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**ANTIOXIDANT ACTIVITY OF *Sabicea brasiliensis* AND ITS EFFECT ON
ADENINE NUCLEOTIDES METABOLISM IN VASCULAR SMOOTH
MUSCLE CELLS**

**PATOS DE MINAS – MG
JULHO DE 2017**

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Artigo Científico apresentado ao Instituto de
Genética e Bioquímica da Universidade
Federal de Uberlândia como requisito final
para a obtenção do título de Bacharel em
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**Orientadora: Profa. Dra. Cristina Ribas
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Antioxidant activity of *Sabicea brasiliensis* and its effect on adenine nucleotides metabolism in vascular smooth muscle cells

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Abstract

This study evaluated antioxidant activity of *Sabicea brasiliensis* root extract and fractions and its effect on adenine nucleotide hydrolyses in vascular smooth muscle cells. Percentage of antioxidant activities of crude extract, ethyl acetate and hydro-methanolic fractions were determined by inhibition of the DPPH and ABTS radical in comparison with BHT standard. Results for DPPH assay showed highest antioxidant activity performance for crude extract (76%), followed by ethyl acetate (46%) and hydro-methanolic (23%) fractions. These results were corroborated by ABTS assay. Total phenolic content was evaluated by Folin-Ciocalteu colorimetric method and showed a significant amount of those molecules in crude extract (140.6 ± 7.7 mg GAE.g⁻¹), followed by ethyl acetate (67.1 ± 4.7 mg GAE.g⁻¹) and hydro-methanolic fraction (13.5 ± 8.2 GAE.g⁻¹). Vascular smooth muscular cells were treated with ethyl acetate fraction at 62.5; 125; 250 and 500 µg·mL⁻¹ and demonstrated an inhibition on ATP hydrolysis (56,65%), evidencing that the *Sabicea brasiliensis* can modulate purine levels in vascular tissue.

Keywords: *Sabicea brasiliensis*. Antioxidant activity. Ethyl acetate fraction. ATP hydrolysis. Vascular smooth muscle cells.

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Introduction

Sabicea brasiliensis is a shrub of approximately 80 cm height, popularly known as “blood-of-Christ”, with high occurrence in Cerrado regions of Brazil [1]. Primarily used as a food, it is also employed in popular medicine to treat inflammation, cardiovascular diseases and infections in women genital organs [2].

Phytochemical characterization of methanolic extract performed by Batista et al [3] isolated molecules from *S. brasiliensis* roots with antioxidant properties such as scopoletin, ursolic acid, cafeoylquinic acids and triterpenes. In the same study, ethyl acetate and butanol fractions presented significant anti-inflammatory activity in cell migration assay.

Several functions for extracellular purines such as ATP, ADP and adenosine in various organs and tissues have been described. In the vasculature, ADP is the main promoter of platelet aggregation, whereas adenosine is a potent inhibitor of this aggregation and an important modulator of vascular tone [4-7]. In addition, it is well established that ATP is involved in pro-inflammatory functions such as stimulation and proliferation of lymphocytes and cytokine release, while adenosine exhibits potent anti-inflammatory and immunosuppressive actions [8,9].

Nucleotides and nucleosides exert their functions by activating purinergic receptors, which are classified as P1 and P2 purinergic receptors. P1 receptors are exclusively responsive to the nucleoside adenosine while P2 receptors are divided into two distinct groups: P2X and P2Y [10-12]. P2X are ion channels activated by ATP and are widely distributed, especially between excitable cells. P2Y family is activated by ATP, ADP, UTP, UDP and UDP-glucose, consists of G protein-coupled receptors, and are distinguished in eight members with a broad distribution in vascular, nervous and cardiac systems [13-15].

