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Profile of *Maytenus aquifolium* action over free radicals and reactive oxygen species

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Reactive oxygen species (ROS) and free radical species have been implicated in initiating, accompanying or causing many diseases in living organisms; there is thus, a continual need for antioxidants molecules to inactivate ROS/free radicals. Many studies of plants crude extracts have demonstrated free-radical scavenging and antioxidant action. Maytenus *species have long been used, in several countries, as traditional medicines against gastric ulcers, dyspepsia and others gastric problems and for their anti-inflammatory properties. In this study,* Maytenus aquifolium *(Celastraceae) root bark ethanol extract was assessed for its ability to scavenge free radicals and reactive oxygen species. The results were expressed as percentage inhibition of the active species. The extract was efficient against studied reactive species: DPPH radical (obtained inhibition = 35.5 ± 1.3 %), $ABTS^{\bullet+}$ (IC_{50} = 0.0036 ± 0.0003 mg/mL), HOCl (IC_{50} = 0.002 ± 0.0001 mg/mL), $O_2^{\bullet-}$ (obtained inhibition = 36.0 ± 2.1 %), and $NO^{\bullet}$ (obtained inhibition = 18.3 ± 0.4 %).*

Uniterms
- Oxidant species
- Free radicals
- *Maytenus aquifolium*
- Oxidative damage

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INTRODUCTION

Free radicals are species that contain unpaired electrons. The oxygen radicals, such as superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and non-free radical species that participate on oxidative processes, such as H_2O_2 and singlet oxygen ($^1O_2$), are various forms of Reactive Oxygen Species (ROS). They are generated in many physiological or pathological REDOX processes. Usually, there are different systems that trap and destroy these species, such as the enzymes superoxide dismutase, catalase and glutathione peroxidase. Over-production of free radicals associated to low degrees of A, C and E Vitamins and a reduced level of the above mentioned enzymes is considered to be the main contributor to oxidative stress (Halliwell, 1995; Banerjee, Dasgupta, De, 2005).

Superoxide, produced on Oxidative Burst by the enzyme complex NADPH oxidase, is converted by the

enzyme Superoxide dismutase (SOD) in Hydrogen peroxide (H_2O_2). Despite the fact that H_2O_2 is not a free radical, it is an important and lesive O_2 metabolite since it leaves to Hydroxil Radical ($OH^{\bullet}$) production (Ferreira, Matsubara, 1997). The $^{\bullet}OH$ oxidizes sulfidryl groups of proteins and promtes DNA mutations by changing purines and pirimidines (Ferreira, Matsubara, 1997). It can initiate too a lipoperoxidation process on cellular membranes what causes cellular death (Halliwell, Gutteridge, 1986). The enzyme Myeloperoxidase (MPO; donor hydrogen peroxide oxidorreductase, EC 1.11.1.7) uses hydrogen peroxide and chloride ions as substrates producing hypochlorous acid (HOCl), an important component on bacterial killing, but an extremely strong oxidant that attack important biomolecules such as amines, amides, thiols, aminoacids and nucleotides (Arnhold, 2004; Auchére, Capellère-Blandin, 1999; Eaton, 1993; Halliwell, Gutteridge, 1989; Weiss, 1989), and originates some other oxidant species (Mutze *et al*., 2003; Lapenna, Cuccurullo, 1996).

In order to fight reactive species, there are important molecules that can delay or inhibit an oxidative process (e.g. lipoperoxidation) when in low concentrations, being defined as antioxidants (Atoui *et al*., 2005; Chun *et al*., 2005). Different dietary antioxidants play important roles in delaying the development of chronic diseases, such as cardiovascular diseases, cancer, inflammatory reactions, and Alzheimer's. Phenolic antioxidants from plants secondary metabolism are good sources of natural antioxidants agents (Chun *et al*., 2005). Natural antioxidants from plant extracts have attracted increasing interest due to consumer concern about the safety of synthetic antioxidants in foods. So, the search for natural antioxidant sources among plants turns necessary.

