Sabicea brasiliensis Wernham: Antioxidant Activity, Proliferative Effect and Modulation of Vascular Adenine Nucleotides Metabolism

Douglas Souza Oliveira¹, Mikaelle Costa Correia¹, Bruna Juber de Araújo¹, Fernanda Cardoso da Silva¹, Paula Marynella Alves Pereira Lima¹, Marcos de Souza Gomes², Thaise Gonçalves de Araújo¹ and Cristina Ribas Fürstenau³*

¹Instituto de Biotecnologia, Universidade Federal de Uberlândia - Campus Patos de Minas, Minas Gerais, Brazil.
²Instituto de Química, Universidade Federal de Uberlândia - Campus Patos de Minas, Minas Gerais, Brazil.
³Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André, São Paulo, Brazil.

Authors’ contributions

This work was carried out in collaboration among all authors. Author DSO designed the first step of the study, performed the extraction and fractioning experiments, carried out the antioxidant measurements, performed the statistical analyses and contributed in the writing of the manuscript. Author MCC designed and performed the in vitro assays with A7R5 cells and contributed in the writing of the manuscript. Authors DSO and MCC contributed equally as first authors to the development of this study. Authors BJA and FCS helped with the extraction and samples preparation and ectonucleotidases assays. Author MSG helped to perform the antioxidant assays, statistical analyses and to construct the graphs. Author PMAPL contributed in cellular viability measurements. Author TGA helped in the design of the study, the discussion of results and writing the final version of the manuscript. Author CRF coordinated the study, obtained the financial support to develop the study, performed the conception and the design of the study, follow all steps in the conduction of the experiments, did the critical analysis of data and wrote the manuscript. All authors read and approved the final manuscript.

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*Corresponding author: E-mail: c.furstenau@ufabc.edu.br;
ABSTRACT

Aims: Study addressed the antioxidant activity (AA) of Sabicea brasiliensis roots crude extract (CE), ethyl acetate (EAF), and hydro-methanolic (HMF) fractions, and its impact on cell viability and adenine nucleotide hydrolysis in vascular A7r5 cells.

Materials and Methods: AA of CE, EAF and HMF were determined by the inhibition of the DPPH and ABTS radicals. Total phenolic content was evaluated by Folin-Ciocalteau. Cell viability was determined by MTT assay at different concentrations (62.5; 125; 250 and 500 μg·mL\(^{-1}\)) of EAF and HMF after 24, 48 and 72 h. Ectonucleotidase activities were evaluated by colorimetric methods after 48 h EAF or HMF treatment.

Results: The highest AA was observed for CE (76%), followed by EAF (46%) and HMF (23%). Phenolic content followed the same pattern. After 48 h, EAF increased A7r5 vascular cells viability by 40%, 40%, 62% and 25% at distinct concentrations, respectively; while HMF augmented it by 50% (500 μg·mL\(^{-1}\)). Finally, after 48 h EAF (500 μg·mL\(^{-1}\)) decreased about 50% of ATP and ADP metabolism while HMF inhibited 56 and 59% the hydrolysis of NPP substrate (at 125 and 250 μg·mL\(^{-1}\)).

Conclusion: Study confirmed the high AA of S. brasiliensis, which influences vascular cells proliferation and purines metabolism, pointing to potential cellular pathways that may support the popular use of this plant.

Keywords: Brazilian savanna; blood of Christ; antioxidant activity; proliferative effect; purines metabolism; vascular smooth muscle cells.

ABBREVIATIONS

| Abbreviation | Description |
|--------------|-------------|
| AA           | Antioxidant activity |
| CE           | Crude extract |
| EAF          | Ethyl acetate |
| HMF          | Hydro-methanolic |
| NPP          | Ectonucleotide pyrophosphatase |
| ROS          | Reactive oxygen species |
| E-NTPDases   | Diphosphohydrolases; |
| VSMC         | Vascular smooth muscle cells |
| DPPH         | 2,2-diphenyl-1-picrylhydrazyl |
| BHT          | Butylated hydroxytoluene; |
| ABTS++       | Ammonium salt of 2,2’-azinobis (3-ethylbenzenethiazoline-6-sulfonic acid) |
| GAE          | Gallic acid equivalents |
| MTT          | 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide |

1. INTRODUCTION

Reactive oxygen species (ROS) are naturally generated in intra and extracellular environments by oxygen metabolism. ROS are important in biological processes, but their excessive generation may cause oxidative stress, cellular damage and the consequent development of pathologies. Oxidative stress derives from an imbalance between increased ROS and reduced antioxidant enzyme activities [1].

