

EPR technique as a tool for evaluating the antioxidant potential of crude extract of *Pterodon pubescens*

Uso da técnica de RPE para avaliação do potencial antioxidante do extrato bruto de *Pterodon pubescens*

Katia Souza Chagas¹, Claudemir Batalini¹, Jair Pereira de Melo Júnior², George Barbosa da Silva¹

¹ Universidade Federal de Mato Grosso - Campus Universitário do Araguaia, LAPQUÍM - Unidade I, Pontal do Araguaia (MT). E-mail: pirapotimao@msn.com

² Universidade de Rio Verde - Faculdade de Medicina, Departamento de Biofísica - Rio Verde (GO).

Abstract

*We evaluate the antioxidant potential of crude extract from stem bark of *Pterodon pubescens*, a typical plant of the Brazilian Cerrado, using Electron Paramagnetic Resonance (EPR) at X-band. The measurements were performed in different concentrations of the crude extract diluted in the 1.0 mmol.L⁻¹ ethanol solution of the free radical DPPH (2,2'-diphenyl-1-picrylhydrazyl). The analysis was taken to obtain the material capability to neutralize the action of the DPPH radical. We observed a clear relation between the extract concentrations and how the EPR signal decrease. We also observed that small amount of extract, about 14.4 mg.L⁻¹, was enough to reduce the radical DPPH in the solution by 50%, indicating that the crude extract of *Pterodon pubescens* has a great antioxidant property. Some secondary metabolites, such as auronas, chalcones, coumarins, flavanones, phenols, tannins and triterpenes were identified in the sample using the phytochemical analysis. The strong antioxidant activity can be related to the phenolic compounds found in the sample.*

Keywords: Antioxidant; EPR; *Pterodon pubescens*.

Resumo

Avaliamos o potencial antioxidante do extrato bruto da entrecasca do caule de *Pterodon pubescens*, uma planta típica do Cerrado Brasileiro, usando a técnica de Ressonância Paramagnética Eletrônica (RPE), em Banda-X. As medidas foram realizadas em diferentes concentrações de extrato bruto, diluídos em solução etanólica do radical livre DPPH (2,2'-difênil-1-picrilhidrazil) (1,0 mmol.L⁻¹). As análises foram realizadas no sentido de obtenção da capacidade do material em neutralizar a ação do radical DPPH. Observamos uma nítida relação entre a concentração do extrato e o decréscimo no sinal de RPE. Também observamos que uma pequena quantidade de amostra, cerca de 14,4 mg.L⁻¹, foi suficiente para reduzir o radical DPPH na solução em 50%, indicando assim que o extrato bruto das cascas de *Pterodon pubescens* apresenta grande potencial antioxidante. Alguns metabólitos secundários, como auronas, chalconas, cumarinas, flavanonas, fenóis, taninos e triterpenos foram

identificados no extrato por triagem fitoquímica. O elevado potencial antioxidante pode estar relacionado à forte presença de compostos fenólicos na amostra.

Palavras-chave: Antioxidante; RPE; *Pterodon pubescens*.

Introduction

There is a growing interest in the properties of natural antioxidants that can prevent the harmful effect of free radicals in metabolic reactions [1,2]. Some studies indicate that a regular ingestion of fruits and vegetables, that are naturally rich in antioxidants, can help the human body to fight against many diseases, including heart diseases, inflammatory diseases and cancer. There is a large quantity of compounds that can be used as an antioxidant substance, such as vitamins, minerals, natural pigments. Many of these compounds are obtained from stems, bark, leaves, fruits and seeds. In this work we investigated the antioxidant activity from the stem bark of *Pterodon pubescens*, a typical Brazilian plant commonly known by the locals as *sucupira branca*. This plant is easily found in the Cerrado, in the central area of Brazil.

The Cerrado is the second largest Brazilian biome that is second only to the Amazon. With a great biological biodiversity, it presents a wide diversity of its flora, with about 12 thousands listed species, occupying 21% of the Brazilian territory [3,4].

In the Brazilian Cerrado, there is a great diversity of vegetal species from which the locals usually collect some material to produce domestic medicine [5]. As a matter of fact, many species of plants in this ecoregion have a great pharmacological potential, which is explored in abundance by natives of the region, for the prophylaxis, due to its analgesic, antimicrobial, anti-ulcer, anti-inflammatory and anti-tumor effects [6-8]. However, research in this area is still scarce.

