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RESEARCH ARTICLE

ANTIOXIDANT ACTIVITY *IN VITRO* FROM BARK AND WOOD CAPSULE EXTRACTS OF CASTANHA-DO-BRAZIL

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ABSTRACT

In this work we evaluated the antioxidant activity of ethanolic extract and fractions from *Bertholletia excela*, castanha-do-brazil, using the free radical scavenging method of free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). Currently many studies have focused on the inhibition of free radicals, as these are associated with various diseases related to the action of these chemical species. The UV-Visible spectroscopy apparatus was used to read the absorbances. With this data it was possible to obtain the antioxidant activity percentages (AA%) and the free radical inhibition of DPPH (EC_{50}). The extracts and fractions of *Bertholletia excelsa* bark showed lower DPPH inhibition value or approximate value compared to the *Ginkgo biloba* positive control. On the other hand, the wood capsule showed no significant value.

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INTRODUCTION

Bertholletia excelsa H. & B., Lecythidacea family, has 325 types of trees in the American tropics, divided into 15 genera, where the Bertholletia is dominant with 75 species (Brasil, 2002). The species was described first in 1808, when Humboldt and Bompland, and later Kunth, named as the majestic tree in the Amazon rainforest (Menninger, 1964). Large deciduous tree to 50 m tall and leaves simple. Fruits (pysidium) a globose wood capsule, lined with hard fibers; seed acutely trigonoid (10-25); packed in two concentric rings around a core; seed coat woody (Wickens, 1995). In 1961, Brazil regulated the name Castanha-do-Brasil for the purposes of foreign trade (Brasil, 1961). The fruits are one of the main products sold by the extractives (Vieira *et al.*, 2008) and a significant part of the Brazil nut has been exported to other countries (Camargo *et al.*, 1994).

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Chemical investigations show that the dry weight of Brazil nut contains about 17% proteins, 65% fat and 16.5% carbohydrates (Wickens, 1995) and is considered a protein source of fatty acids and fiber (Ferreira *et al.*, 2006). The bark of the tree contains organic acids, reducing sugars, glycosides, phenols and tannins (Campos *et al.*, 2011). Reactive oxygen species (ROS) and electrophiles cause DNA and neuronal cells damage, resulting in the development of malignant and neurodegenerative diseases (Lee and Johnson, 2004; Ferreira and Matsubara, 1997). The oxidative stress is no more than the imbalance between oxidants and antioxidants molecules that results in the induction of cell damage by free radicals, which can cause damage and cell death (Bianchi and Antunes, 1999). In a healthy cell the level of ROS is regulated by the antioxidant defense system, however, to the environmental stress or cellular damage the system do not detoxify the ROS generated and may suffer the oxidative stress (Liu *et al.*, 2007). Thus, the search for molecules that have antioxidants activity and low side effects has grown worldwide (Reddy *et al.*, 2012).

The literature reports chemical studies and antioxidant activity of the nuts from *Bertholetia excelsa* (John and Shahidi, 2010; Buratto *et al.*, 201; Pires, 2011), however the antioxidant activity of stem bark and wood capsule were not found in the literature, this work relates the first occurrence for the study of these parts of this plant. In this work we evaluated the antioxidant activity *in vitro* of the ethanolic extract and the fractions (ethyl acetate and acetone) from *Bertholletia excelsa* stem bark and the wood capsule by the method of DPPH (2,2-diphenyl-1-picryl-hydrazyl) free radical scavenging assay.

MATERIALS AND METHODS

The absorption measurements were determined on a UV-VIS 2450 Spectrometer, (Shimadzu, Tokyo, Japan) using as radiation source in the visible region (360-800 nm) a tungsten lamp and the ultraviolet region (190-360 nm) a deuterium lamp, with a dual beam for minimizing experimental deviations. The solvents were analytical PA grade (Vetec, Rio de Janeiro, Brazil or Synth, São Paulo, Brazil) and the chromatography column was used silica gel 60 (35-70 mesh) (Merck, Rio de Janeiro, Brazil), with the glass support columns varying diameter according to the amount of the ethanolic extract. *Ginkgo biloba* reagent was purchased commercially from GinkoLab 80 mg from the drug (Laboratory Multilab, São Paulo, Brazil), the radical DPPH was purchased from Sigma-Aldrich Co (São Paulo, Brazil). Cytotoxicity assays were realized in Biology Laboratory of Malaria and Toxoplasmosis (LaBMAT), Department of Microbiology and Parasitology, Bioscience Center, Universidade Federal do Rio Grande do Norte (UFRN). The animals were obtained from the animal house of UFRN, with an approval of the Ethics Committee for Animal Use (CEUA - UFRN n ° 043/2010). Black adult females mice were used for *in vivo* testing and received water and food *ad libitum* for used later in the tests.