Concentration and availability of adenine nucleotides in the extracellular medium is regulated by ectonucleotidases [16]. Ectonucleotidases are enzymes that are anchored in the cell membrane, having their active site facing the extracellular medium or are present in the soluble form in the circulation [17]. This enzyme group consists of the ectonucleoside triphosphate diphosphohydrolases (E-NTPDases) family, ectonucleotide pyrophosphatase /phosphodiesterases (E-NPPs), ecto-5'-nucleotidase and alkaline phosphatases. NTPDases hydrolyzes ATP and ADP to AMP, while E-NPP enzymes are

responsible for hydrolyzing 5'-phosphodiester bonds in nucleotides and their derivatives, resulting in the production of monophosphate nucleotide. AMP resulting from the action of NTPDase and E-NPP is subsequently hydrolyzed to adenosine by ecto-5'-nucleotidase [18,19].

Numerous studies have sought to infer the involvement of molecules in the modulation of ectonucleotidases activity and, consequently, in the concentration of adenine nucleotides in extracellular medium. Recently, a study showed that these enzymes are sensitive to phenolic compounds as resveratrol and curcumin [20,21]. Other study showed that acid caffeic may change the hydrolyses of adenine nucleotides in platelets [22]. Also, another author demonstrated the alteration in nucleotides hydrolyses in central nervous system of rats treated with caffeine and caffeic acid [23].

Furthermore, considering the presence of phenolic compounds already characterized in *Sabicea brasiliensis* and the established medicinal properties of this plant, the objective of this study was to evaluate the antioxidant activity of *Sabicea brasiliensis* root and its effect on adenine nucleotide hydrolyses in vascular smooth muscle cells (VSMC).

Material and Methods

Plant samples and extraction

Sabicea brasiliensis roots were collected in February, 2017 in Lagoa Formosa – MG, Brazil (18°41'41.8"S 46°27'51.9"W). Voucher specimen of *S. brasiliensis* (74.313) was identified and stored at the Herbarium of the Institute of Biology of the Universidade Federal de Uberlândia, MG, Brazil.

Plant samples were cleaned with water to remove soil and further dried at 40 °C for 30 hours. Dried samples were then ground to powder by knife mill. The root powder samples (190 g) have been macerated exhaustively at room temperature with 500 mL of methanol and water solution (1:1, v/v) for 15 days, with the replacement of the extracting solution after each three days. At the end of maceration, all the extracted solution was filtered in Whatman No. 2 filtering paper (Whatman International Ltd., Kent, England,

UK) twice, concentrated using a rotary evaporator (801 Fisatom® Ltda) and then lyophilized (Liotop® L101 lyophilizator) to obtain the crude extract (19,86 g).

After the extraction, 12 g of crude extract were dissolved in methanol water solution (9:1, v/v) and partitioned exhaustively with n-hexane and ethyl acetate into a separatory funnel. Each sample was concentrated in rotary evaporator to obtain hexane fraction (0.3 g), ethyl acetate fraction (6.8 g) and residue of partition that was considered a hydro-methanolic fraction (4.9 g). All samples were stored at -80°C until further analysis.

Antioxidant activity by DPPH method

Antioxidant activity of the crude extract (CE) and both ethyl acetate (EAF) and hydro-methanolic (HMF) fractions was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) method as described in a previous study by Lopes-Lutz et al. [24] with some modifications. Initially, an ethanol solution of DPPH was prepared at a concentration of $40 \mu\text{g} \cdot \text{mL}^{-1}$. To each test tube was added 2.7 mL of the DPPH solution, followed by 0.3 mL of crude extract and fractions dissolved in water at concentrations of 62.5; 125; 250 and $500 \mu\text{g} \cdot \text{mL}^{-1}$. Butylated hydroxytoluene (BHT) standard was used as a positive control. Negative control was a solution containing all the reagents except the crude extract and fractions. The reaction was kept in the dark at room temperature for one hour. Absorbances were measured at 517 nm (Gehaka® UV 340G spectrophotometer). Antioxidant Activity (AA%) was calculated by the following equation:

$$\text{AA\%} = [(A_{\text{CN}} - A_{\text{sam}}) / A_{\text{CN}}] * 100$$

Where:

A_{CN} = Absorbance of the negative control;

A_{sam} = Absorbance of the sample;

Values were compared with those of the BHT standard at concentrations of 31.25; 62.5; 125; 250 and $500 \mu\text{g} \cdot \text{mL}^{-1}$.