The use of plants with pharmaceutical properties has received increased interest nowadays from both homeopathic and allopathic branches. Besides, these medicinal plants play an important role in public health, especially in developing countries. Celastraceae family comprises 55 different genera with 850 species spread throughout the tropics and sub-tropics. The *Maytenus* genus is one of the greatest in this family: i) 77 species have been found in Brazilian flora (Mossi *et al*., 2004), and ii) different compounds have been isolated, such as flavonoids, cathechins, condensed tannins, quinonemethide triterpenes, triterpenes and sesquiterpene pyridine alkaloids (Vellosa *et al*., 2006).

Numerous compounds isolated from *Maytenus ilicifolia* have been tested for their antitumoral activity, however, the infusion of the leaves from *M. ilicifolia* have been used in Brazil by the folk medicine for their antiacid and antiulcerogenic effects (Queiroga *et al*., 2000). Souza-Formigoni *et al*. (1991) proved the protective action of the tea prepared by pouring boiling water on fresh or dried leaves ("abafado") of *Maytenus ilicifolia* and *Maytenus aquifolium* against the experimental development of ulcer in rats, both orally (p.o.) and intraperitoneally (i.p.). The presence of phenolic metabolites could justify the usage of some species of *Maytenus* as anti-inflamatory and antiulcerogenic agents (Jorge *et al*., 2004).

Extracts of fruits, vegetables, cereals, and their by-products have showed effective antioxidant activities in different models system (Sun, Ho, 2005). As soon as different plants used as food have been already characterized as antioxidant sources, and may have a possible application on cancer treatment and *Maytenus* genus plants have a popular and diffused use in Brazil and others countries against gastric ulcers, dyspepsia and others gastric problems, we decide to establish its profile against reactive species (free radical or not).

MATERIALS AND METHODS

Experimental apparatus and analytical condition

This study was undertaken in order to assess the antioxidant and antiradical activity *M. aquifolium* root bark crude ethanolic extract (MaEtOH) by determining its capacity to scavenge free radicals and some reactive oxygen species. All assays were done with a HP 8453 Diode Array Spectrophotometer. Various doses of plant extract were assayed and their scavenger capacities against oxidant species were calculated as mean values of triplicates assays and expressed as percentage of radical or ROS scavenged (% inhibition) calculated for HOCl assay by Eq.1, and by Eq.2 for DPPH, $ABTS^{\bullet+}$, $NO^{\bullet}$ assays.

$$\text{Inhibition (\%)} = \left(1 - \left(\frac{A_0 - A_T}{A_0 - A_1}\right)\right) \times 100 \qquad \textbf{Eq.1}$$

where $\mathbf{A_0}$ is test absorbance at 412 nm without HOCl or sample, $\mathbf{A_1}$ is test absorbance at 412 nm with HOCl, but no sample and $\mathbf{A_T}$ is test absorbance at 412 nm with HOCl and sample.

$$\text{Inhibition (\%)} = \left(1 - \left(\frac{A_{\text{sample}}}{A}\right)\right) \times 100 \qquad \textbf{Eq.2}$$

where **A** is test absorbance without sample and $\mathbf{A_{sample}}$ is test absorbance with sample.

Chemicals

ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)], DTNB [5,5'-dithiobis(2-nitrobenzoic acid)], phenazine methosulfate (PMS), NADH, NBT (nitrobluetetrazolium), and DPPH (2,2-diphenyl-1-picrylhydrazyl) were purchased from Sigma Chemicals Co. Griess Reagent was kindly provided by Professor Iracilda Zeppone Carlos (Clinical Immunology Lab. of the School of Pharmaceutical Sciences – UNESP at Araraquara, SP, Brazil). All other reagents were analytical grade and commercially available.

DPPH radical scavenging activity

DPPH is a free radical that, when dissolved in ethanol, has purple color. Loss of this color indicates radical scavenging activity. Ethanol solution of *M. aquifolium* extract at various concentrations was evaluated against 60 μM DPPH. The reaction mixture (total volume 1.0 mL) was shaken vigorously and allowed to react at room temperature. After 15 min, remaining DPPH was determined colorimetrically at 531 nm, using absolute ethanol as a blank (Soares *et al*., 1997).