Materials collected from the *Pterodon pubescens* has been regularly used as a popular medicine against many diseases because of their anti-rheumatic, analgesic, and anti-inflammatory properties. Some phytochemical studies have revealed that *Pterodon pubescens* contains some natural compounds that can be related to the healing process [9]. However, it is still not clear which of these compounds are responsible for the healing process and how they work in the human body. Although it is not clear which compounds are involved in the healing process, somehow it involves a radical reduction. It is known that there is some correlation between the antioxidant activity and many physiological mechanisms, since the antioxidants reduce the oxidative stress in cells [10].

In this work we evaluate the antioxidant potential of the crude extract of *Pterodon pubescens*, using Electron Paramagnetic Resonance (EPR). The EPR technique is very useful to quantify free

radicals in samples. It is because the technique is highly sensitive to a small quantity of radicals. The EPR has already been applied to investigate antioxidant properties of other Brazilian species [11]. In addition, the phytochemical analysis was performed to find some secondary metabolites which might be associate to the antioxidant activity.

Experimental

Materials and methods

The vegetal material was obtained in the Brazilian Cerrado from the stem bark of *Pterodon pubescens*. The tree is found at 15°55'8.9'' S and 52°16'43.0'' W. After the taxonomy identification, the material was put into a desiccator at 40°C without any incidence of light. The dried material was milled, and the resulting powder was separated to process the crude extract.

Following some strategies for the preparation of the crude extract [12], about 1.0 Kg of the powder was immersed into a hydroethanolic solution at room temperature for 10 days. The solution was prepared using 4.8 L of ethanol and 1.2 L of distilled water. To facilitate the extraction, the powder solution was agitated occasionally. Then the solid material was filtered, and the solvent was recovered using a rotary evaporator under vacuum at 60°C. The crude extract was stored in a desiccator at 50-60°C, and the mass reduction was monitored for seven days. Since there was no longer reduction, the sample was removed from the desiccator and stored at 7-10°C. During all the preparation of the samples, the material was protected from bright light to avoid any degradation. The collected stem barks yielded 85 g of the crude extract, as it is shown in Figure 1.



Figure 1: The crude extract obtained from the stem bark of *Pterodon pubescens*.

To investigate the secondary metabolites, the method described by Matos (2009) [13] was followed, with some adaptations, which is based on color change or precipitate formation. It was prepared two samples of extracts, one hydrophilic and another lipophilic. For the hydrophilic sample it was used 700 mL of ethanol 70% (v/v), while for lipophilic sample it was used 570 mL of chlorophorm (CHCl_3) as solvent. Then 10 g of the dried bast was added to both solvents.

The antioxidant activity test was realized in an X-Band EPR spectrometer (Miniscope MS300 - Magnettech). The samples were prepared using a solution of DPPH (2,2'-diphenyl-1-picrylhydrazyl) at 1.0 mmol.L^{-1} , and at different concentrations of vegetal extract. It was prepared six samples with 200, 150, 100, 75 and 25 mg.L^{-1} of the extract. All samples were prepared from the same DPPH solution that is called in this work as the reference sample. In order to make reproductive EPR measurements, the samples were put into sealed capillaries. The antioxidant effect was observed by measuring the signal amplitude as a function of the time.

Results and Discussion

The EPR of DPPH dissolved in ethanol provides a signal that is shown in the Figure 2.

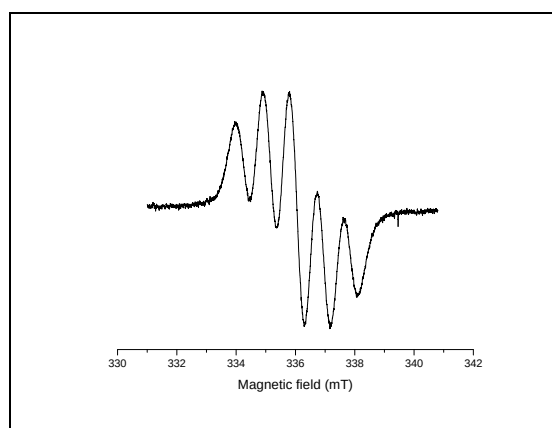


Figure 2: A typical EPR signal of the DPPH diluted in ethanol. The signature is related to the nitrogen hyperfine couplings.

