



Potential of Total Polyphenols from Barbatimão against Different Reactive Oxygen/Nitrogen Species

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Received 13th April 2015; Accepted 30th April 2015

ABSTRACT

The objective of this study was to obtain the polyphenol fraction from *Stryphnodendron adstringens* (Mart.) Coville (Barbatimão) and evaluate their potential effect against different reactive oxygen/nitrogen species. First was studied the extraction parameters (solvents: water, n-hexane, dichloromethane, anhydrous ethanol and ethanol/water solutions; pH: 3.0, 7.0 and 10.0 and temperature: 4.0, 25.0 and 80.0°C). Total polyphenols (TPs) were quantified by Folin-Ciocalteu method while that tannins/proanthocyanidins were determined by Vanillin-HCl method. Barbatimão presented high percentage of TPs (22±1.1%) in stem bark with a major proportion of proanthocyanidins (101±9%). The polyphenolic fraction exercised significant effects in scavenging nitric oxide, peroxy radical (R-COO), superoxide and hydrogen peroxide (H₂O₂). In addition, polyphenolic extracts could prevent the peroxy radical-induced cell damage in ex-vivo cultured liver slices and inhibited protein cysteine-SH oxidation. The results show that Barbatimão may be a rich source of polyphenols, mainly tannins, which are efficiently extracted with low toxicity solvents, neutral pH and room temperatures, having a promising use in food preservation and human health.

Key words: *Stryphnodendron adstringens*, Polyphenols, Extraction, Antioxidants, Barbatimão.

1. INTRODUCTION

Brazil possesses the richest plant biome on the planet, with 55.000 higher plant species distributed in five main biomes: Mata Atlântica, Cerrado, Amazônia, Pantanal and Pampa [1,2]. However, when it comes to market of natural products, the Brazil accounts for only 10% of world total despite the extensive existing natural park [3].

Several researches show that ingestion of natural extracts containing antioxidants in their composition, especially polyphenolic compounds, are associated with lower incidence of coronary heart disease, cancer and diabetes [4]. This important group of compounds contributes to cellular processes by protecting against lipids and protein oxidation [5,6] and the reason may be due to ability to capture and react with free radicals. Additionally, the polyphenols are the most abundant secondary metabolites found in plants and include others classes of compounds such as phenolic acids, anthocyanins, colorful, simple and complex flavonoids [7,8].

In Brazil some plants may be considered rich sources of polyphenols as açai (*Euterpe oleraceae* Mart.), graviola (*Annona muricata* L.), Brazil nut (*Bertholletia excelsa* H.B.K.), cashew (*Anacardium occidentale* L.), and feijoa (*Feijoa sellowiana* Berg.). Barbatimão (*Stryphnodendron adstringens* Mart. Coville), here called of SA, is found principally in savannah regions and the decoctions/infusions of this crude plant is traditionally used to treatment of leucorrhea, diarrhea, anti-inflammatory and antiseptic [9-11]. The extracts of this plant may be obtained from maceration in solvent, however, the knowledge of the extraction behavior is important to select the best conditions in order to maximize the yield and the polyphenolics content of SA extracts. To the best of our knowledge, were found few works in scientific literature about the investigation of antioxidant potential of SA in biological systems, and in this sense, the goal of this work was to determine the parameters that promoted a higher polyphenol extraction from SA, evaluating posteriorly their antioxidant potential and specific free-radical scavenging activity against different reactive species of oxygen/nitrogen.

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2. MATERIALS AND METHODS

2.1. Chemicals

Anhydrous ethanol, dichloromethane, n-hexane and methanol, both purchased from Merck (Germany) with purity higher than 99.0%, were the organic solvents used in extraction step. Folin–Ciocalteu reagent, sodium bicarbonate, epinephrine, purified bovin liver catalase, hydrogen peroxide, Griess reagent, ellagic acid, bovine serum albumin (BSA), (+) – catechin, 5,5-dithiobis 2-nitrobenzoic acid (DTNB), luminol and 2,2'-Azobis (2-methylpropionamide) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). CuZn-superoxide dismutase-2 from bovine erythrocytes was purchased from Roche (USA).