Antioxidants from natural sources (like vitamins, phenolic compounds, flavonoids, carotenoids, among others) are capable of removing free radicals generated during oxidative metabolism in order to maintain the redox balance [2]. Specifically, phenolic compounds are usually found in natural plant products and are extensively studied due to their antioxidant properties against damage caused by oxidative imbalance as they may act as free radical scavengers. In this sense, studies report that phenolic compounds exert important biological activities in the cardiovascular system, including the inhibition of platelet adhesion and aggregation, acting on lipid metabolism and protecting biomolecules, for example [3]. Thus, in addition to the endogenous enzymatic antioxidants, exogenous antioxidants like ascorbic acid and some secondary metabolites of plants like phenolic compounds can also prevent oxidative stress [4] responsible for the genesis and consolidation of different pathologies.

The Brazilian Cerrado has a wide biodiversity housing a varied flora whose bioactive compounds are subject of different studies, highlighting a member of Rubiaceae family Sabicea brasiliensis Wernham, which is a shrub
with approximately 80 cm high, popularly known as "blood of Christ" [5]. Batista and colleagues [6] characterized the extract of *S. brasiliensis* pointing to scopoletin, ursolic acid, caffeoylquinic acids and triterpenes as the bioactive molecules with a preventive role in different diseases, suggesting the basis for its popular use in blood pressure control, treatment of genital infection and inflammation in women [7].

Extracellular adenine nucleotides (ATP e ADP) are widely accepted as signalling molecules mediating neurotransmission, immune responses, cell growth and cell death [8]. Several studies focused on the involvement of extracts and bioactive molecules in purinergic signalling and in the concentration of adenine nucleotides in the extracellular environment, contributing to the availability of adenine nucleotides in the extracellular medium [9,10]. Purines act via purinergic receptors activation [11] and their concentration in the extracellular medium are regulated by ectonucleotidases [12].

Among these enzymes, the family of ectonucleoside triphosphatase diphosphohydrolase (E-NTPDases) can hydrolyse ATP and ADP in AMP and the family ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPs) is responsible for the hydrolysis of 5′-phosphodiester bonds in nucleotides and their derivatives, resulting in the production of monophosphate nucleotides. Once released into the bloodstream, ATP and ADP may influence vasomotion, platelet aggregation, growth of vascular smooth muscle cells (VSMC), and mediation of inflammatory responses [13,14]. ATP can increase the proliferation of VSMC [13], which may lead to the development of atherosclerosis, pulmonary hypertension and restenosis. However, once controlled, this effect may assist in wound healing, remodelling and neovascularization [15].

Considering: 1) the presence of phenolic compounds already characterized in *Sabicea brasiliensis* Wernham; 2) the established medicinal properties of this plant; and 3) that the antioxidant activity displayed by natural phenolic compounds regulate the availability of nucleotides in the extracellular environment, which may increase VSMC proliferation, this study evaluated the antioxidant activity (AA) of *S. brasiliensis* Wernham roots crude extracts and solvent fractions, its influence on cell viability and its effects on adenine nucleotide hydrolysis in vascular cells of A7r5 lineage.

