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Acacia mangium Willd (Acácia) Extract Antioxidant and Antiproliferative Activities

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Abstract: Introduction: According to World Health Organization about 80% of the world's population use medicinal plants to healing diseases, including those caused by free radicals, formed during biological oxidative processes or by exposure to exogenous factors. Plants like *Acacia mangium* Wild (Fabaceae) must have their antioxidant activity aiming at the discovery of new drugs. **Objective:** Considering that studies integrating the phytochemical, antioxidant and antiproliferative aspects of medicinal plants are scarce, the present work aimed to characterize the antioxidant performance of the leaf ethanol extract of *Acacia mangium* Willd. **Methods:** The leaf ethanol extract of *A. mangium* Willd. had its antioxidant activity by free radicals scavenging by ABTS method, by ferrous ion (Fe^{2+}) chelating activity and by the β-carotene/linoleic acid system, correlating this performance to the chemical components of this plant, as well as the evaluation of antiproliferative activity (*in vitro*) that was analyzed through the MTT assay in tumor lineage cells Sarcoma 180. **Results:** *Acacia mangium* Willd. has antioxidant, antifungal and mutagenic activities and its extract presented high flavonoid and tannin indices and exhibited an important antioxidant action, both by the ABTS method and by the β-carotene method, however, this activity was not observed by the Fe^{2+}quelation method. These results reinforce the thesis, already defended by other authors, that flavonoids and tannins are probably the holders of the antioxidant action of this species. The results of antiproliferative activity assays showed that the extract was effective in inhibiting the proliferation, in vitro, of Sarcoma 180 cells, which is an unprecedented data regarding the species *A. mangium* Willd. **Conclusion:** The leaf ethanol extract of *Acacia mangium* showed an important antioxidant and antiproliferative activities.

Key words: Biological activities, Antioxidants, Antiproliferative activity, Plant extract, Phenol.

1. Introduction

Reactive oxygen species (ROS) have been considered for years as major responsible for cardiovascular diseases, cancer, decline of the immune system, and aging. In living beings, those who control the production and elimination of ROS are antioxidants. Antioxidants are able to stabilize or neutralize these molecules before they damage the cell, inducing diseases [1].

ROS, such as hydroxyl radical (•OH), superoxide radical anion (O2•–), hydroperoxide (ROO•) and hydrogen peroxide (H_2O_2), are normally formed during cell metabolism, assisting in the regulation of homeostasis and controlling cell activity modulating signaling pathways [2]. However, when there is an overproduction of ROS, cells can enter a state known as oxidative stress and if there are no antioxidants available in vivo, genetic damage by DNA modification, leading to a mutagenesis and carcinogenesis, or oxidation of lipids and proteins [3].

The antioxidant cell system controls ROS levels in a cell and protects it from oxidative stress. In general, antioxidants are chemicals that, when present in low concentrations, compared to the oxidisable substrate, delay or inhibit oxidation of the substrate effectively [4]. Endogenous antioxidants can be enzymatic, such as superoxide dis-mutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), and include

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NADPH (Nicotinamide Adenine Dinucleotide Phosphate) and ubiquinol-10. In turn, those from exogenous sources are ascorbic acid, α-tocoferol, vitamin A, carotenoids, licopen and phenolic compound [5, 6]. However, due to the possible adverse effects of synthetic antioxidants, the food and pharmaceutical industries have turned their attention to finding natural products with antioxidant activity in order to replace synthetic ones or make an association between them [6, 7]. Important organisms that are thoroughly investigated due to their significant antioxidant activity and the wide variety of classes of associated phytochemicals are plants [8, 9].

Currently several scientific studies try to understand the mechanism of action of secondary metabolites present in plants and relate them to medicinal actions, such as phenolic compounds that are potent antioxidants. These bioactive compounds of plant origin have an important demonstration in the bibliography as a good alternative of protection for the human body against damage induced by reactive species [10]. However, it should not be forgotten that the antioxidant efficiency of these compounds depends on their chemical structure and their concentration, which can vary from plant to plant, since environmental conditions and genetic factors influence the quantification of these substances in vegetables.