2.2. Plant Material

The stem bark of SA (Mart.) was collected in Caetit /BA/Brazil and identified. SA stem bark are showed in Figure 1a and a voucher specimen was deposited (number RSPF 12495) in herbarium of Universidade de Passo Fundo/RS/Brazil. The samples were dried in air-circulation oven at $30^{\circ}\text{C}\pm 0.5^{\circ}\text{C}$ for 24 h, ground in a cyclone mill (Marconi, MA-020) and separated on 20 mesh sieves. After this procedure all content obtained was stored in glass containers under controlled temperature until further use. The bromatological characterization of the SA stem barks was evaluated according to methods described by Mac do (2005) [12].

2.3. Extraction Procedure

The polyphenolic fraction was extracted individually with distilled water, anhydrous ethanol, ethanol/water solution (75:25 and 50:50 (v/v)), n-hexane and dichloromethane. Ethanol was selected as organic polar

solvent based on fact that, when mixed in water, may improves the solubility of the bioactive component. In addition, ethanol presents as a safe biological solvent unlike others organic solvents conventionally used in extraction step [8]. N-hexane and dichloromethane were used to evaluate the extraction behavior of polyphenolic fraction with non-polar organic solvent. The best extraction concentration was investigated in a range of 0.005-0.5 g/mL (plant/solvent [w/v]), temperature of 4.0, 25.0 and $80.0^{\circ}\text{C}\pm 1^{\circ}\text{C}$ and pH (3.0, 7.0 and 10.0). The results were expressed as percentage of total polyphenols (TPs). To the extraction process, the systems containing SA + solvents were macerated manually for 10 min and the resulting solution was kept in the dark for 1 h. All material was rapidly filtrated. An aliquot of 50-100 μL from this solution was used to analyze the polyphenols content and the remaining solution was lyophilized to study *in vitro* and *ex-vivo* behaviors.

2.4. Determination of TP Content

TPs were determined by Folin–Ciocalteu procedure [13] with modifications. Briefly, aliquots of 50-100 μL (from extracts) were transferred into test tubes and the volume completed to 5 ml with distilled water. Was added 0.20 ml Folin–Ciocalteu reagent, 0.5 ml saturated aqueous sodium carbonate solution and then, the tubes were vortexed and absorbance measured after 20 min at 765 nm. The TPs was calculated as ellagic acid equivalents (from the calibration curve with ellagic acid standard solution) and expressed as % polyphenols (g of ellagic acid equivalents/100 g SA stem bark). All measurements were performed in duplicate.

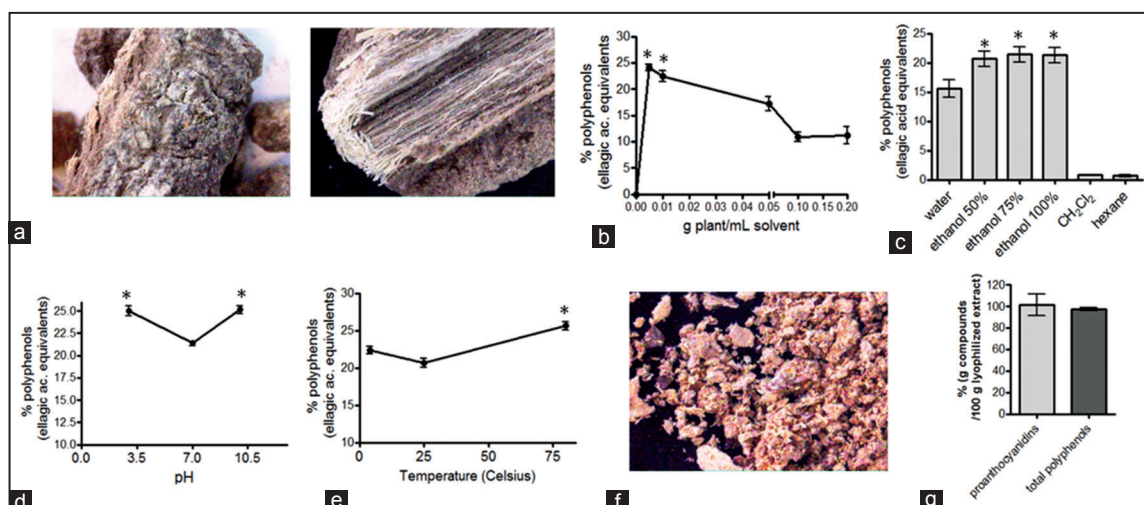


Figure 1: Polyphenols presents in *Stryphnodendron adstringens* (SA): (a) SA stem barks ($\times 6.5$ magnification), (b) effect of plant/solvent ratios (g/mL), (c) effect of solvents, (d) effect of pH on SA polyphenols extraction, (e) effect of temperature, (f) lyophilized SA polyphenolic extract ($\times 6.5$ magnification), (g) percentage (g compounds/100 g dry stem bark) of proanthocyanidin and total polyphenols. *Different from other groups of the same experiment; $p < 0.05$, ANOVA.