### 2. MATERIALS AND METHODS

#### 2.1 Plant Samples and Extraction

*Sabicea brasiliensis* Wernham roots were collected in February 2017, in Lagoa Formosa – MG, Brazil (18°41′41.8"S 46°27′51.9"W). *S. brasiliensis* voucher specimen was identified under the number 74.313 and stored at the Institute of Biology herbarium of the Universidade Federal de Uberlândia, MG, Brazil. Plant samples were cleaned to remove soil and further dried at 40°C for 30 h. Dried samples were ground to powder by a knife mill. The root powder samples (190 g) were then exhaustively macerated at room temperature with 500 mL of methanol and water solution (1:1, v/v) for 15 days, with replacement of the extracting solution after each three days. At the end of the maceration period, 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 (Liotta® L101) to obtain 19,86 g of crude extract (CE) (η=10,45%). After the extraction, 12 g of CE 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 a rotary evaporator to obtain hexane fraction (0.3 g η=2.5%), ethyl acetate fraction (EAF) (6.8 g, η=56.6%) and the residue of partition that was considered as a hydro-methanolic fraction (HMF) (4.9 g, η=40.83%). All samples were stored at -80°C until further experiments.

#### 2.2 Total Phenolic Content Determination

Total phenolic content of CE, EAF and HMF were determined using the Folin-Ciocalteau colorimetric method, based in the formation of a blue complex produced by the reduction of the reagent caused by phenolic compounds present in samples [16]. A volume of 0.5 mL of CE, EAF and HMF dissolved in water at concentrations of 62.5; 125; 250 and 500 μg·mL⁻¹ were placed into test tubes to which were added 2.5 mL of 10% (v/v) Folin-Ciocalteau solution and 2.0 mL 7.5% (w/v) sodium carbonate (Na₂CO₃). The mixture was kept in a water bath at 50 °C for 5 min. Samples were allowed to rest at room temperature and the absorbance was measured at 760 nm in a spectrophotometer (Gehaka® UV 340G spectrophotometer). Total phenolic content measurements of extracts and solvent fractions were 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 of gallic acid.

2.3 DPPH Method

Antioxidant activity (AA) of CE, EAF and HMF was determined by the DPPH (2,2-diphenyl-1-pirclylhydrazyl) method as previously described by Lopes-Lutz et al. [17], with some modifications. Initially, an ethanol solution of DPPH was prepared at a concentration of 40 μg·mL⁻¹. To each test tube was added 2.7 mL of the DPPH solution, followed by 0.3 mL of CE, EAF and HMF dissolved in water at concentrations of 62.5; 125; 250 and 500 μg·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 solvent fractions. The reaction was kept in the dark at room temperature for one hour. Absorbances were measured at 517 nm (Gehaka® UV 340G spectrophotometer). AA% was calculated by the following equation:

\[
AA\% = \frac{(ACN - A_{sam})}{ACN} \times 100
\]

Where \( A_{CN} \) = Absorbance of the negative control; \( A_{sam} \) = Absorbance of the sample. Values of AA% obtained for CE, EAF and HMF were compared with those of the BHT standard at 0; 62.5; 125; 250 and 500 μg·mL⁻¹.

2.4 ABTS Method

The ABTS⁺ (ammonium salt of 2,2’-azinobis (3-ethylbenzenethiazoline-6- sulfonic acid)) assay was performed according to Prior et al. [18]. ABTS radical was formed by the reaction of 7 mM ABTS⁺ solution with 2.4 mM potassium persulfate and the reaction mixture was kept in dark at room temperature for 16 h. 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, EAF or HMF 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 CE, EAF and HMF was calculated according to the following equation:

\[
\% \text{ Effect on radical capture} = \frac{(ACN-A_{sam})}{ACN} \times 100
\]

Where \( A_{CN} \) = Absorbance of the negative control; \( A_{sam} \) = Absorbance of the sample.

2.5 Cell Culture and Treatments

A7r5, an embryonic rat aorta smooth muscle cell line (American Type Culture Collection, ATCC®), were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% FBS (Invitrogen), 100 U/mL penicillin (Gibco), 100 g/mL streptomycin (Gibco) at 37 °C, 5% CO₂, in an humidified incubator. For the experiments, 1 x10⁵ cells were plated on 24 or 96-well plates and cultured until reaching 80-90% confluence. A7r5 cells were then made quiescent by serum deprivation for 3 h prior to treatment with EAF or HMF at 62.5; 125; 250 and 500 μg·mL⁻¹ in DMEM medium supplemented with 5% FBS for 24, 48 and 72 h for MTT assays and for 48 h for measurement of ectonucleotidase activities. All experiments were carried out in triplicates with cells between 3rd to 5th passage.