ABTS method

The ABTS•+ method [ammonium salt of 2,2'-azinobis (3-ethylbenzenethiazoline-6- sulfonic acid)] was performed according to the method described by Guerreiro et al. [25]. ABTS radical was formed by the reaction of 7 mM ABTS•+ solution with 2.4 mM potassium persulphate and the reaction mixture was kept in dark at room temperature for 16 hours. Once formed, the ABTS radical was diluted with ethanol to obtain the absorbance (from 0.7 to 0.72) at 735 nm. For the assay, 10 µL of CE and fractions dissolved in water at concentrations of 62.5; 125; 250 and 500 µg·mL⁻¹ were added in 990 µL of the ABTS radical solution. The BHT standard was used as a positive control. Absorbances were measured at 735 nm (Gehaka® UV 340G spectrophotometer).

Percentage of inhibition of the ABTS•+ radical by the samples was calculated according to the following equation:

$$\% \text{ Effect on radical capture} = [(A_{\text{CN}} - A_{\text{sam}}) / A_{\text{CN}}] * 100$$

Where:

A_{CN} = Absorbance of the negative control;

A_{sam} = Absorbance of the sample;

Determination of total phenolic content

Total phenolic content of CE, EAF and HMF were determined using the Folin-Ciocalteu colorimetric method, based in the formation of a blue complex produced by the reduction of the reagent caused by phenolic compounds present in samples.

As previously described [26], 0.5 mL of each extract and fractions dissolved in water at concentrations of 62.5; 125; 250 and 500 µg·mL⁻¹ were collected and placed in test tubes, to which were added 2.5 mL of 10% (v/v) Folin-Ciocalteu solution and 2 mL 7,5% (w/v) sodium carbonate (Na₂CO₃). The mixture was then kept in water bath at a temperature of 50 °C for 5 minutes. Samples were allowed to rest at room temperature and the absorbance was measured at 760 nm in a spectrophotometer (Gehaka® UV 340G). Total phenolic content of extracts was carried out in triplicate and results were expressed as mean of mg of gallic acid equivalents (GAE) per 1 g of respective sample, using a standard curve prepared with gallic acid.

Cell culture and treatment

A7r5 embryonic rat aorta smooth muscle cells (American Type Culture Collection, ATCC®) were typically plated on to 24-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin, 100 g/ml streptomycin at 37 °C, 5% CO₂ in a humidified incubator. All experiments were carried out with cells between 3 to 5 passages. After reaching 80-90% of confluence, A7r5 cells were made quiescent by serum deprivation for 3 hours and then treated with EAF at 62.5; 125; 250 and 500 µg·mL⁻¹ in DMEM medium supplemented with 5% FBS for 48 hours.

Analysis of NTPDase activities in A7r5 cells

Adherent cells in 24 well plates were incubated with nucleotides (ATP and ADP 500 µM) in 0.5 mL of a reaction medium containing [in mM, final concentration] 80 Tris, 5.0 CaCl₂, 72.5 NaCl (pH 7.4) at 37°C. After 20 minutes incubation, the reaction was stopped by transferring an 0.2 mL aliquot to a tube containing 0.2 mL of 10% trichloroacetic acid (TCA) and kept on ice for few minutes. Then, 1 mL of malachite green reagent was added to the tubes. The released inorganic phosphate (Pi) was measured at 630 nm (Gehaka® UV 340G spectrophotometer) according to Baykov et al. (1988) [27]. Controls used to correct for non-enzymatic hydrolysis of the substrates were performed by adding nucleotides to the mixture after the reaction had been stopped with malachite reagent.

Protein determination

After the hydrolysis assay, cells were dried, solubilized with 100 µL of 1.0 N NaOH and frozen overnight. The next day, 50µL of cells solution were aliquoted from each well, transferred to a tube and the protein concentration of samples was determined by the Coomassie Brilliant Blue method according to Bradford [28], using bovine serum albumin as the standard.