Several plants and their derivatives have been studied and characterized due to the growing economic interest and the wide use of plant products for phytotherapeutic purposes. It is known that many compounds present in herbal medicines may exhibit antioxidant activity, while they can also induce DNA damage and generate chromosomal mutations. Thus, studies that elucidates or demonstrates the potential antioxidant, antimutagenic or antiproliferative actions of a medicinal plant are of great importance in the field of phytotherapeutic research.

Acacia mangium Willd. is a Fabaceae family member from Australia, Papua New Guinea and Indonesia. This genus is known to include many fast-growing species with various biological activities [11]. It has antioxidant and antifungal activities [12], mutagenic and cytotoxic property [13], especially for human breast carcinoma [14] as well, in recent years; it has been used to obtain tannins because of its richness in polyphenolic compounds.

Thus, considering the importance of research integrating phytochemical, antioxidant and anticancer aspects from medicinal plants, we see the need to improve existing studies as well as the proposition of new investigations on the functionality of the species in question.

2. Methods

2.1 Ethanolic Extract Preparation

The experiments were conducted in the Laboratory of Plant Genetics and Toxicology, located at the Federal University of Espírito Santo (UFES). For the preparation of ethanol extract leaves of *Acacia mangium* Willd (Fabaceae) were collected at Parque Estadual de Itaúnas, a protected area in Conceição da Barra city, Espírito Santo, Brazil and the crude extract was obtained by exhaustive maceration in absolute ethanol P.A. (99.3%) at room temperature (25-30 °C), protected from light for five days. Subsequently, the material was filtered and concentrated on a rotary vacuum evaporator. A voucher specimen was deposited in the Herbarium of UFES – VIES at Vitória, Brazil (registration number: 38066).

2.2 Total Flavonoids Compounds (TFC)

Total flavonoid content (TFC) was measured using the colorimetric method [15] with modifications. In sealed tubes, 1.5 mL of methanol solution of $AlCl_3 \cdot 6H_2O$ (2% w/v) was added to 0.5 mL of extract. After 10 minutes in the dark, the absorbance at 430 nm was detected in a spectrophotometer for microplate (Epoch Microplate Spectrophotometer - BioTek®). The methanolic $AlCl_3 \cdot 6H_2O$ solution was used as blank and the experiment was performed in triplicate. Methanolic

dilutions series of rutin were prepared and assayed. The amount of flavonoid in extract was expressed in milligram of rutin equivalent flavonoid per gram of dry matter of extract.

2.3 Total Tannin Compounds (TTC)

Total tannin content (TTC) was determined by the Folin-Denis method [16] with minor modifications. An ethanol extract 500 μg mL^{-1} (500 μL) was added to 500 μL of Folin-Denis reagent. After 3 min, 500 μL of Na_2CO_3 solution (8%) was added, mixed and maintained for 2 h at room temperature and in the dark. The material was centrifuged at 2,000 rpm for 5 minutes, the supernatant was added in microplate, and the absorbance measured at 725 nm. The ethanol was used as blank and the experiment was performed in triplicate. Distilled water dilutions series of tannic acid were prepared and assayed. The results were expressed as tannic acid equivalents per gram of dry weight.

2.4 Antioxidant Activity by Free Radicals Scavenging by ABTS Method

Total antioxidant activity of *A. mangium* Willd. extract was measured by capturing $ABTS^+$ radical derived from 2,2-azinobis-(-3-ethylbenzothiazoline)-6-sulfononic acid, using the method described by Re et al [17] with modifications. The $ABTS^+$ radical was generated by mixing 5 mL of solution ABTS (7 mM) with 88 uL of potassium persulfate solution (140 mM), and allowed to react for 16 h at room temperature in the dark. Then, the $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.7 (±0.05) at 734 nm. In a microplate, 200 μL of $ABTS^+$ solution was added to 40 μL of the *A. mangium* Willd crude extract at different concentrations. After 6 minutes the start of the reaction, at room temperature and protected from light, the absorbance was immediately taken by ELISA Epoch BioTek® reader at 734 nm. The experiment was performed in triplicate and the percentage of scavenging inhibition capacity of $ABTS^+$ of the *A. mangium* Willd extract was calculated and compared with a standard (Trolox) and a control (without antioxidant), by the following formula:

$$\%\ \text{scavenging} = \frac{(\text{Abs0} - \text{Abs1})}{\text{Abs0}} \times 100$$

where Abs0 = absorbance of control and Abs1= absorbance of the sample.