$ABTS^{\bullet+}$ radical scavenging activity

$ABTS^{\bullet+}$ was prepared by reacting 5 mL of 7 mM ABTS aqueous solution with 88 μL of 140 mM potassium persulphate (molar ratio 1:0.35) and the mixture allowed to stand in the dark at room temperature for 12–16 h before use (Pellegrini *et al*., 1999). Prior to assay this $ABTS^{\bullet+}$ stock solution was diluted with KH_2PO_4/K_2HPO_4 (100 mM, pH 7.0, diluted 1:10 before use) buffer solution (ratio 1:88) to give an absorbance at 734 nm of 0.414±0.013 (n=40). One milliliter $ABTS^{\bullet+}$ was then added to glass test tubes containing various concentrations of each extract and mixed for 15 sec. Tubes were incubated for 30 min and then read at 734 nm.

HOCl scavenging activity

TNB (5-thio-2-nitrobenzoic acid) was produced from DTNB, as described by Ching and co-workers (1994). TNB (80 μM) is oxidized to DTNB by HOCl (22 μM) causing the absorbance at 412 nm to fall while DTNB absorbance (325 nm) appeared. Samples were incubated with HOCl for 5 min in KH_2PO_4/K_2HPO_4 (50 mM, pH 7.0) buffered solution. TNB was then added, following 15 min incubation on 25 °C (Ching *et al*., 1994).

Superoxide radical scavenging activity

Superoxide radicals, produced by NADH and PMS, reduce NBT and produce a formazan compound. The intensity of color is inversely proportional to the antioxidant concentration (Kakkar *et al*., 1984). The assay was carried out in sodium pyrophosphate buffer (0.025 M, pH 8.3) and the mixture contained 25 μL of 372 μM PMS, 75 μL of 600 μM NBT, 50 μL of 1560 μM NADH, plant extract (several volumes) and buffer to complete 1 mL final volume. Reactions were started by adding NADH. After incubation at 25 °C for 90 sec, 100 μL of glacial acetic acid and 900 μL of sodium pyrophosphate buffer were added. After vigorous homogenization, the color intensity of mixture was measured at 560 nm.

Nitric oxide radical scavenging activity

For this assay, several concentrations of MaEtOH were assayed in the test tubes with sodium nitroprusside solution (25 mM) in a final volume of 1 mL, and the tubes were incubated at 25 °C for 1.5 h. An aliquot (0.25 mL) of the solution was then withdrawn and diluted with 0.15 mL Griess Reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore, produced during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride, was immediately read at 570 nm. Sodium nitroprusside is known to decompose in aqueous solution at physiological pH, producing $NO^{\bullet}$. Under aerobic conditions, $NO^{\bullet}$ reacts with oxygen to produce the stable products nitrate and nitrite, and the nitrite can be determined with Griess reagent. The final absorbance at 570 nm is diminished by an $NO^{\bullet}$ scavenger, because less nitrite is produced to form the chromophore (Yen *et al*., 2001).

RESULTS AND DISCUSSION

The presence of previously isolated phenolic metabolites such as flavonoids, cathechins, condensed tannins, quinonemethide triterpenes, triterpenes and sesquiterpene pyridine alkaloids justify the observed effects of that are presented below.

DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule; because of its odd electron, the ethanolic solution shows a strong band at 531nm. As DPPH reacts with suitable reducing agents, this electron becomes paired off and the solution loses colour stoichiometrically with the number of electrons taken up. Such reactivity has

been widely used to test either the ability of several compounds to act as free radical scavengers or the antioxidant activity of plant extracts (Soares *et al*., 1997). DPPH reduction was evaluated by decolorization of the radical solution (absorbance at 531nm) in the plant extract presence. Figure 1 shows the results of DPPH scavenging dose-response from MaEtOH which did not promote 50% inhibition at assayed concentrations (obtained inhibition = 35.5 ± 1.3 %).