This signal has a typical signature characterized by the well-reported nitrogen hyperfine couplings [14]. Our study is based on the capacity of the extract scavenge the radical. When some amount of the radical is scavenged, the EPR signal is reduced proportionately. In this reduction, the line shape remains the same. Therefore, there is a direct relation between the decreased amplitude peak to peak and the scavenged DPPH radical.

The Figure 3 shows the antioxidant effect of the vegetal extract on the 1.0 mmol.L^{-1} DPPH solution. The amplitude peak to peak (A_{pp}) was monitored for each five minutes, starting from the reference sample.

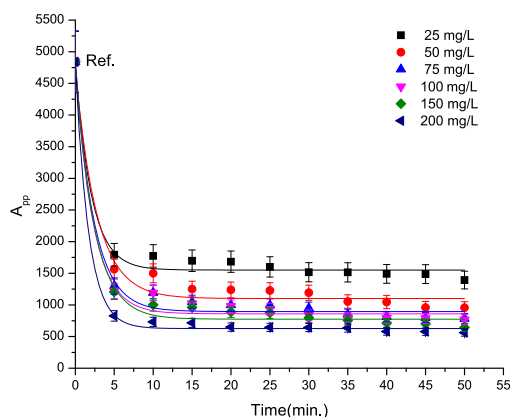


Figure 3: The signal amplitude peak to peak (A_{pp}) of DPPH solution. The amplitude decreases because the reaction between the DPPH and the extract. The exponential decay is a function of the concentration of the extract.

As can be seen in the Figure 3, the signal amplitude decays very quickly. It is necessary only few minutes for the signal achieve steady states. Since each magnetic field scan lasts one minute, it is inappropriate to use time interval much less than five minutes.

An exponential curve was fitted for each set of data as are shown in Figure 3. The exponential function used is each fitting obeys the following function:

$$A(x, t) = B(x) + C(x)e^{-kt} \quad (\text{Eq. 1})$$

where $A(x, t)$ is the signal amplitude and it is a function of the extract concentration x and time t ; while k represents the decay constant. For long time, the amplitude A becomes equal to the parameter B of the fitting curve. Thus, the parameter B represents the stabilized signal related to the reactions under the equilibrium condition. As there are many compounds in the vegetal extract that can contribute to the scavenging, it is not possible to understand detailed kinetic reactions in the process.

The Table 1 shows all the parameters of the curve fitted in the experimental data.

Table 1: Fitting results. The exponential curves (see Eq. 1) were fitted to the EPR amplitudes for each concentration x of the plant extract in the 1.0 mmol.L^{-1} DPPH solution. The parameter B represents the amplitude of the signal for a long time of reaction.

$x \text{ (mg.L}^{-1}\text{)}$	B	C	$k \text{ (min}^{-1}\text{)}$
25	1567	3271	0.49
50	1140	3694	0.36
75	926	3911	0.42
100	890	3997	0.44
150	805	4032	0.41
200	636	4204	0.60

As can be seen, the decay constant does not change very much. It is important to consider that the first ten minutes of the reaction are very determinative to the decay constant. However, the few data in this time region do not allow us to determine the decay constant precisely. Not surprisingly, the parameter B also decays exponentially as the extract concentration increases. The Figure 4 shows the parameter B as a function of the concentration, and the related fitting curve. We found that our stabilized signal can be modeled as:

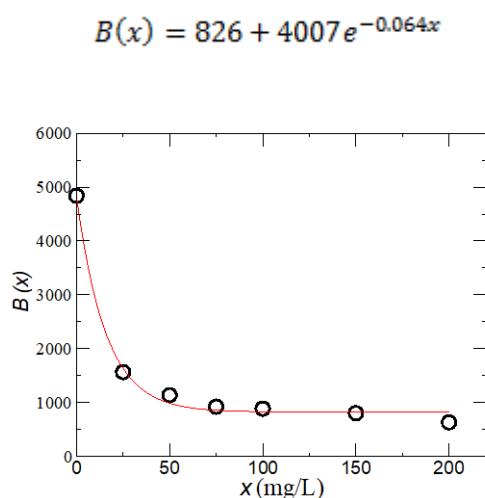


Figure 4: The amplitude of the stabilized signal according to the fitting results. This amplitude is modeled as an exponential decay.