2.6 Cellular Viability

A7r5 cells were treated with EAF and HMF at concentrations mentioned before for 24, 48 and 72 h and their viability were evaluated by the MTT colorimetric assay [19]. Absorbances were read at 560 nm (Robonik® spectrophotometer). Cellular viability was determined as follows:

\[
\text{Cell Viability} \ (\%) = \frac{(Abs_{test} - Abs_{blank})}{(Abs_{control} - Abs_{blank})} \times 100
\]

Where \( Abs_{test} \) = Absorbance of treated cells (cells + treatment); \( Abs_{blank} \) = Absorbance of blank wells (DMSO only since it was the diluent used); \( Abs_{control} \) = Absorbance of control cells (cells with no treatment).

2.7 Analysis of NTPDase Activities in A7r5 Cells

Adherent A7r5 cells in 24 well plates were treated with EAF or HMF for 48 h, then 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 and kept on ice for a few minutes. Then, 1.0 mL of malachite green reagent was added to the tubes. The released inorganic phosphate (Pi) was measured at 630 nm (Gehaka® UV 340G spectrophotometer).
2.9 Statistical Analyses

According to Baykov et al. [20], 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. After the hydrolysis assay, cells were solubilized with 100 µL of 1.0 N NaOH and frozen overnight. The next day, 50µL of the cell suspension were aliquoted from each well, transferred to a tube and the protein concentration of samples was determined according to Bradford method [21], using bovine serum albumin as the standard. Enzymatic activities were expressed as nmol Pi released per minute per milligram of protein.

2.8 Analysis of NPP Activity in A7r5 Cells

To measure NPP activity, after treatment with the HMF for 48 h, A7r5 cells adhered to 24-well plates were incubated with the artificial substrate marker for the enzyme p-Nph-5'-TMP (5'-TMP, 500 µM) in 0.2 mL of a reaction medium containing (in mM, final concentration): 50 Tris, 2.0 CaCl₂, 120 NaCl, 5.0 KCl, 10 Glucose pH 8.9, at 37°C for 40 min. The reaction was stopped by transferring an 0.1 mL aliquot to a tube containing 0.1 mL of 0.2 N NaOH on ice. Then, the amount of p-nitrophenol formed was measured at 405 nm in a plate reader (Robonik®), using the molar extinction coefficient of 18.8 x 10⁻³ M⁻¹ cm⁻¹. The controls used to correct for non-enzymatic hydrolysis of the substrate were performed by adding 5'-TMP after the reaction had been stopped. Total protein content was determined by Bradford method [21] as mentioned before. Enzymatic activities were expressed as nmol p-nitrophenol per minute per milligram of protein.

2.9 Statistical Analyses

For evaluation of the AA (DPPH and ABTS methods) the experiments were arranged in a completely randomized design in 3 x 5 factorial (samples (CE, EAF, HMF) x concentrations), with three replications. For the test of total phenolic content, the experiment was arranged in a completely randomized design, with three replications. Data were submitted to analysis of variance and means were compared by Scott-Knott test at 5% probability using the SISVAR program (version 5.0). Pearson product moment correlation was used to examine the correlations. Results for cellular viability were expressed as the mean ± standard deviation (SD) and subjected to analysis of variance (ANOVA) and Bonferroni’s test. Results for nucleotide hydrolysis were expressed as mean ± SD and analyzed by Student’s t test of independent samples. p < 0.05 was considered statistically significant. Analyses and graphics were performed using GraphPad Prism version 6.0.

3. RESULTS AND DISCUSSION

Plant polyphenols, phenolic and flavonoid compounds are of special interest since they exhibit beneficial health effects as antioxidant, anti-inflammatory, antibacterial, antidiabetic and cancer preventive agents [22,23]. Results show a significant phenolic content (mg GAE/g) in CE (140.6±7.7), followed by EAF (67.1±4.7) and HMF (13.5±8.2) (Fig. 1).