Statistical analyses

For evaluation of the antioxidant activity (DPPH and ABTS methods) and total phenolic compounds, the experiments were arranged in a completely randomized design (CRD) in 3 x 5 factorial (samples x concentrations), with three replications. Data were submitted to analysis of variance and means were compared by Scott-Knott test at 5% probability using SISVAR program (version 5.0). Results for nucleotide hydrolysis were expressed as the mean $\pm$ standard deviation (SD) and subjected to analysis of variance (ANOVA) and Tukey's test. $P < 0.05$ was considered statistically significant. Statistical analyses for ATP and ATP hydrolysis and all graphics in this study were performed using GraphPad Prism version 6.0.

Results and Discussion

Antioxidant activity

Plant secondary metabolites including polyphenols, phenolic and flavonoid compounds have gained interest because they exhibit beneficial health effects due to their potential antioxidant, anti-inflammatory, antibacterial, antidiabetic and cancerpreventive properties [29-32]. More recently, the interest has significantly increased in finding natural antioxidants from natural sources to replace pharmaceutical antioxidants drugs due to their toxicity/carcinogenicity [33,34].

Results for the antioxidant activity (AA) determined by scavenging of the DPPH free radical by *S. brasiliensis* roots extract and fractions are shown in Figure 1. The percentage of AA increased in a concentration-dependent manner for all samples tested. Thus, the highest percentage of antioxidant activity was obtained at a concentration of 500 $\mu\text{g}\cdot\text{mL}^{-1}$. Highest antioxidant activity performance was detected for CE (76%) followed by EAF (46%) and HMF (23%), respectively.

Data from the evaluation of the antioxidant activity through the method involving inhibition of the ABTS radical are shown in Figure 2. The percentages of the antioxidant activity of all the samples were similar to those observed with the previous method. However, HMF had the lowest antioxidant potential, and its activities at concentrations of 62.5, 125 and 250 $\mu\text{g}\cdot\text{mL}^{-1}$ were statistically similar. Moreover, the ABTS method confirmed that there was an increase in the antioxidant activity with increasing

concentrations of the CE and EAF, corroborating a dose-dependent effect. From ABTS test, percentages of AA were 33% for CE, 30% for EAF and 7% for HMF at the highest concentration. All results were statistically significant. The best performance of CE and EAF regarding antioxidant activity may be explained by the presence of molecules with recognized antioxidant properties such as scopoletin, ursolic acid, cafeoylquinic acids and triterpenes already described by BATISTA et al (2014) in ethyl acetate fraction of *S. brasiliensis* root [3].

Furthermore, results for total phenolic content showed a significant amount of those molecules in CE 140.6 ± 7.7 mg GAE/ g of extract, followed by EAF 67.1 ± 4.7 mg GAE/ g and HMF 13.5 ± 8.2 GAE/ g (Figure 3). Additionally, DPPH and ABTS activity of *S. brasiliensis* extract and fractions exhibited a strong and positive correlation with its total phenolic content ($r = 0.99$ and $r = 0.90$, respectively to $p < 0.05$), suggesting that the probable reduction of the DPPH radicals comes by the hydrogen donating ability of phenolic and polyphenolic compounds present in these samples. By scavenging free radicals, *S. brasiliensis* antioxidants may play a preventive role in different diseases and may support its popular use in the control of blood pressure, treatment of genital infection and inflammation in women [2].

ATP and ADP hydrolysis

EAF was chosen to perform the functional biological tests based on its antioxidant potential as well as on the presence of 5-*O*-caffeoylquinic, 4,5-*O*-dicafeoylquinic and 3,5-*O*-dicafeoylquinic, previously reported [3]. These molecules are derived from caffeic acid and may be found in numerous plants including fruits, vegetables and coffee. Studies have reported a decrease in the risk of inflammation and cardiovascular diseases in treatments with these molecules [35]. Furthermore, 5-caffeoylquinic acid reduces oxidative stress and improves nitric oxide bioavailability by inhibiting excessive production of reactive oxygen species in the vasculature, and leads to the attenuation of endothelial dysfunction, vascular hypertrophy, and hypertension in spontaneously hypertensive rats [36].