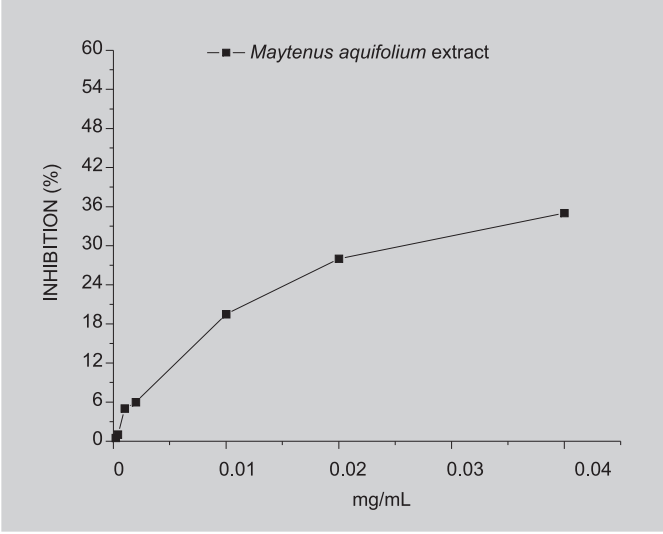

FIGURE 1 - Anti-radical property of *M. aquifolium* crude extract evaluated by DPPH method.

The $ABTS^{\bullet+}$ assay has been used to screen the relative radical-scavenging capacities of flavonoids and phenolics, which act as electron- or H-donating agent or plant extracts that contains these substances (Pellegrini *et al*., 1999; Vellosa *et al*., 2006; Oliveira *et al*., 2007). The green cation radical $ABTS^{\bullet+}$ has absorbance peaks at 630, 734 and 812 nm. On interaction with antioxidants the radical is reduced, suppressing the absorbance of the green radical cation in a dose-dependent way. From the data in Figure 2 MaEtOH has an IC_{50} value equal to 0.0036 ± 0.0003 mg/mL, wich is larger than those previously obtained to *M. ilicifolia* - 0.0020 mg/mL, Trolox - 0.0007 mg/mL and uric acid - 0.0012 mg/mL (Vellosa *et al*., 2006).

In biological systems, hypochlorous acid is the most toxic and abundant oxidant agent produced by PMN (Lapenna, Cuccurollo, 1996; Vellosa *et al*., 2007). It can attack important biological molecules and generate other harmful ROS (Weiss, 1989; Eaton, 1993). This oxidant specie reacts with ammonia produced by *Helicobacter pylori* in human stomach to generate the microbicide monochloramine, which is also related to stomach injury observed in gastric ulcers (Lapenna *et al*., 1996). Hence, it

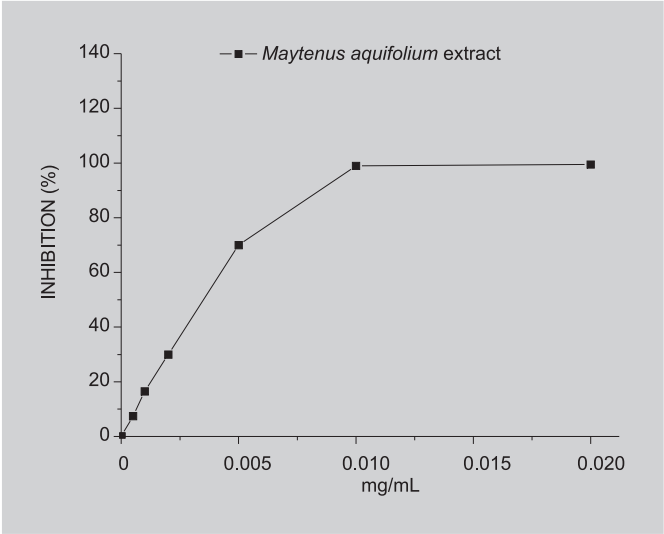

FIGURE 2 - Anti-radical property of *M. aquifolium* crude extract evaluated by $ABTS^{\bullet+}$ method.

is important to discover drugs and plant extracts that are able to fight HOCl. Here, we evaluated the potential of MaEtOH to scavenge HOCl. From the results in Figure 3, the IC_{50} value can be obtained and characterize the extract as HOCl scavenger (IC_{50} = 0.002 ± 0.0001 mg/mL). Although this value is bigger than the uric acid one - 0.00032 mg/mL (Vellosa *et al*., 2006), it is smaller than Trolox – 0.0031 and equal to *M. ilicifolia* – 0,0019, previously determined (Vellosa *et al*., 2006). This low IC_{50} is very encouraging, because *M. aquifolium* is currently used in Brazilian folk medicine against gastric ulcers and any gastric protection obtained may be explained, at least partly, by scavenging HOCl.