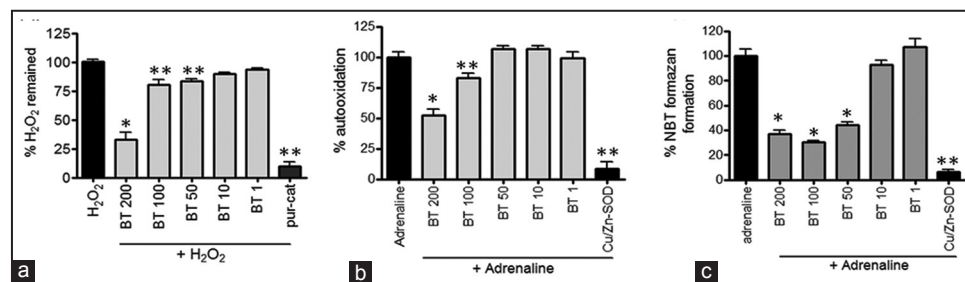


Figure 2: Scavenging activity of *Stryphnodendron adstringens* (SA) extracts against hydrogen peroxide and superoxide anion: (a) Remaining H₂O₂, (b) percentage of superoxide production, (c) percentage of superoxide-mediated nitroblue tetrazolium (NBT) reduction as assessed by NBT reduction assay. Legends: BT (Barbatimao polyphenolic extracts) at different concentrations ($\mu\text{g/mL}$). *Different from hydrogen peroxide alone (a) or adrenaline auto-oxidation alone (b); **different from hydrogen peroxide alone (a) or adrenaline auto-oxidation alone (b) and from BT 200; $p < 0.05$, ANOVA.

2.5. Vanillin-HCl Assay

Condensed tannins (proanthocyanidins/flavon-3-ol compounds) were determined using vanillin-HCl assay as previously described by Sun *et al.* (1998) [14]. Lyophilized extracts were redissolved in absolute methanol to a final concentration of 10 mg/mL and aliquots of 10, 30 and 100 μl were used for quantification. (+)-catechin (0-300 $\mu\text{g/mL}$ in methanol) was used as standard for calibration curve. To 1 ml of catechin solution or test solution (extracts) in a test tube, 2.5 ml of methanol (blank tube) or 1% vanillin solution in methanol (sample) was added. After, 2.5 ml of 8% HCl in methanol was added for blank and sample tubes giving a final volume of 6 ml. The mixture was incubated for 20 min at 30°C and the absorbance at 500 nm was measured. Proanthocyanidin content was calculated and expressed as percentage (g of proanthocyanidin/100 g lyophilized extract).

2.6. Determination of Superoxide Scavenging Activity (Adrenochrome and Nitroblue Tetrazolium [NBT] Assays)

2.6.1. Adrenochrome assay

The ability of SA to scavenge superoxide anion was measured as previously described by Bannister and Calabrese (1987) [15]. The extract at different concentrations was mixed with purified catalase (100 U/mL stock solution) in glycine buffer (50 mM, pH 10.2) to a final volume of 195 μl . Superoxide generation was initiated by addition of 5 μl of adrenaline 60 mM, and adrenochrome formation was monitored at 480 nm for 5 min at 32°C in a microplate reader. Data were expressed as percentage of superoxide production.

2.6.2. NBT assay

Superoxide scavenger activity was also measured by monitoring of superoxide-dependent reduction of NBT to the blue formazan at 560 nm in the presence of different extract concentrations [16]. Briefly, 0.1 mM of NBT and 1.25 mM adrenaline were mixed with

phosphate buffer saline (pH 7.4) in the presence or absence of the extracts, reaching a final volume of 200 μl . NBT reduction was monitored for 1 h/37°C and the rate of superoxide formation was expressed as percentage of NBT formazan formation.