EAF could inhibit ATP hydrolysis in A7r5 embryonic rat aorta smooth muscle cells (Figure 4a). Non-treated (control group) cells presented an activity of ATP hydrolysis equal to $21.64 \text{ Pi} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ while the activity was decreased to $12.26 \text{ nmol Pi} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at $500 \mu\text{g} \cdot \text{mL}^{-1}$ of EAF, thus presenting a significant inhibition of 56,65 %

at the maximal concentration of EAF tested ($P < 0.05$). The same pattern of EAF effect was found for ADP hydrolysis. Results, however, were not statistically significant even at the highest concentration tested ($500 \mu\text{g} \cdot \text{mL}^{-1}$) in comparison to control group (Figure 4b). It is noteworthy that treatment with $500 \mu\text{g} \cdot \text{mL}^{-1}$ EAF inhibited ADP hydrolysis ($P < 0.05$) when compared to $62.5 \mu\text{g} \cdot \text{mL}^{-1}$ concentration, evidencing that the responses to EAF treatment may work the same way for both nucleotides. Besides, at lowest concentrations, EAF seems to increase both ATP and ADP hydrolysis. However, additional experiments are necessary to confirm these results.

It is noticed that between members of NTPDase family, NTPDase1 hydrolyzes ATP and ADP about equally well [37,38]. Thus, the same pattern observed for EAF treatment regarding ATP and ADP hydrolysis in A7r5 cells is probably due to an effect of this fraction on NTPDase1 specific activity. Membrane-bound NTPDase1 is the major ectonucleotidase in the vasculature [39] and hydrolyzes ATP almost directly to AMP with the transient production of minor amounts of free ADP [38]. Inhibition of this enzyme, at high EAF concentrations for example, may lead to ADP, a potent platelet aggregating molecule, accumulation in the extracellular medium. ADP is the most potent factor that may cause platelet activation. Furthermore, NTPDase1 is accepted to be a potent antithrombotic agent because rapidly metabolizes ADP, terminating further platelet recruitment and aggregation [40, 41]. In addition, ATP in vascular smooth muscular causes vasoconstriction via P2 receptors mainly activating P2X_1 that is a Ca^{+2} channel, and ADP has the same function activating P2Y receptors. On the other hand, these nucleotides have a contrary response in endothelial cells, where they cause local vasodilation via oxide nitric through P2 activation [42].

Another aspect to be considered is that Chen et al. [43] found that 3,4-di-O-caffeoylquinic acid, a phenolic isolated from *Elephantopus mollis*, minimized the loss of cell viability induced by oxidative stress and ROS-induced impairment of endothelium-dependent relaxation to acetylcholine in rat aorta through an antioxidant property in cultured endothelial human cells. Therefore, the popular medicinal use of *S. brasiliensis* in the control of blood pressure may be through protecting the endothelium from oxidative stress by the antioxidant power of caffeoylquinic acids also present in *S. brasiliensis* despite the purinergic system participation.

Moreover, caffeic acid has also been reported to chelate metals [44]. Da Silva et al. [45] also suggests that the chelating property of flavonoids may diminish NTPDase, ecto-5'-nucleotidase and Na^+/K^+ -ATPase activities in cortical membrane preparation. It

is well known that NTPDase1 and all others surface located NTPDases require Ca^{2+} or Mg^{2+} ions in the millimolar range for maximal activity and are inactive in their absence [37, 38]. Thus, the inhibition observed on ATP and ADP hydrolysis by high concentrations of EAF may also be a response to the antioxidant molecules capacity in chelating metal ions.

Conclusion

Finally, we conclude that crude extract and fractions of *Sabicea brasiliensis* roots present significant antioxidant power. Total phenolic content confirmed the high antioxidant activity of this brazilian plant. Furthermore, ethyl acetate fraction alters ATP hydrolysis modulating the balance of purine levels in vascular smooth muscular cells.