2.5 Antioxidant Activity by Chelating Activity on Fe^{2+} Ions

Ferrous ions (Fe^{2+}) chelating activity was measured by inhibition of ferrous–ferrozine complex formation after treatment with *A. mangium* Willd crude extract with Fe^{2+}, using the method of Jayakumar and Murugan [18]. Iron ion may lose their pro-oxidant properties when chelate. Briefly, 1 mL of extract methanolic solution (31.25, 62.5, 125, 250 and 500 μg mL^{-1}) was added to 50 μL of $FeCl_2$ solution (2 mM) and incubated at room temperature for 5 minutes. The reaction was initiated by the addition of 200 μL ferrozine (5 mM) in distilled water, mixed and the absorbance measured at 562 nm after 20 min in a micro-plate reader. The percentage ferrous ion chelating effects of the *A. mangium* Willd extract and standard (EDTA) were calculated using the following equation:

$$\text{Chelating activity}\ (\%) = \frac{\text{Abs0} - \text{Abs1}}{\text{Abs0}} \times 100$$

where Abs0 is the absorbance of Fe^{2+} reaction control and Abs1 is the absorbance of the sample. The assay was carried out in triplicate.

2.6 Antioxidant Activity by β-carotene/linoleic acid assay

The antioxidant activity was also evaluated using the β-carotene/linoleic acid system according to Duarte-Almeida et al [19]. Briefly, the system solution was prepared by adding 1 mL β-carotene solution (0.5 mg/mL of chloroform), 80 μL of linoleic acid and 530 μL of Tween 40. Subsequently, the chloroform was completely evaporated with oxygenator, after which 50 mL of oxygenated water was mixed with the

solution and the system solution was protected from light. Then, 40 μL of *A. mangium* Willd crude extract ethanolic solution or standard Trolox was added to 250 μL of the system solution. Absorbance was measured at 470 nm immediately (t = 0 min) and after 120 min of incubation at 50 ℃. The results were calculated as the percentage inhibition of oxidation, by the following formula:

$$\% \text{ Inhibition of oxidation} = \frac{\text{Abs0} - \text{Abs1}}{\text{Abs0}} \text{ x } 100$$

where Abs0 is the "absorbance initial - absorbance final of control" and Abs1 is the "absorbance initial - absorbance final of sample". The assay was carried out in triplicate. Percentage of oxidation inhibition of extract and Trolox was expressed as percentage at the concentration of 500 μg.mL^{-1}.

2.7 In Vitro Sarcoma 180 Anticancer by MTT Bioassay

Sarcoma 180 cells (murine sarcoma) were acquired from Cells Bank of Rio de Janeiro and the protocols were approved by the Research Ethical Committee of Universidade Federal do Espírito Santo under process number 89/2015. Sarcoma 180 were cultured with RPMI 1640 (Cultilab) supplemented with antibiotic gentamicin (50 mg.L^{-1}), anti-fungal amphotericin B (2 mg.L^{-1}), 10% fetal bovine serum (Gibco) and cells were maintained at 37 ℃ and CO_2 5% saturation. Cells were maintained under these conditions 24 hours before the beginning of treatments. Sarcoma 180 cells were plated in 96-well plates, 2 x 10^5 cells/well and treated with *A. mangium* Willd extract diluted with water at 10.0, 50.0 or 100.0 μg.mL^{-1}. Cells were cultured with *A. mangium* Willd extract for 24 h or 48 h to evaluate its anticancer activity.