Plant Material

The fruits of the *Bertholletia excelsa* were collected at the municipality of Canutama-Amazonas-Brazil (8°13’49”S; 64°01’51”W) and a voucher specimen (n. 903) was deposited at the Herbarium Rondoniensis (RON) of the Universidade Federal de Rondônia.

Extraction and Fractionation

The barks (1.34 kg) and the wood capsules (2.10 kg) were cut and dried to 40 °C, providing 1.2 kg and 1.9 kg of the dry material, respectively. The extract was prepared by percolation with EtOH 95% in starring. The solvent was evaporated to yield 326.48 g of bark and 15.84 g of wood capsule extracts. The ethanolic extracts were subjected to column chromatography, using silica gel 60 (35-70 mesh) eluting with solvents in polarity gradient Hexane, $CHCl_3$, EtOAc (ethyl acetate), ACT (acetone) and MeOH. It was realized tests for the steroids, terpenes, flovonoids and saponins (Silva *et al.*, 2010; Falkenberg *et al.*, 2009) with the extracts and fractions.

Cytotoxicity Assay

Cell viability was performed with the extracts and fractions used in this study. RAW cells (mouse macrophages) were used, maintained in continuous culture in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% bovine fetal serum in microaerophilic atmosphere (5% CO_2, 2% O_2 and N_2 balance) at 37 °C. The cytotoxicity of the extracts and fractions of *B. excelsa* was determined using the colorimetric assay metiltiazoltetrazólio (MTT) (Mosmann, 1983). For the macrophages assay, the cells were trypsinized, washed, suspended in RPMI without FBS, and the macrophages were counted in a Neubauer chamber. The cells were distributed into 96 wells per plate (1x105 cells/well) and then incubated in CO_2 atmosphere for 24 h at 37 °C. After this time, the extracts and fractions were diluted in distilled H_2O and were tested in range concentrations between 100-1,5μg mL^{-1}, in triplicate. In parallel, we performed a control group consisting of RPMI 1640 medium without fetal bovine serum. After a period of 24 h incubation of materials, 100 μL of MTT (5 mg mL^{-1} in RPMI 1640 medium without FBS and without phenol red indicator) was added to each well. After 3 h incubation in CO_2 incubator at 37 °C, the supernatant was removed and added to 100 µL DMSO in each well. The absorbance of each well was measured on spectrophotometric reading at 570 nm filter. Cytotoxic concentration values (CC_{50}) were obtained by the drug concentration-response curves where de results were expressed in mean±SEM determined from three separate experiments.

Antioxidant Activity

The antioxidant activity (AA) was performed with the ethanolic extract and the ethyl acetate and acetone fractions, with same adaptations from Mensor *et al.* (2001) methodology. *Ginkgo biloba* (GBL) was used as positive control. The drug was extracted with MeOH from medicine GinkoLab 80 mg®, yielding active ingredient GBL. Analyzes were performed in 100 mg L^{-1} and 50 mg L^{-1} concentrations, using MeOH as solvent. The stock solution of DPPH was used at a concentration of 100 mg.L^{-1}. The antioxidant activity was carried out with the ethanolic extract and the fractions ethyl acetate and acetone at concentrations of 100 mg L^{-1} and 50 mg L^{-1}. EC_{50} calculation was based on linear regression, from the results obtained of the antioxidant activity percentage (%AA), performing analyzes with different concentrations in order to get five different percentages of antioxidant activity between 10-90%. The absorbance readings were conducted setting the spectrophotometer at 600-450 nm mode, with fast speed and the deviation 1.0. Before reading in UV/VIS, the absorbance was discounted with MeOH, using the Auto Zero appliance. All analyzes were performed in triplicate, using as a blank a solution of 2.5 mL of the sample solubilized with 1.0 mL of MeOH. The reading of the samples (ethanolic extract and fractions) and GBL (positive control) with DPPH (0.25 mM) was performed separately with 2.5 mL of each sample (GBL, extract and fractions) and 1.0 mL of DPPH solution. This mixture was left in contact for 30 min for to react. Absorbance values were measured at 517 nm. The assay was performed in triplicate for all samples. As negative control was used MeOH (2.5 mL) in 1 mL of DPPH (0.25 mM) and the lecture was read in 517 nm region.