![Fig. 1. Total phenolic content of CE, EAF and HMF of S. brasiliensis Wernham roots.](image)

Results were given in mg of GAE/gram of the respective extract or fraction. Bars represent mean ± SD (n=3) and different letters indicate statistically significant differences at 5% probability by the Scott-Knott test

It is already described the presence of caffeic acid derived molecules in Sabicea brasiliensis roots, such as 5-O-caffeoylquinic, 4,5 and 3,5-dicaffeoylquinic acids [6]. Caffeoylquinic acids are the most widely available polyphenols consumed and exhibit antioxidant potential and anti-inflammatory effects [23,24]. They are found in abundance in the Rubiaceae family, especially in coffee and tea [25]. The antioxidant effects of these molecules have been reported in plants like Achillea tenorii [26] and Hypericum hircinum L [27]. In the vasculature, 3,4-di-O-caffeoylquinic acid from the Taiwanese folk medicine Elephantopus mollis minimized the loss of cell viability induced by oxidative stress in cultured human umbilical vein endothelial cells [28]. In murine VSMC, the same acid minimized angiotensin II-induced cellular disturbance which was related to its antioxidant properties [29].
Antioxidants are compounds that hinder oxidative processes and thereby delay or prevent oxidative stress. There is a growing interest in finding antioxidants from natural sources to replace pharmaceutical antioxidants drugs due to their toxicity and carcinogenic effects [2]. AA was determined by scavenging the DPPH radical in response to S. brasiliensis Wernham roots extract and solvent fractions (Fig. 2a). AA increased in a concentration-dependent manner and the highest antioxidant activity was found for CE (76%), followed by EAF (46%) and HMF (23%) at 500 µg·mL⁻¹. ABTS results confirmed the increase in AA (Fig. 2b), with 33% for CE, 30% for EAF and 7% for HMF at the highest concentration. Thus, CE, EAF and HMF may directly react with free radicals by electron donation and radical scavenging, thereby inhibiting ROS-induced damage.

Besides, the AA of S. brasiliensis CE, EAF and HMF exhibited a strong and positive correlation with their phenolic content (DPPH, r = 0.99 and ABTS, r = 0.90, respectively to p < 0.05), suggesting that the reduction of free radicals comes from the ability of phenolic and polyphenolic compounds present in these samples in donating hydrogen atoms. The preservation of the already characterized caffeic acid derived molecules in EAF samples, such as 5-O-cafeoylquinic, 4,5 and 3,5-dicaffeoylquinic acids [6] may account for the best performance of CE and EAF regarding AA and the positive correlation with total phenolic compounds.

Some plants from the Brazilian Cerrado are reported to present antiulcerogenic, anti-diabetic, anti-inflammatory and antitumoral activities based on the action of their bioactive compounds as free radical scavengers. Extracts obtained from H. speciosa, C. adamantium, and S. adstringens demonstrated a powerful AA that was attributed to the high presence of phenolic and flavonoid compounds in such species [30–32]. Phytochemical studies showed the presence of flavonoid compounds in the methanolic extract of S. brasiliensis Wernham leaf’s derived from kaempferol [33]. A study with methanolic extract of its roots also revealed the presence of scopoletin, ursolic acid, triterpenes, phenolic compounds and flavonoids [6]. These molecules have great antioxidant activity and are used to treat several conditions [34,35]. After fractionation, Batista and collaborators characterized many phenolic compounds derived from caffeic acid in the EAF of S. brasiliensis [6]. Accordingly, we also showed a high AA in the CE and EAF may be due to the presence of these compounds.

The A7r5 cellular viability was evaluated after treatment with EAF (Fig. 3a-c) and HMF (Fig. 3d-f) over 24, 48 and 72 h. Both fractions showed a proliferative potential in A7r5 cells after 48 h of treatment. EAF increased cell viability by 40% at concentrations 62.5 and 125 µg·mL⁻¹, 62% by 250 µg·mL⁻¹ and 25% to 500 µg·mL⁻¹ (Fig. 3b). In addition, HMF induced an increase in A7r5 viability of 50% at 500 µg·mL⁻¹ (Fig. 3e). No differences in cell viability to both EAF and HMF were observed in other times of treatment. Different natural substances are considered as prototypes in the search for drugs with pharmacological activity and therapeutic applicability. S. brasiliensis Wernham solvent fractions demonstrated promising activity in VSMC of A7r5 lineage.