The MTT (3-(4,5- dimethyl-2- thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide) assay was performed to assess cell viability. After the last treatment, the plates were centrifuged at 860 rcf for 10 minutes, the supernatant was discarded, 20 μL of MTT (mg.mL^{-1}) were added to each well and the cells were incubated for an additional 3 h. The plates were centrifuged at 860 rcf for 5 minutes, the supernatant was discarded and 100 μL of DMSO were added. The absorbance was measured at 630 nm by ELISA reader (Epoch – BioTech®). The experiment was performed in triplicate and the relative cell viability was calculated using negative control cells as 100% of cell viability, calculated with the following equation:

$$\% \text{ Cell viability} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \text{ x } 100$$

2.8 Statistical Analysis

It was performed from the two-way ANOVA Analysis of Variance followed by the Sidak test, for the analysis of antioxidants ($p < 0.05$).

3. Results

Total content of flavonoids and tannins in leaves ethanolic extract of *A. mangium* Willd foliar were 483.70 ± 3.08 mg.g^{-1} (rutin equivalent) and 116.75 ± 9.24 mg.g^{-1} (tannic acid equivalent), respectively.

Figure 1 shows the results of the determination of antioxidant activity by the $ABTS^+$ radical method. It can be noted that at concentrations of 500; 250 and 125 μg.mL^{-1}, there was no significant difference between the extract of *A. mangium* Willd and the Trolox pattern, while at concentrations of 62.5 and 31.25 μg.mL^{-1}, there was a significant difference, and the extract exhibited higher activity than the standard in all concentrations. These results indicate an important antioxidant activity for the species, a condition that favors its medicinal use.

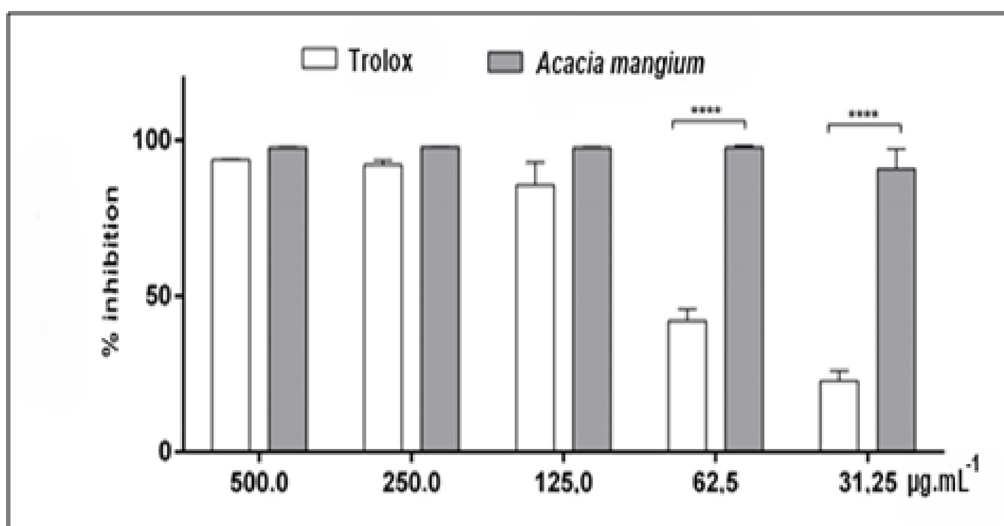

Fig. 1 Percentage of inhibition of $ABTS^+$ radical from different concentrations of ethanol extract of *A. mangium* Willd., compared to the Trolox pattern. Results expressed on average ± standard error. Two-Way ANOVA, Sidak test, $p < 0.0001$.

The chelating activity of Fe^{2+} of the ethanol extract of *A. mangium* Willd. (Figure 2) was significantly lower for all concentrations compared to the EDTA standard.