Statistical Analysis

The absorbance obtained were converted in percentage of antioxidant activity, as described by Mensor *et al* (2001) equation.

$$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100]/ Abs_{contro}\}$$

Where Ab_{sample}, Ab_{blank} $Abs_{control}$ representing respectively, the absorbance of the reaction of the sample with DPPH,

absorbance of the sample with the solvent and the absorbance of DPPH with solvent.

We used the ORIGIN 6.1 program to plot the linear regression to calculate of EC_{50} value. For cellular viability assay used was one-way ANOVA test using the Student-Newman-Keuls test, comparing different concentrations of the same compound relative to untreated control.

RESULTS

The qualitative test of substance classes for the bark of *B. excelsa* showed that the ethanolic extract, ethyl acetate and acetone fractions showed flavonoids, saponins and terpenes. As for the wood capsule was only observed the presence of terpene in the extract and fractions, with no occurrence of flavonoids and saponins. The standardization of DPPH showed that the concentration of 0.25 mM presented the best results; therefore, this concentration was utilized for all experiments.
When DPPH is consumed changes its color from purple to yellow, this consumption of DPPH is an index to estimate the antioxidant capacity, evaluating the scavenger the free radicals in the middle (Brand-Williams *et al*., 1995). The reading of DPPH in methanol showed a greater absorbance in 515 to 516 nm, characteristic of the alcoholic solution of DPPH (Sousa *et al*, 2007). The spectra of the samples (EtOH extract and fractions) used as white, showed a decay of absorbance with decreasing concentrations, as expected. The analysis of the samples with DPPH showed a higher absorbance in the range 515 and 520 nm in relation to their respective spectra of the blank analysis; it was observed that there was the addition of DPPH in the sample. The lower absorbance in the range, between 515 and 520 nm, the greater reduction of DPPH of the samples. The analysis of the AA percentage was performed initially with the concentrations of 100 mg L^{-1} and 50 mg L^{-1} (Table 1). Based on these results, analyzes were performed with different concentrations, in order to obtain five different percentages of AA between 10-95%, which was used for the calculation of EC_{50}.

Table 1. Percentage of antioxidant activity of the ethanolic extract and fractions of *Bertholetia excels*

Plant	Extract/Fraction	C (mg mL^{-1})	AA±SD
	EtOH	100	94,61±0,97
B. excelsa		50	93,95±0,77
(Bark)	EtOAc	100	95,37±0,12
		50	93,92±0,65
	ACE	100	93,42±1,15
		50	93,17±0,21
B. excelsa	EtOH	100	26,78±0,34
(Woody capsule)		50	24,65±0,62
	EtOAc	100	29,00±0,21
		50	17,10±0,05
	ACE	100	43,93±1,04
		50	24,91±0,68
G. biloba	-	100	94,70±0,13
		50	77,68±0,64b

AA% = Antioxidant activity percentage; AA±SD = Antioxidant activity, SD standard deviation

According to the Table 1 it is possible, observe that the extract and the fractions of the wood capsule from *B. excelsa* had lower antioxidant activity values than the positive control, *G. biloba* (32.04±0.17 µg.mL^{-1}). However, the bark of plant had performed promising results, especially for the concentration of 50 µg.mL^{-1} that showed AA% higher than *G. biloba*. The calculation of EC_{50} (Concentration needed to inhibit the oxidative reaction by 50%) showed that the ethanol extract and fractions from the bark of *B. excelsa* present excellent results compared with the wood capsule. The values of the EC_{50} of the extract and fractions from bark were lower than the standard *G. biloba*, since the samples from wood capsule exhibited EC_{50} greater than the standard *G.biloba* for both the extract and fractions (Table 2).

Table 2. Antioxidant activity of the *Bertholletia excelsa*

	C (µg mL^{-1})	%AAO	EC_{50} (µg mL^{-1})
Bark			
EtOH	10	81,18±0,37	
	7	78,12±1,20	
	5	55,88± 1,33	6,77±0,05
	3	36,12±0,66	
	12	20,80±1,28	
EtOAc	10	82,66±0,64	
	6	70,62±1,83	
	5	57,78±3,59	4,58±0,17
	3	36,34±2,95	
	2	24,91±1,27	
ACET	10	92,59±1,33	
	7	73,48±1,05	
	5	49,72±2,92	4,85±0,14
	3	34,34±2,07	
	2	23,76±1,38	
Woody capsule			
EtOH	300	79,90±1,12	
	250	73,22±1,85	
	200	47,24±3,55	166,21±12,51
	150	38,53±1,19	
	100	26,78±0,34	
EtOAc	400	80,93±0,64	
	300	69,68±0,64	206,11±2,80
	250	62,47±1,57	
	200	47,07±0,93	
	100	29,00±0,21	
ACET	250	82,42±2,05	
	200	66,20±1,36	132,31±0,32
	150	56,81±0,09	
	100	43,93±1,04	
	50	24,91±0,68	

Value obtained from regression lines. EC_{50} is defined as a concentration sufficient to obtain 50% of a maximum effect estimated in 100%; SD standard deviation.