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Figures captions

Fig.1 Percentage of the antioxidant activity of crude extract, ethyl acetate and hydro-methanolic fractions of *S. brasiliensis* roots by DPPH radical inhibition method. Mean values by the same letter in uppercase to compare the concentration in the sample (extract, fractions or BHT) analyzed and in lowercase for comparison between the concentrations of the same sample do not differ significantly at 5% probability by the Scott-Knott test.

Fig.2 – Percentage of the antioxidant activity of crude extract, ethyl acetate and hydro-methanolic fractions of *S. brasiliensis* roots by the method of inhibiting the ABTS radical. Mean values by the same letter in uppercase to compare the concentration in the sample (extract, fractions or BHT) analyzed and in lowercase for comparison between the concentrations of the same sample do not differ significantly at 5% probability by the Scott-Knott test.

Fig.3 – Total phenolic content of crude extract, ethyl acetate and hydro-methanolic fractions of *S. brasiliensis* roots. Results were given in mg of GAE/ gram of the respective extract or fraction. Bars represent mean $\pm$ standard deviation ($n = 3$) and different letters indicate statistically significant differences at 5% probability by the Scott-Knott test.

Fig.4 – ATP (A) and ADP (B) hydrolysis in A7r5 cell line treated with different concentrations (from 62.5 to 500 $\mu\text{g.mL}^{-1}$) of *Sabicea brasiliensis* roots EAF. Bars represent mean $\pm$ SD of three independent experiments. (*represents significant difference from control group (non-treated cells) and #represents significant difference from 62.5 $\mu\text{g.mL}^{-1}$, $p < 0.05$).