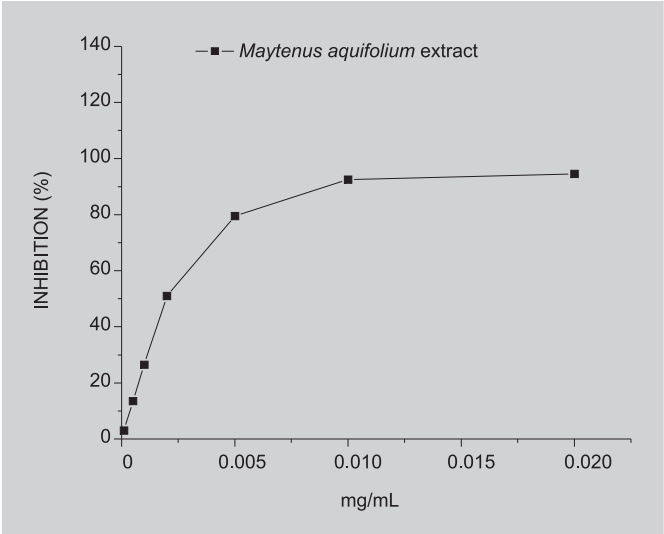

FIGURE 3 - HOCl scavenger action of *M. aquifolium* crude extract evaluated by TNB method.

We have also evaluated the superoxide anion scavengering potential of MaEtOH by a non-enzymatic superoxide generation method. The radical is generated by reacting phenazine metasulphate with NADH, and revealed by NBT reduction. We used it to evaluate if the sample tested (Figure 4) is able to antagonize superoxide anion *in vitro*. It is important to notice that in this method, NBT must be in excess for evaluating the real potential from samples in scavenge superoxide anion. The superoxide scavenger potential found for MaEtOH $O_2^{\bullet-}$ (obtained inhibition = 36.0 ± 2.1%) may be expected to contribute to a possible protective action in different tissues, including stomach.

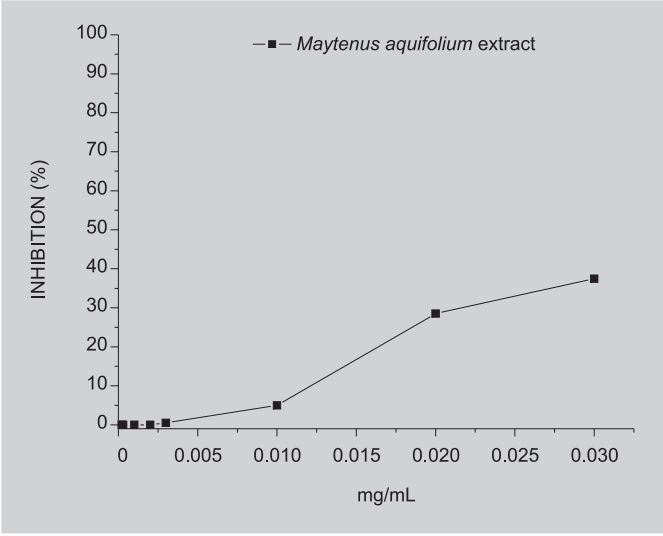

FIGURE 4 - Superoxide radical scavenger action of *M. aquifolium* crude extract evaluated by the superoxide anion non-enzymatic method.

Recent studies have shown that reactive nitrogen intermediates, such as nitric oxide ($NO^{\bullet}$), peroxynitrite ($ONOO^{\bullet-}$) and nitrogen dioxide (NO_2), also play an important part in the inflammatory process and possibly in carcinogenesis (Yen *et al*., 2001). We constructed an analytical curve with sodium nitrite to calculate the nitric oxide level in the sodium nitroprusside method described above. In this method, using sodium nitroprusside as a source of nitric oxide, control tubes (without scavengers) had absorbance values of 0.428±0.02 (n=40), representing about 21 mM $NO^{\bullet}$. We evaluated MaEtOH as a potential source of nitric oxide scavengers due to its inhibitory effect on Griess Reagent oxidation by nitric oxide (obtained inhibition = 18.3 ± 0.4 %; Figure 5).