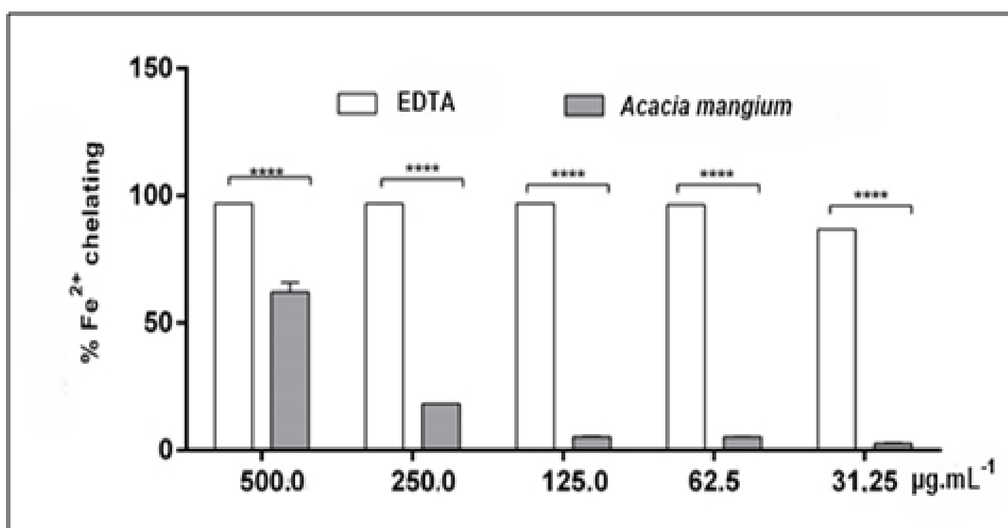

Fig. 2 Percentage of Fe^{2+} quelation of different concentrations of ethanol extract of *A. mangium* Willd. compared to the EDTA standard. Results expressed on average ± standard error. Two-Way ANOVA, Sidak test, $p < 0.0001$.

The extract of *A. mangium* Willd. showed a high protection activity to the β-carotene/linoleic acid system compared to the Trolox standard at the highest concentrations (500 and 250 $\mu g.mL^{-1}$). The antioxidant condition is maintained in the other concentrations tested, even showing a tendency to fall with action comparable to that displayed by the pattern (Figure 3).

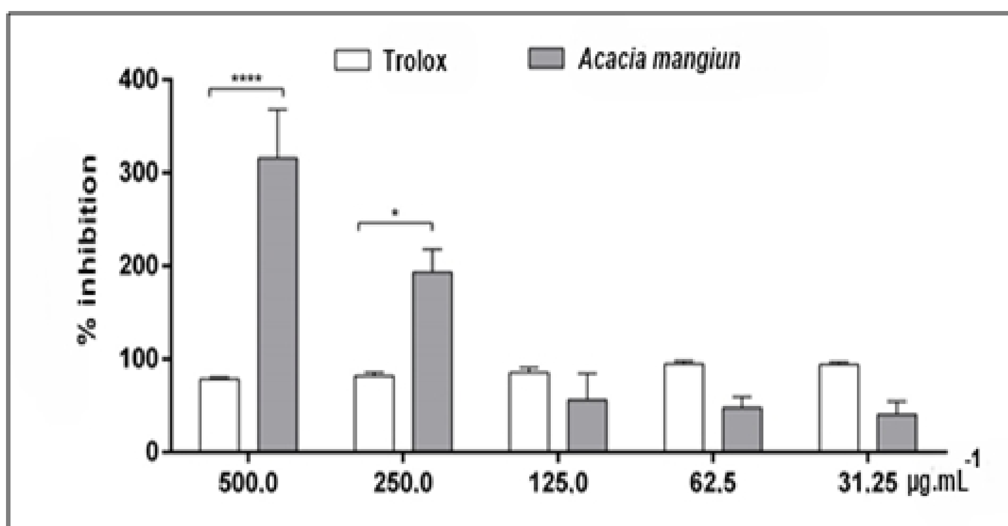

Fig. 3 Antioxidant activity of the ethanol extract of *A. mangium* Willd. and the Trolox pattern at different concentrations by the β-carotene/linoleic acid method. Results expressed on average ± standard error. Two-Way ANOVA, Sidak test, $p < 0.0001$.