Despite the growing number of studies looking for molecules able to prevent the excessive VSMC proliferation [36], results showing proliferative agents are also becoming relevant to accelerate vascular remodelling in response to angioplasty; increase the thickness of the intima layer during arterial diseases; and to grow new vessels during the healing of wounds and tumours [15,37]. Therefore, results show that S. brasiliensis Wernham increases A7r5 proliferation and this effect may be beneficial in processes of vascular regeneration.

Finally, the effect of EAF (Fig. 4a and 4b) and HMF (Fig. 4c) was evaluated on nucleotide hydrolysis. EAF inhibited 51.85% of ATP hydrolysis in A7r5 cells (Fig. 4a). The same pattern of EAF effect was found for ADP hydrolysis (Fig. 4b). Despite HMF had no effect on ATP and ADP hydrolysis (data not shown), this fraction was able to inhibit the hydrolysis of NPP artificial substrate at 125 and 250 µg·mL⁻¹ by 56 and 59%, respectively (Fig. 4c). The inhibition observed in ATP and ADP hydrolysis potentially leads to an accumulation of these nucleotides in the extracellular medium. ATP presents both pro- and antiapoptotic effects depending on the cell type activation [38,39]. In VSMC cultures, ATP and ADP demonstrated a mitogenic effect, inducing DNA and protein synthesis acting via P2Y receptors, being recognized as two potent stimulators of VSMC.
Fig. 2. CE, EAF and HMF antioxidant activity evaluation. Percentage of DPPH (a) and ABTS radical inhibition (b). Mean values represented by the same letter in Uppercase were used to compare the concentration between the treatments (CE, EAF, HMF or BHT) and those represented by letters in lowercase were used for comparison between the concentrations in the same group of treatment. Equal letters indicate that values do not differ significantly at 5% probability by the Scott-Knott test (n=3).

Fig. 3. Effect of EAF and HMF on A7r5 viability. Results were obtained by MTT assay after treatment with EAF at different concentrations (62.5, 125, 250 and 500 μg.mL⁻¹) for (a) 24, (b) 48 and (c) 72h. Cells were also treated with HMF at the same concentrations for (d) 24, (e) 48 and (f) 72 h.

Data are expressed as mean ± SD of cellular viability in percentage, considering control group as 100% (n=3).

* p< 0.05; ** p<0.01; **** p<0.0001 vs. control

Fig. 4. ATP (a) and ADP (b) hydrolysis in A7r5 cells submitted to EAF treatment at different concentrations (0, 62.5, 125, 250 and 500 μg.mL⁻¹) for 48 h. (c) p-Nph-5′-TMP hydrolysis in A7r5 cells after HMF treatment for 48 h.

Data are presented as mean ± standard deviation (n=3).

* p< 0.05; ** p< 0.01 vs. control
proliferation and progression factor [13,40], the modulation of ectonucleotidases was already explored in response to extracts of *Uncaria tomentosa* [41,42], *Thymus vulgaris* [10], *Hibiscus sabdariffa* L. [43] and lingonberry extract [9]. These works suggest that plant extracts may alter the enzymatic profile of ectonucleotidases, influencing the extent of purinergic receptors activation.

4. CONCLUSION

Results suggest that *S. brasiliensis* Wernham favours a less oxidizing environment due to its high phenolic content and antioxidant activity. Also, this plant seems to cause the accumulation of adenine nucleotides. Together, these features may contribute to the proliferative effect observed in A7r5 cells which may be important for angiogenesis, tissue remodelling and homeostasis maintenance. Hence, cell proliferation and purinergic signalling are potential cellular pathways to be better investigated to support the popular use of this plant.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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