CONCLUSION

There is evidence concerning the participation of

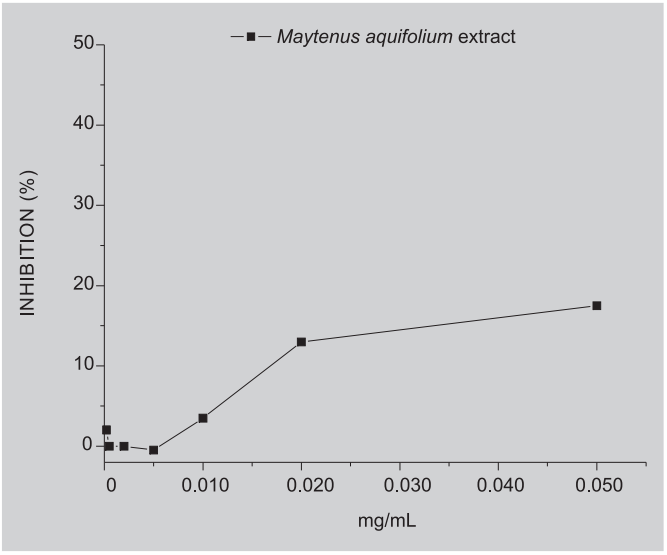

FIGURE 5 - Nitric oxide scavenger action of *M. aquifolium* crude extract evaluated by sodium nitroprusside and Griess Reagent Method.

reactive oxygen species in the etiology and physiopathology of human diseases, such as neurodegenerative disorders, inflammation or digestive system disorders like gastric ulcers. The role of these ROS in several diseases and the potential antioxidant of protective effect of natural compounds on affected tissues are topics of highest current interest. To consider a natural product or a drug as an antioxidant substance it is necessary to investigate its properties *in vitro* and then to evaluate its functions in biological systems.

We demonstrated that *M. aquifolium* ethanolic extract was able to scavenge different species by studying its action over hypochlorous acid, superoxide anion and nitric oxide, which are able to be generated in living organisms, and DPPH and $ABTS^{\bullet+}$, models used in this kind of study. We conclude that MaEtOH is efficient against different oxidant species assayed at different levels. The results present here dose dependent action profile of *M. aquifolium* over different reactive species, radicalar or non-radicalar, and provide useful information to justify further biological studies that must clarify this plant therapeutic potential on oxidative stress involved on pathogenesis of different diseases.

So, the results provide useful pharmacological information related to free radicals and oxidants species. It is possible that the usage of this plant against different pathologies could prevent or fight tissue damage when are established an oxidative stress. Of course further studies are necessary, including: **i)** isolation and characterization of molecules from this plant; **ii)** *in vivo* studies of biological

properties that confirm the possibility here expressed, and **iii)** toxicological studies to evaluate the safety of this plant as medicinal agent.

RESUMO

Perfil de ação da *Maytenus aquifolium* sobre radicais livres e espécies reativas do oxigênio

Espécies Reativas do Oxigênio (ERO) e radicais livres têm tido implicações na iniciação e evolução de muitas doenças ou nas causas das mesmas em organismos vivos; há portanto, necessidade contínua por moléculas antioxidantes para inativar ERO/radicais livres. Estudos sobre extratos brutos de plantas têm demonstrado suas ações antioxidante e seqüestradora de radicais livres. Espécies do gênero Maytenus *são utilizadas, em vários países, como medicamentos tradicionais no combate a úlceras gástricas, dispepsia e outras desordens gástricas, bem como por suas propriedades antiinflamatórias. Neste estudo, o extrato bruto etanólico da raiz da* Maytenus aquifolium *(Celastraceae) foi avaliado quanto à sua habilidade em seqüestrar radicais livres e outras espécies reativas do oxigênio. Os resultados são expressos como porcentagem de inibição das espécies ativas. O extrato foi eficiente contra as espécies estudadas: radical DPPH (inibição alcançada = 35,5 ± 1,3 %), $ABTS^{\bullet+}$ (IC_{50} = 0,0036 ± 0,0003 mg/mL), HOCl (IC_{50} = 0,002 ± 0,0001 mg/mL), $O_2^{\bullet-}$ (inibição alcançada = 36,0 ± 2,1 %), and $NO^{\bullet}$ (inibição alcançada = 18,3 ± 0,4 %).*

UNITERMOS: Espécies oxidantes. Radicais livres. Maytenus aquifolium. *Dano oxidativo.*

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