Cell Viability Assay and Cytotoxicity

The *in vitro* cytotoxicity assay with the EtOH extract and fractions (EtOAC and ACE) of the bark of *B. excelsa* did not show toxicity in the *in vitro* assay. The ethanolic extract showed 91% of viability at the less concentration, 1.5 µg mL^{-1}. The EtOAc fraction showed that, particularly at the highest concentrations there was a cell growth higher than the control. In the concentration of 100 µg.mL^{-1} to 1.5 µg mL^{-1} the viability varied between 97% to 100%. In any of the tested concentrations were no statistically significant differences in relation to the control. The acetone fraction showed moderate cytotoxicity in the higher concentration (100 µg mL^{-1}), with a viability of 73% and CC_{50} 530 µg mL^{-1} in relation to the control. At lower concentrations (6.2, 3.1 and 1.5 µg mL^{-1}) revealed a significant viability 93, 95 and 98%, respectively.
In relation to the EtOH extract of wood capsule, there was a significant statistical difference in relation to the control. The viability was 87 to 91% at concentrations of 50 to 1.5 µg mL^{-1}, respectively. The EtOAc fraction in concentrations of 12.5; 6.2; 3.1 to 1.5 µg mL^{-1} demonstrated the viability of 90, 91, 92 and 89%, respectively. In this case, the three highest concentrations had significant statistically differences in relation to the control ($p>0.05$), showing cytotoxicity in 880 µg.mL^{-1}. The acetone fraction was less cytotoxic in more dilute

concentrations, 12.5; 6.2; 3.1; 1.5 µg mL^{-1}, with a viability of 85, 89, 88 and 84%, respectively.

DISCUSSION

Studies of antioxidant activity with plants, including extracts and active compounds has been widely disseminated since the diversity of the flora allows to discover active ingredients that may inhibit the action of free radicals in the human body. The human body undergoes constant action of ROS and RNA (Reactive Nitrogen Species) generated in inflammatory processes, by some biological dysfunction or from foods (Harris, 1992). Many compounds derived from plants are capable of reacting with free radicals, without developing the proliferation of these species, eliminating the risk of chain reaction. These are known as antioxidants that react directly with free radicals and reduce the stress oxidative and cell damage (Manach *et al.*, 2004). Currently, there is increasing interest in the identification of antioxidant compounds that are pharmacologically active and have low side effects. These compounds have been used in preventive medicine and food industry because these antioxidants help the body to fight the damage caused by oxidative stress from free radicals (Reddy *et al.*, 2012). There is a preference for natural antioxidants instead of the synthetic antioxidants source (Abdalla and Roozen, 1999), which has led an increase parallel of the utilization of methods to estimate the efficiency of the compounds as antioxidants (Schwarz *et al.*, 2001; Sánchez - Moreno, 2002). Mensor *et al.* (2001) analyzed the antioxidant activity of 15 plants collected in Brazil, using different plant parts. The assay was performed using the DPPH method, having as patterns *Ginkgo biloba* standardized and pure compound of rutin. In some cases, rutin behaved better than *Ginkgo biloba*, however, six plants assayed scored better against *Ginkgo biloba*, as in the case of *Anadenanthera peregrina* and *Pseudopiptadenia contorta*. The results were attributed to secondary metabolites present in plants. In the method of free radical DPPH, antioxidant efficiency is measured at room temperature, thus eliminating the risk of thermal degradation of the molecules tested. However, the reaction mechanism between the antioxidant and DPPH depends upon the structural conformation of the antioxidant molecule. The number of molecules of DPPH that are reduced seems to be correlated with the amount of hydroxyl radical available (Brand-Williams *et al.*, 1995). This method favors a quick and easy evaluation for antioxidant activity (Mensor *et al.*, 2001). The action mechanism of DPPH against antioxidants molecules is already known and has been proposed by Bondet *et al.*, (1997) in reaction with the BHT (butylated-hydroxytoluene) antioxidant.