2.7. Determination of Hydrogen Peroxide Scavenging Activity

To assess the *in vitro* reactivity of SA extracts against hydrogen peroxide, different extract concentrations were mixed with 0.02 M phosphate buffer (pH 7.4) to a final volume of 195 μl . After, 5 μl of H₂O₂ was added to microplate wells and incubated during 10 min. The remaining concentration of H₂O₂ was then determined using an extinction coefficient value (E 240 nm) of 43.6 M⁻¹ cm⁻¹ [17,18]. Data were expressed as %H₂O₂ remained compared to controls (H₂O₂ without extracts).

2.8. Peroxyl Radical Scavenging Activity (Total Reactive Antioxidant Potential [TRAP] and Thiobarbituric Acid Reactive Species [TBARS] Assay)

2.8.1. TRAP assay

In vitro scavenging activity of extracts against peroxyl radicals was estimated by the TRAP as previously described [19,20]. Briefly, the reaction mixture containing AAPH and luminol in 0.1 M glycine buffer (pH 8.6) was incubated at room temperature during 1 h. Thermal decomposition of water-soluble azobis produces peroxyl radicals (ROO•) at a known steady rate. Peroxyl radicals react with luminol, resulting in chemiluminescence (CL). The addition of extracts at different concentrations decreases the CL proportionally to its antioxidant potential. TRAP profile was obtained by measuring the CL emission in a liquid scintillation counter Wallac 1409. CL intensity was monitored for 30 min after addition of the extracts. The CL curve profiles were used to statistically compare the scavenging potential of extracts against peroxyl as compared to control. Data were expressed as % CL.

2.8.2. TBARS

In vitro TBARS assay was employed to indirectly quantify the peroxy radical scavenging activity of SA extracts [21]. Briefly, egg yolk was homogenized in 20 mM phosphate buffer; 1 ml of this solution was sonicated and then homogenized with 0.1 ml of SA extracts at different concentrations. Lipid peroxidation was induced by addition of 0.1 ml of AAPH solution. Positive control was performed by incubation of egg lipids with AAPH in the absence of extracts. Reactions were carried out for 60 min/37°C. Samples (0.3 ml) were centrifuged with 0.18 ml of trichloroacetic acid (50%) at 10,000 g/10 min. An aliquot of 0.1 ml from supernatant was mixed with 0.1 ml TBA in a microplate and heated at 95°C/45 min. After cooling, the absorbance of samples was measured using a microplate reader at 532 nm. Data were compared to controls (egg lipids in the absence of AAPH) and expressed as percentage.

2.9. Nitric Oxide (NO•) Scavenging Activity

NO• was generated from spontaneous decomposition of sodium nitroprusside (SNP) in 20 mM phosphate buffer (pH 7.4). Once generated, the NO• interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [22]. The reactional mixture containing 10 mM SNP in phosphate buffer and SA at different concentrations were incubated at 37°C/1 h. An aliquot of 0.5 ml was taken and homogenized with 0.5 ml Griess reagent. A volume of 0.2 ml of this homogenate was added to a microplate and the absorbance of chromophore was measured at 540 nm. Percentage of inhibition of NO•-derived nitrite formation was measured by comparing the absorbance values of positive controls (only 10 mM SNP and vehicle) and extract plus NPS co-incubations.

2.10. Protein Sulphydryl Oxidation Assay (DTNB Assay)

To analyze oxidative alterations in sulfhydryl protein groups, 500 µg of BSA dissolved in phosphate buffered saline (pH 7.4) was exposed to 2.5 mM of hydrogen peroxide or 2.5 mM AAPH in the presence or absence of the polyphenolic extracts. To measure the remaining levels of reduced protein thiol (-SH) in the samples, 40 µl of boric acid-ethylene-diamine-tetraacetic acid (EDTA) buffer (0.1 M boric acid, 0.2 mM EDTA, pH 8.5) was added followed by addition of 30 µl of 10 mM DTNB. Ethanol was added to produce the intense yellowish color between the sulfhydryl (cys-SH) groups and DTNB. After 20 min, cys-SH levels were determined by spectrophotometer at 412 nm [23]. Results are expressed as percentage compared to non-oxidized BSA.

