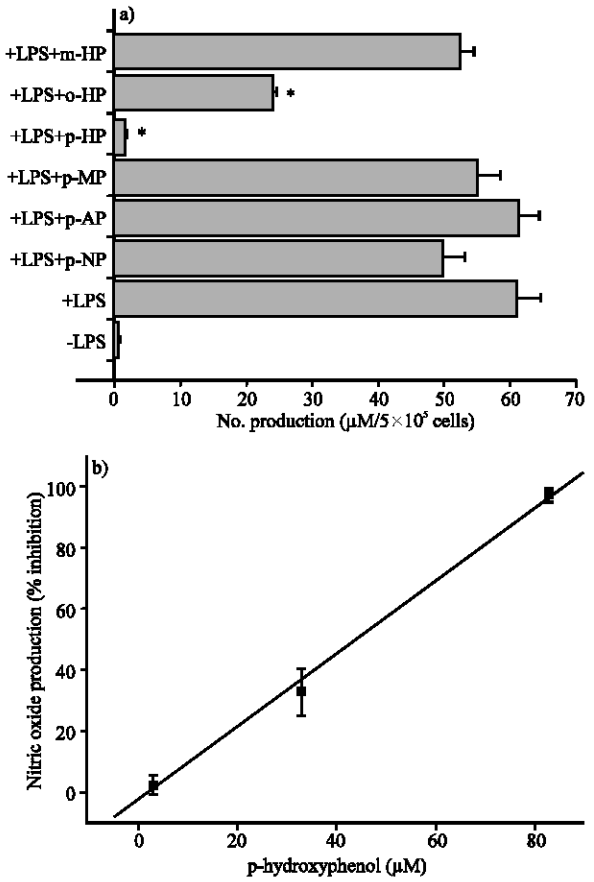
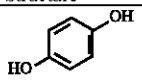
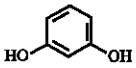
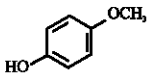
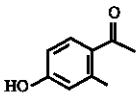
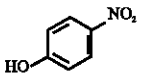
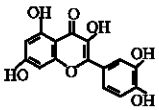
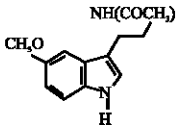
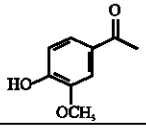


Fig. 2: (A) Effect of substituted phenols on NO production by LPS-stimulated macrophages. Adherent cells w e re incubated for 24 h with 1 mg mL^{-1} LPS in the presence or absence (positive control) of 83 μM substituted phenols. The cell-free supernatants were submitted to NO analysis (materials and methods). The results are mean and SEM of at least five experiments. Substituted phenols were: *p*-Nitrophenol (p-NP), *p*-Acetylphenol (p-AP), *p*-Methoxyphenol (o-MP), *p*-Hydroxyphenol (p-HP), o-Hydroxyphenol (o-HP) and *m*-Hydroxyphenol (m-HP). *p<0,05 by Kruskal-Wallis One Way Analysis of Variance on Ranks followed by multiple comparisons versus Control Group (Dunn's Method). (B) Dose-response curve for *p*-hydroxyphenol

Table 1: Structure and physicochemical parameters for the phenols and antioxidants

	E° (V)#	σ_p^*	Structure
p-hydroxyphenol (hydroquinone)	0.46	-0.38	
o-hydroxyphenol (catechol)	0.53	--	
m-hydroxyphenol (resorcinol)	0.81	--	
p-methoxyphenol	0.73	-0.28	
p-acetylphenol	1.06	0.47	
p-nitrophenol	1.23	0.81	
Quercetin	0.33	--	
Melatonin	0.95	--	
Apocynin	0.67	--	

Single-electron reduction potential at pH 7.0 (refs), *Hammett sigma constant for substituents at para position (ref)

The phenols studied here are structurally very similar (Table 1), the only and important difference is the oxidizability, which is influenced by the electronic effect of the substituent group. In this study, the compounds bearing high electron-withdrawing groups as 4-nitrophenol and 4-acetylphenol are representative of phenols with high reduction potential; on the other hand, hydroquinone and catechol, due to its electron-donating groups, are representatives of low reduction potential compounds. Table 1 shows the reduction potential and the Hammett sigma constant for substituents at *para* position (σ_p) for the phenols studied. The Hammett sigma constant is the physicochemical parameters for measuring the electronic effect of the substituents groups in the aromatic ring. A positive value of σ_p indicates an electron-withdrawing group and a negative value an electron-donating group. The presence of electron-withdrawing groups causes an increase in the reduction potential and on the other hand, electron-releasing groups increase the oxidizability by decreasing the reduction potential.

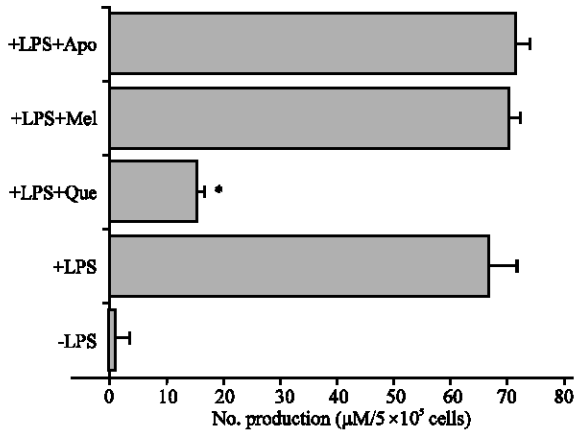


Fig. 3: Effect of antioxidants on NO production by LPS-stimulated macrophages. Adherent cells were incubated for 24 h with 1 mg mL^{-1} LPS in the presence or absence (positive control) of 33 μM substituted phenols. The cell-free supernatants were submitted to NO analysis (materials and methods). The results are mean and SEM of at least 5 experiments. Antioxidants were: Quercetin (Que), Melatonin (Mel), Apocynin (Apo). *p<0.05 by Kruskal-Wallis One Way Analysis of Variance on Ranks followed by multiple comparisons versus Control Group (Dunn's Method)

The results depicted in Fig. 2 show clearly that the oxidizability of the phenols derivatives are linked to the inhibition of NO production. Indeed, the best inhibitors were hydroquinone and catechol, which have reduction potential of 0.46 and 0.53 V, respectively.

To reinforce this point of view, we compared the effect of quercetin (E° = 0.33 V), apocynin (E° = 0.66 V) and melatonin (E° = 0.95 V). These substances were chosen as they are among the most studied molecules concerning their immunomodulatory, antioxidants and anti-inflammatory properties. Again it was able to observe that quercetin; the substance with lower reduction potential was the best inhibitor (Fig. 3).

CONCLUSION

In conclusion, our results show that, irrespective of its chemical structure, the redox potential of a molecule might be the main factor regarding its effect as an inhibitor of the production of NO by activated macrophages.

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