This result allows us to determine the IC_{50} of the extract. The IC_{50} is the amount of material necessary to reduce the radical quantity by 50%. Thus the IC_{50} can be determined only by interpolating the above function at $B(0)/2$. In this work, we found IC_{50} for the extract is equal to 14.4 mg.L^{-1} . The small value for the IC_{50} indicates that the crude extract of *Pterodon pubescens* has a great antioxidant potential.

Some secondary metabolites that were found in the sample by phytochemical analysis are shown in Table 2. As can be seen, there were found aurones, chalcones, coumarins, flavanones, phenols, tannins and triterpenes.

Table 2: Chemical analysis of the secondary metabolites of the sample of *Pterodon pubescens*.

Secondary metabolites	Hydrophilic extract	Lipophilic extract
Aglyclones - flavonoids	(-)	(0)
Aglyclones - steroids and terpenoids	(-)	(0)
Alkaloids	(+)	(-)
Anthocyanins and anthocyanidins	(-)	(-)
Anthranols	(-)	(-)
Aurones and chalcones	(+)	(-)
Catechetical tannins	(+)	(+)
Coumarins	(+)	(+)
Flavanones	(+)	(-)
Flavones, flavonoids, flavononols and xanthones	(-)	(-)
Flavones, flavonoids, flavononols and xanthones -	(-)	(-)
Confirmation		
Free steroids	(+)	(-)
Leukanthocyanidins	(-)	(-)
Pentacyclic triterpenoids	(+)	(-)
Phenols	(+)	(-)
Pyrogalic tannins	(-)	(-)
Quaternary bases	(+)	(-)
Quinones	(-)	(-)
Resins	(+)	(0)
Saponins	(+)	(0)
Strong fixed acids	(+)	(-)

Legend: (+) = positive ; (-) = negative ; (0) = not accomplished.

As discussed by other authors [15,16], the secondary metabolites in the crude extract obtained from the *Pterodon pubescens* can explain the great antioxidant capacity of the sample. As it is shown in the phytochemical analysis, the sample is rich in phenolic compounds. The antioxidant activity can be promoted by flavonoids, tannins and other metabolites that scavenge free radicals and act as metal chelator [17]. Each phenolic compound can exert antioxidant activity in a particular way. However, it was not possible to investigate the detailed mechanism for the radical reduction.

Conclusions

In this work we used the Electron Paramagnetic Resonance to evaluate the antioxidant property of the crude extract of *Pterodon pubescens*, a typical Brazilian plant that is used by the local population for domestic medicine. We found that IC₅₀ for this extract is equal to 14.4 mg.L⁻¹, which is the amount necessary to scavenge 50% of the radical DPPH. This result indicates that extract of *Pterodon pubescens* has a great antioxidant potential. The phytochemical analysis provides important information about the presence of some secondary metabolites such as flavonoids and tannins that can be related to the strong antioxidant activity of the sample. Although it was not possible to correlate each natural metabolite to the antioxidant mechanism, this work provides some information that can be useful for many pharmacological purposes.

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References

- [1] S. C. Lourenço; M. Moldão-Martins; V. D. Alves. Antioxidants of natural plant origins: from sources to food industry applications, *Molecules*, 24 (22), (2019), 4132-4157. Doi: 10.3390/molecules24224132.
- [2] C. Zehiroglu; S. B. O. Sarikaya. The importance of antioxidants and place in today's scientific and technological studies, *Journal of Food Science and Technology*, 56 (11), (2019), 4757-4774. Doi: 10.1007/s13197-019-03952-x.
- [3] C. A. Klink; R. B. Machado. Conservation of the Brazilian Cerrado, *Conservation Biology*, 19 (3), (2005), 707-713. Doi: 10.1111/j.1523-1739.2005.00702.x.
- [4] E. E. Sano; A. A. Rodrigues; E. S. Martins; G. M. Bettiol; M. M. C. Bustamente; A. S. Bezerra; A. F. Couto Jr.; V. Vasconcelos; J. Schöler; E. L. Bolfe. Cerrado ecoregions: a spatial framework to assess and prioritize Brazilian savana environmental diversity for conservations, *Journal of Environmental Management*, 232, (2019), 818-828. Doi: 10.1016/j.jenvman.2018.11.108.
- [5] T. M. A. Alves; A. F. Silva; M. Brandão; T. S. M. Grandi; E. F. A. Smânia; A. S. Júnior; C. L. Zani. Biological Screening of Brazilian Medicinal Plants, *Memórias do Instituto Oswaldo Cruz*, 95 (3), (2000), 367-373. Doi: 10.1590/s0074-02762000000300012.
- [6] I. M. P. Violante; L. Hamerski; W. S. Garcez; A. L. Batista; M. R. Chang; V. J. Pott; F. R. Garcez. Antimicrobial activity of some medicinal plants from the Cerrado of the central-western region of Brazil, *Brazilian Journal of Microbiology*, 43 (4), (2012), 1302-1308. Doi: 10.1590/S1517-83822012000400009.