2.11. Ex-vivo Evaluation of Oxidant-Induced Toxicity in Liver Slices

Antioxidant activity of SA extracts was also evaluated *ex vivo* by using AAPH as oxidant. Rat liver slices

were pre-incubated with different concentrations of polyphenol-enriched extracts at 37°C/30 min. After pre-incubation, 5 mM AAPH was added and liver slices were incubated for additional 1.5 h. The medium was removed and toxicity of the oxidant to liver cells was evaluated by measuring lactate dehydrogenase (LDH) activity into the incubation medium as estimative of losses in cell membrane integrity (LDH kit, Labtest). Data were compared to untreated liver slices and expressed as percentage.

2.12. Statistical Analysis

Results are expressed as the mean ± standard error of the mean (±SD); p values were considered significant when p<0.05. The differences between the experimental groups were analyzed by one-way ANOVA analysis followed by Tukey's *post-hoc* test.

3. RESULTS

3.1. Polyphenols Extraction from SA

Table 1 shows the previously published data for TPs. For example, using the Phenol-Explorer database, Pérez-Jiménez *et al.* (2010) [24] identified the 100 richest food sources of polyphenols, being the *Syzygium aromaticum* the major source of these compounds. In our assays, SA showed higher content of TPs (22±1.1%) compared to other species. Taking into consideration that SA has not been much studied and possesses a high content of polyphenolics, we decided to use it in the sequential experiments and the Table 2 presents the characterization of SA stem barks used in this work.

Figure 1 shows the stem barks used as raw material for polyphenols extraction (a and b), the effect of plant/solvent ratio (b), solvents (c), pH (d) and temperature (d) on polyphenols extraction. The results indicated that the best concentration was 0.005 g of plant/mL solvent. To higher ratios, the efficiency of polyphenols extraction was decreased, possibly due to solvent saturation. Regarding to the solvent effects, was observed that anhydrous ethanol and its hydroalcoholic solutions (50:50 and 75:25 (ethanol: water) were equally efficient for TPs extraction, proving the fact that non-polar solvents were are not able to extract polyphenolic compounds. In acidic and alkaline systems (pH=3.0 and 10.0, respectively) the efficiency of polyphenols extraction was slight higher when compared to neutral pH. Yet, extremes pH increased the polyphenols extraction in approximately 10-13% when compared to pH=7.0. An increase of about 23% in the content of extracted polyphenols was observed at high temperatures (80°C/1 h) in comparison with other temperatures. However, high temperatures can promote losses and decrease the antioxidant activity of polyphenols [25]. Based in these results, the extraction conditions that promoted a higher quantity of extracted polyphenols were: 25°C, 50:50 ethanol/water (v/v), pH=7.0 and extraction of 1h

in dark room. The extracts were lyophilized in this conditions and the resulting powder is also shown in Figure 1f. TPs content in the lyophilized extract was 98±3% (g of polyphenols/100 g of extract; Figure 1g). Quantification of condensed tannins showed that proanthocyanidins are the major constituents of the SA extract (101±9%, g catechin equivalents/100 g lyophilized extract).

3.2. Scavenging Activity of SA Extracts against Hydrogen Peroxide and Superoxide Anion

To access the possible antioxidant role of SA extracts, was evaluated the *in vitro* scavenging potential of extracts against different reactive species. Figure 2a shows a significant reactivity between SA extracts and the non-free radical oxidant hydrogen peroxide, which were observed from 50 µg/mL and being more pronounced at the concentration of 200 µg/mL. The scavenging activity of SA extracts against superoxide anion was determined by two different assays. In the first approach (adrenochrome assay) was tested the effect of the extracts in superoxide-mediated adrenaline autoxidation and the results showed that concentrations from 100 µg/mL were effective to scavenge superoxide (Figure 2b). The aforementioned

assay was performed at alkaline environment, suitable for rapid adrenaline autoxidation. In an alternative assay system, we incubated the extracts in phosphate buffered saline at pH=7.0 and adrenaline autoxidation-derived superoxide was incubated in the presence of NBT (Figure 2c). In the NBT/neutral pH system, superoxide generation by adrenaline auto-oxidation is not as rapid as in pH 10 and the incubation time was higher than in adrenaline/glycine system. The results showed that SA extracts may efficiently inhibit superoxide-mediated adrenaline autoxidation (from 100 µg/mL) and a more robust effect was detected in inhibiting superoxide-mediated NBT formazan blue formation (from 50 µg/mL). All experiments were performed in quadruplicate (n=4) and the results are expressed as the mean ± standard deviation.