Fig.1

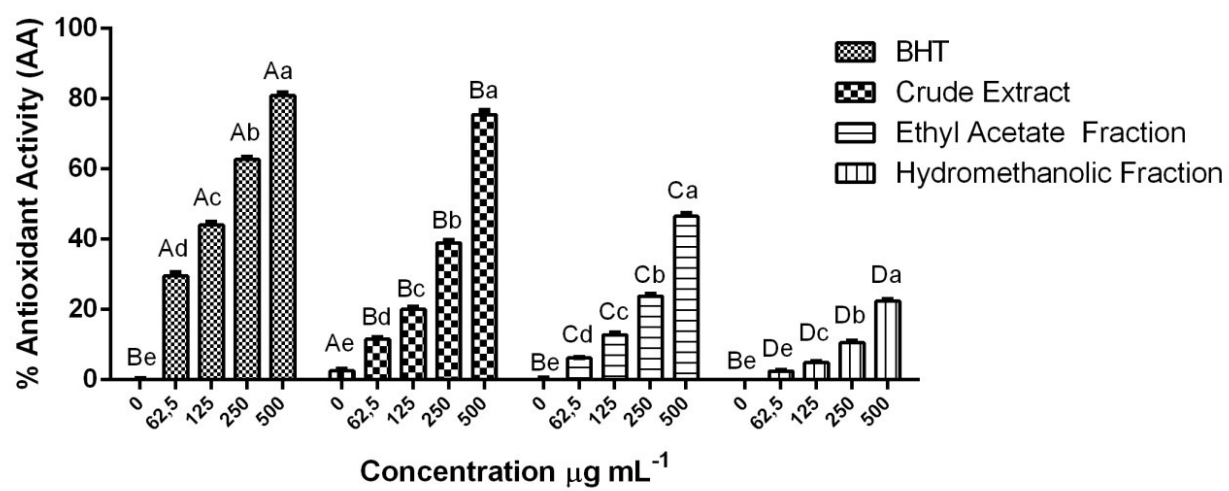


Fig.2

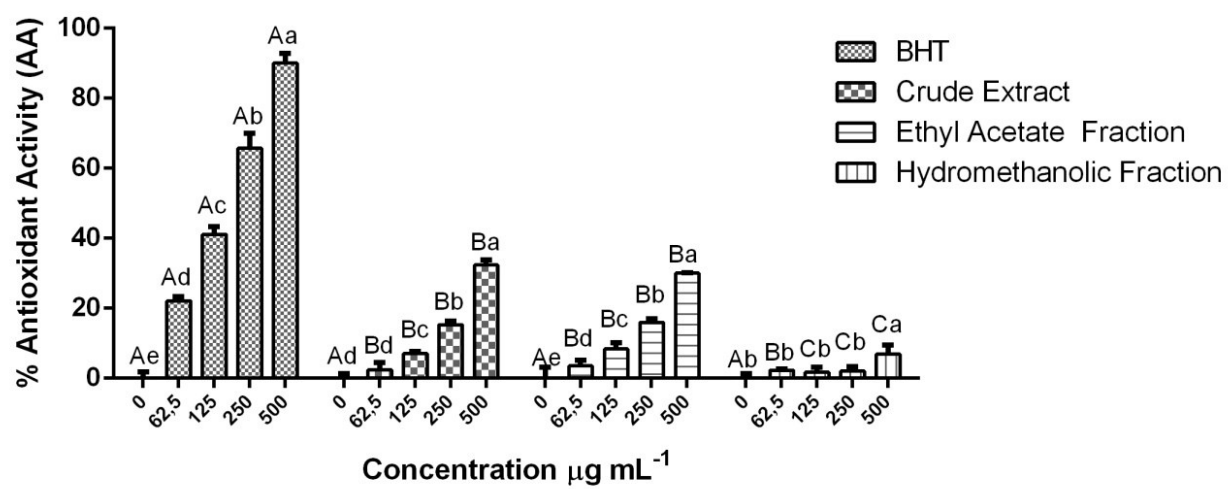


Fig.3