- [7] L. I. Silva; K. Arunachalam; F. Miyajima; I. M. P. Violante. Antimicrobial and antioxidant activities of selected plants used by populations from Jurueña Valley, Legal Amazon, Brazil, *International Journal of Pharmacy and Pharmaceutical Sciences*, 9 (5), (2017), 179-191. Doi: 10.22159/ijpps.2017v9i5.17086.
- [8] L. Santos; A. M. Viel; L. F. Tarosso; L. S. Momesso; D. A. Palmieri; K. D. Spera. Medicinal plants of the Brazilian Cerrado: knowing to preserve, *Bioscience Journal*, 36 (2), (2020), 556-567. Doi: 10.14393/BJ-v36n2a2020-42784.
- [9] D. Hansen; M. Haraguchi; A. Alonso. Pharmaceutical properties of 'sucupira' (*Pterodon* spp.), *Brazilian Journal of Pharmaceutical Sciences*, 46 (4), (2010), 607-616. Doi: 10.1590/S1984-82502010000400002.
- [10] M. Valko; J. Leibfritz; J. Moncol; M. T. D. Cronin; M. Mazur; J. Telser. Free radicals and antioxidants in normal physiological functions and human disease, *International Journal of Biochemistry and Cell Biology*, 39 (1), (2007), 44-84. Doi: 10.1016/j.biocel.2006.07.001.
- [11] A. B. Santos; D. H. S. Silva; V. S. Bolzani; L. A. Santos; T. M. Schmidt; O. Baffa. Antioxidant properties of plant extracts: An EPR and DFT comparative study of the reaction with DPPH, TEMPOL and spin trap DMPO, *Journal of the Brazilian Chemical Society*, 20 (8), (2009), 1483-1492. Doi: 10.1590/s0103-50532009000800015.
- [12] V. C. Filho; R. A. Yunes. Estratégias para a obtenção de compostos farmacologicamente ativos a partir de plantas medicinais. Conceitos sobre modificação estrutural para otimização da atividade, *Química Nova*, 21 (1), (1998), 99-105. Doi: 10.1590/s0100-40421998000100015.
- [13] F. J. A. Matos. *Introdução à fitoquímica experimental*, 3. ed. Fortaleza: EUFC, 2009.
- [14] N. S. Dalal, D. E. Kennedy; C. A. McDowell. Electron paramagnetic resonance (EPR) and electron-nuclear double resonance (ENDOR) of hyperfine interactions in solutions of α , α' -diphenyl- β -picryl hydrazyl (DPPH), *The Journal of Chemical Physics*, 59 (6), (1973), 3403-3410. Doi: 10.1063/1.1680483.
- [15] D. R. Vianna; G. Bubols; G. Meirelles; B. V. Silva; A. Rocha; M. Lanznaster; J. M. Monserrat; S. C. Garcia; G. Von Poser; V. L. Eifler-Lima. Evaluation of the antioxidant capacity of synthesized coumarins, *International Journal of Molecular Sciences*, 13 (6), (2012), 7260-7270. Doi: 10.3390/ijms13067260.
- [16] C. Jain; S. Khatana; R. Vijayvergia. Bioactivity of secondary metabolites of various plants: A review, *International Journal of Pharmaceutical Sciences and Research*, 10 (2), (2019), 494-504. Doi: 10.13040/IJPSR.0975-8232.10(2).494-04.
- [17] C. I. G. Tuberoso; C. D. Orrú. Phenolic compounds in food, *Progress in Food Chemistry*, chap. 1, p. 1-45, 2008. Doi: 10.1021/bk-1992-0507.ch001.