3.3. SA Extracts Scavenge NO• and Peroxyl Radicals

To test the effect of SA extract in scavenging peroxyl radicals were used two different approaches. In the CL detection (TRAP assay) the peroxyl-mediated luminol CL was significantly inhibited by extracts from 1 µg/mL (Figure 3a). For TBARS assay, the

Table 1: TPs found in different plants.

Species	Folk name	Part of the plant	TP (%)	Reference
<i>Cuphea carthagenensis</i> (Jacq.)	Sete Sangrias	Total plant	5.9	-
<i>Hibiscus sabdariffa</i>	Hibiscus	Flowers	4.7	-
<i>Psidium cattleianum</i> Sabine	Araçá	Fruit	2.2	-
SA (Mart)	Barbatimão	Stem bark	22	-
<i>Bauhinia microstachya</i> RADDI	Escada-de-macaco	Leaves	2.5	Da Silva <i>et al.</i> , 2007
<i>Theobroma cacao</i>	Cacau	Seeds	6.7	Padilla <i>et al.</i> , 2008
<i>Syzygium aromaticum</i>	Cravo-da-índia (cloves)	Flower buds	15.0	Pérez-Jiménez <i>et al.</i> , 2010
<i>Ilex paraguayensis</i> St. Hil.	Erva-mate (mate tea)	Leaves	10-18	Heck <i>et al.</i> , 2008
<i>Camellia sinensis</i>	Chá-verde (Green tea)	Leaves	14-21	Anesini <i>et al.</i> , 2008
<i>Vitis vinifera</i> (different varieties)	Uva (grape wine)	Leaves	4.6 to 18.9	Schneider <i>et al.</i> , 2008

TP=Total polyphenol, SA=*Stryphnodendron adstringens*

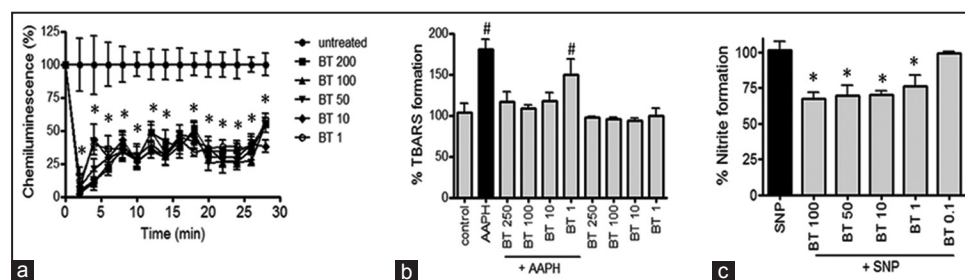


Figure 3: Scavenging activity of *Stryphnodendron adstringens* (SA) polyphenolic extracts against peroxyl radical and nitric oxide, (a) Total reactive antioxidant potential assay, (b) SA polyphenols inhibit peroxyl radical mediated lipid peroxidation *in vitro*, (c) remaining nitrite determined by Griess reaction. Legends: BT (Barbatimao extracts) at different concentrations (µg/mL); sodium nitroprusside (SNP). *Statistically different from 2,2'-Azobis (2-methylpropionamide) dihydrochloride alone group (a) or SNP alone group (c); #different from control group (b); p < 0.05, ANOVA.