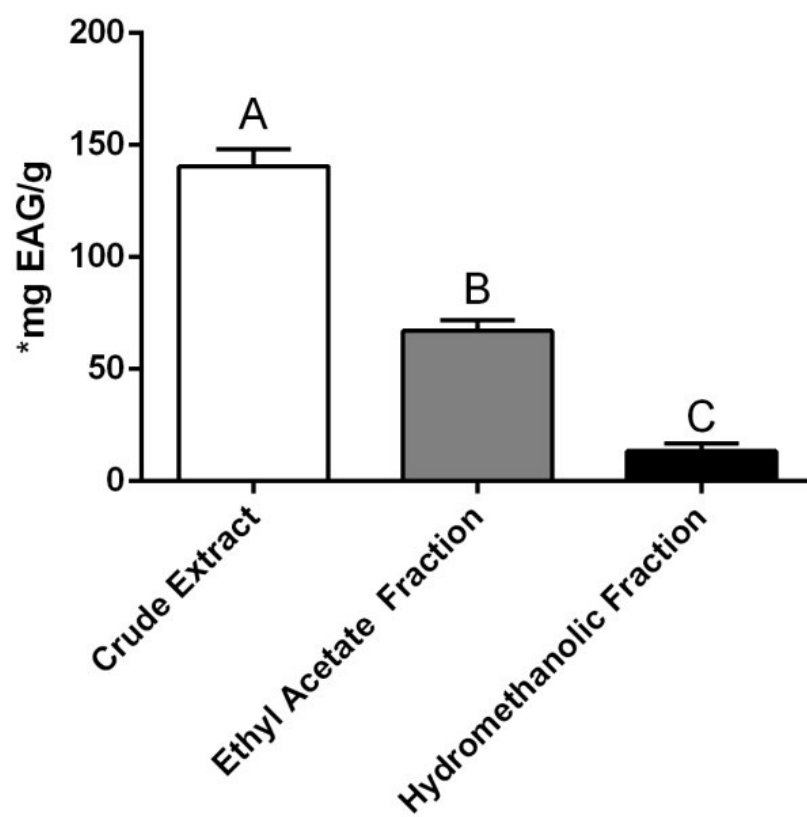
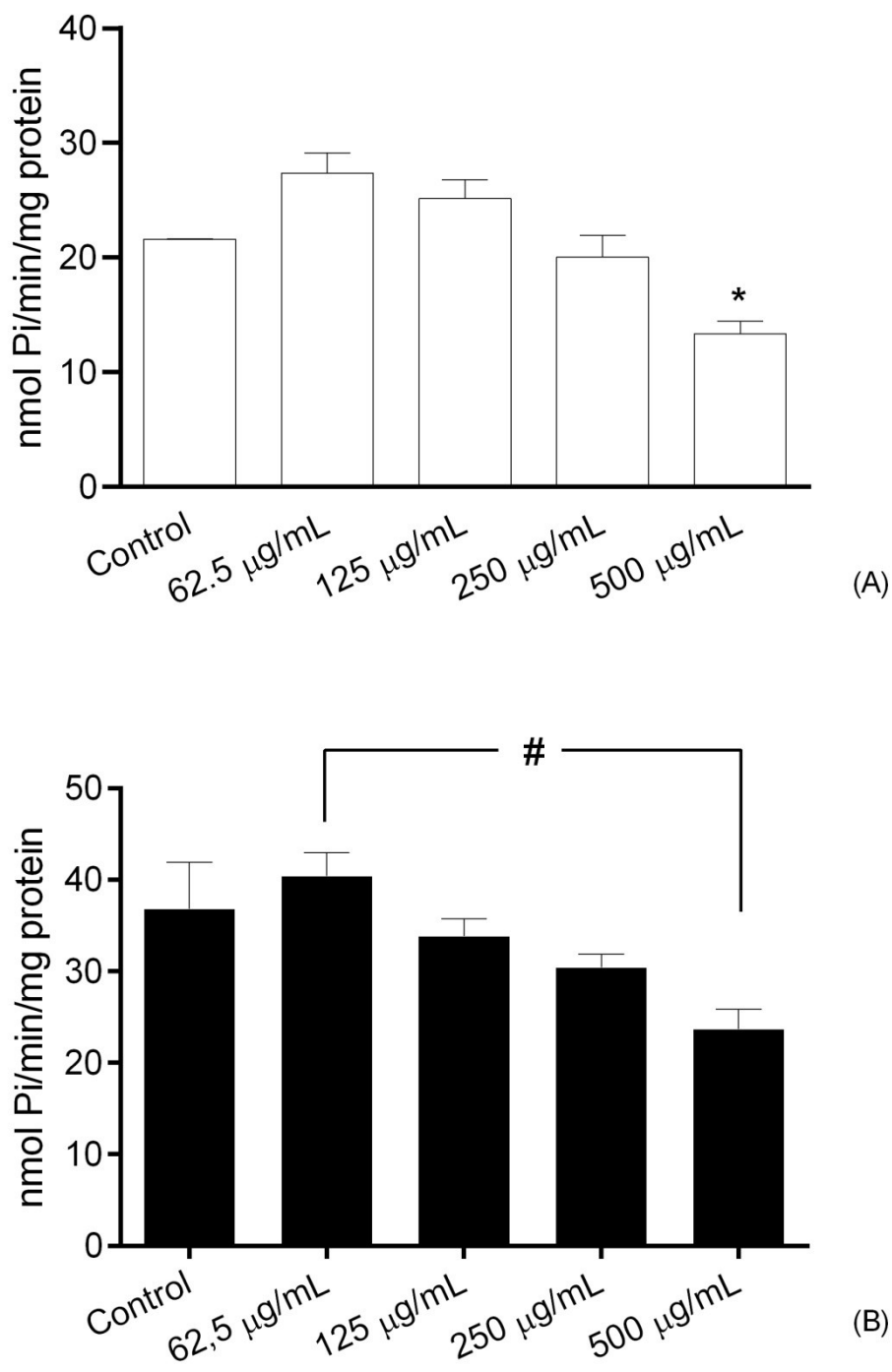


Fig.4



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