The result of the antioxidant activity by the stable radical DPPH method can be expressed in various ways. For example, one may express the results as the ability to scavenge/decrease the percentage of DPPH at EC_{50}, that is the amount of antioxidant substance required to reduce by 50% the initial concentration of DPPH and also by antioxidant or antiradical power, which expresses the inverse relationship of EC_{50} (Brand-Williams *et al.*, 1995). DPPH is a stable free radical that reacts with compounds, which are able to donate a hydrogen atom (Blois, 1958; Brand-Willians *et al.*, 1995). There are some compounds which are used as standard control antioxidants from natural or synthetic source, where the most used are vitamin C and E (Bianchi and Antunes, 1999), *Ginkgo biloba* (Mensor *et al.*, 2001), gallic acid and rutin (Cerqueira *et al.*, 2007; Sousa *et al*, 2007) and BHT (Melo *et al.*, 2003). The oil from the nuts of *B. excelsa* is rich in α– and γ–tocoferois, which are isomers observed in relatively constants levels in all oils evaluated in samples from different geographic areas of the Amazon region. The tocopherols profile extracted from the nuts can be appointed as chemical markers in control of quality and authenticity (Funazaqui, 2013). The antioxidant activity of chestnuts (Burato, 2011; John and Shahidi, 2010), may be associated with tocopherols found in the chemical composition of this part of the plant. In this work, the *B. excelsa* analyzes were performed with extracts and fractions of bark and wood capsule, separately. The EtOH extract and the fraction ACT and EtOAc of the bark showed the AA values for concentrations of 100 mg L^{-1} and 50 mg L^{-1} greater than 93% and EC_{50} values of these samples were better than that found for the *G. biloba* (Table 1).

In contrast, the results of the EtOH extract and fractions (EtOAc and ACT) from the wood capsule at the same concentrations were not significant as oxidation inhibitors, displaying much higher than standard. The literature reports the evaluation of the antioxidant activity only of the nuts from *B. excelsa*. John and Shahidi (2010) analyzed the whole almonds, the epidermis that involve the nut and the nucleus of the Brazilian nut. The brown skin showed the highest concentration of phenolic compounds and showed higher antioxidant activity than the whole almond. Buratto *et al.* (2011) examined the EtOH extract of almonds *B. excelsa* as the antioxidant activity, getting a better result than BHT used. This same study evaluated the antibacterial activity, where the extract did not show activity. Almonds of *B. excelsa*, have high selenium content and high amounts of phenolic compounds, with significant results for antioxidant activity (Pires *et al.*, 2011). There are few studies reporting the activity of bark *B. excelsa*. Among them we can mention the work that relates the biological activity of the bark done by Campos *et al.* (2011), which identified the metabolic groups for the hydroalcoholic extract of the bark, organic acids, reducing sugars, phenols and tannins, saponins and depsídios and depsidonas. This extract showed antimicrobial activity in assay front of *Klebsiella pneumoniae* and *Escherichia coli* microoganism where the first is more susceptible to the inhibition by the extract. Natural antioxidants are mainly originated from plants in the form of phenolic compounds (flavonoids, phenolic acids, alcohols, stilbenes, tocopherols, tocotrienols), carotenoids and ascorbic acid (Reddy *et al.*, 2012). The amount of antioxidant molecules, particularly flavonoids and carotenoids are compounds of particular interest, as they are commonly found in food and are relevant for human health (Racchi, 2013). This work presented the first report on the antioxidant activity of the bark and the wood capsule of B. *exlcesa*, in addition to reporting the cytotoxic activity of these extracts and fractions. The high performance of the antioxidant activity of the bark may be related to the presence of flavonoids, both in EtOH extract and, ethyl acetate and acetone fractions. The flavonoids are known to be related to oxidation processes (Rocha *et al.*, 2010) and are electron donors, causing the neutralization of free radicals involved in this process (Machado *et al.*, 2008). In addition, we emphasize the cytotoxic activity to the array of study where the extracts and fractions showed satisfactory cell viability. The data from our study demonstrate the potential antioxidant activity of the bark of B. exlcesa towards DPPH radical, with a better performance in comparison to the standard, *Ginkgo biloba*.

Conclusion

Our study data demonstrated that the bark of *B. excelsa* showed highest antioxidant activity in respect to DPPH radical, and all samples analyzed showed better antioxidant potential than *Ginkgo biloba*. The wood capsule of *B. excelsa* did not show satisfactory results compared to the *G. biloba*, with EC_{50} greater than 130 $\mu g.mL^{-1}$. The data of this research demonstrated the antioxidant potencial of *B. exlcesa* bark compared to the wood capsule. It is important to report that this is the first study describing the antioxidant activity of bark and wood capsule of *B. excelsa*, Castanha-do-Brasil.

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