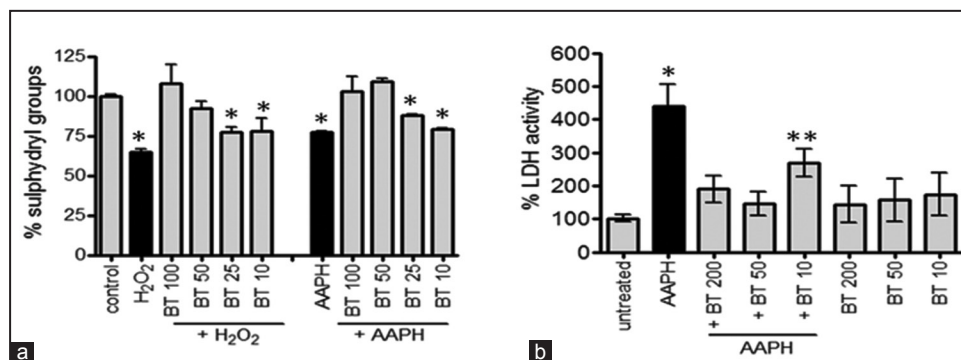


Figure 4: *Stryphnodendron adstringens* polyphenols prevent protein cysteine oxidation and inhibit liver tissue damage mediated by 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) - produced peroxy radicals: (a) Protein cysteine-SH groups, (b) quantification of lactate dehydrogenase. *Different from control/untreated groups; **Different from untreated and from AAPH group; $p < 0.05$, ANOVA.

Table 2: Characterization of SA stem barks.

Parameter	%
Dry mass	86.95
NDF	37.96
Gross protein	3.96
Incineration residue	1.32
Lipids (petroleum ether soluble fraction)	0.36
Titrated acidity	10.24
pH	4.71

NDF=Neutral detergent fiber, SA=*Stryphnodendron adstringens*

SA extracts (250, 100 and 10 $\mu\text{g}/\text{mL}$) were able to quench the peroxy radical generated by AAPH decomposition, as shown by a decrease in the peroxy-mediated lipoperoxidation (Figure 3b). In contrast to TRAP assay, 1 $\mu\text{g}/\text{mL}$ concentration had no statistically significant effect against peroxy radical in TBARS assay. SA extracts (100, 50, 10 and 1 $\mu\text{g}/\text{mL}$) were also able to scavenge $\text{NO}\cdot$, decreasing nitrite formation from the oxidation of $\text{NO}\cdot$ as showed in Figure 3c. Was not observed dose-dependence in none of the assays for peroxy free radical or $\text{NO}\cdot$. All experiments were performed in quadruplicate ($n=4$) and the results are expressed as the mean \pm standard deviation.

3.4. Extracts Inhibited Hydrogen Peroxide and Peroxyl-mediated Protein Sulphydryl Oxidation

The protein sulphhydryl groups (cys-SH) are important to maintenance of protein conformation and function. During an oxidative insult can undergo oxidation leading to formation of disulphide bounds or sulphinic, sulphenic and sulphonic acid formation [26]. In this assay was observed that SA extract were able to inhibit peroxy and hydrogen peroxide-mediated albumin sulphhydryl oxidation from 50 $\mu\text{g}/\text{mL}$ as determined from quantification of reduced sulphhydryl (cys-SH) groups by DTNB assay (Figure 4a).

3.5. SA Extract Inhibit Peroxyl-Induced Tissue Damage in Liver Slices

To test the able of SA extract to prevent oxidative damage in a mammalian tissue system, rat liver slices were pre-incubated with SA extracts and subsequently exposed to AAPH. From determination of LDH release into the incubation medium was observed that polyphenol-enriched extracts from SA (50, 10, 1 $\mu\text{g}/\text{mL}$) inhibited peroxy-induced cell membrane rupture in liver slices, suggesting that this extract was cytoprotective against oxidative stress at the tested concentrations (Figure 4b). To cite, we also tested the effect of the polyphenolic extracts on viability of cancer cells. After treating of C6 cells, a highly proliferative glioblastoma cell line, with 0.1-100 $\mu\text{g}/\text{mL}$ of polyphenols for 72 h, we observed only a 30% decrease in cell viability compared to untreated cells at 100 $\mu\text{g}/\text{mL}$, suggesting that our isolated polyphenols did not exhibit a major anticancer potential (data not showed).

4. CONCLUSION

This work investigated the effects of SA extracts against different reactive oxygen/nitrogen species. In general, it was possible obtain a high polyphenol content in mild extraction conditions and the extracts showed good results in the systems, i.e., from *in vitro* studies, it seems plausible consider the use of these extracts as a potential source of free radical scavengers in benefits of human health.

5. ACKNOWLEDGMENTS

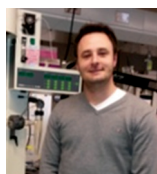
The authors gratefully acknowledge IFRS, CNPq and CAPES for the financial support and scholarships.

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