Analyzing the antiproliferative activity test, it is observed that the leaf ethanol extract of *A. mangium* Willd. in all concentrations (10; 50; 100 µg.mL^{-1}) and tested times (24; 48; 72 hours) presented significant cytotoxic activity for Sarcoma 180 cells in relation to the control. In the negative control, when the evaluation times were over, compared to the 24-hour time, in which there were 100% of viable cells, there was an increase of 33.61% and a decay of 29.88% of these cells, at 48 and 72 hours, respectively. Regarding the treatments, the dose of 50 µg.mL^{-1} was the most cytotoxic for all times tested, and the 24-hour dose presented an inhibition of 77.73% of the cells, while in the time of 48h the inhibition reached 86%, that is, only 14% of viable cells (Figure 4).

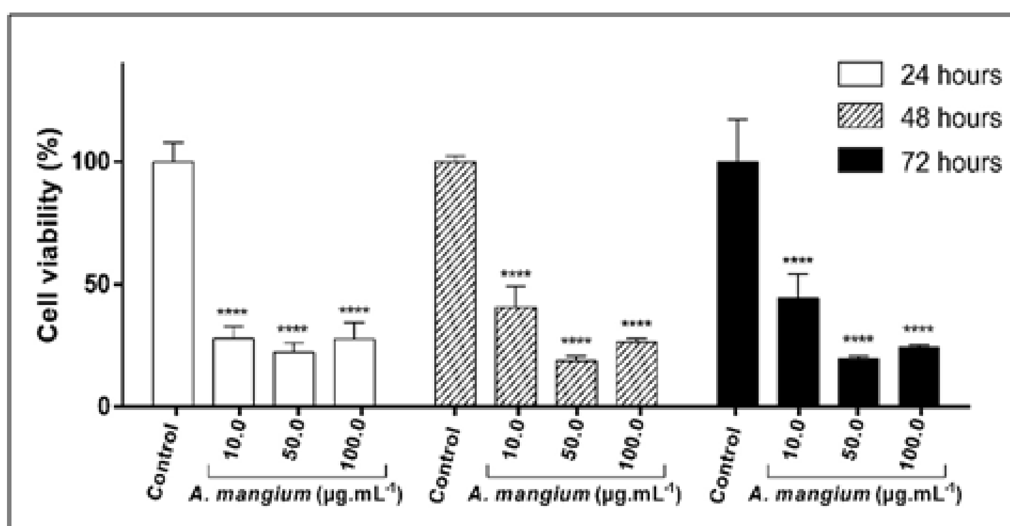

Fig. 4 Evaluation of the viability of Sarcoma 180 cells at different times of treatment with ethanol extract of *A. mangium* Willd. compared with control group cells (not treated) by the MTT method. Two-way ANOVA post hoc Dunnett's multiple comparisons test ($p < 0.05$).

4. Discussion

Many compounds present in plants have the ability to capture reactive oxygen species and, among these substances, one can highlight those with phenolic nucleus, as is the case of flavonoids and tannins [20]. The result found here indicates that *A. mangium* Willd has considerable total flavonoid content, corroborating information cited by Andrade et al [20] that consider the genus *Acacia* an important source of phenolic compounds. These also correlated the contents with the antioxidant activities exhibited by different species of the genus.

In the genus *Acacia*, there are also reports of the presence of tannins in different species, with variations in their contents and the types found in each of them [21, 22], including records in the species *A. mangium* Willd [23], reinforcing the results presented here. Studies also show that tannins and other phenolic compounds derived from plants are responsible for antioxidant and antimicrobial activities against fungi and bacteria [12, 24].

Among the various methods for the evaluation of antioxidant activity, the scavenging of free radical ABTS has been one of the most used, due to its high sensitivity index, speed and stability. This assay can be used to measure the activity of hydrophilic and lipophilic compounds [25, 26].

When evaluating the antioxidant activity of *Acacia hydaspica*, by the $ABTS^+$ radical method [27] detected a better activity of some of the fractions tested in relation to the ascorbic acid pattern, which reinforces its potential use in this sense. In addition, these authors also indicated a direct significant correlation between the elimination activity of the $ABTS^+$ radical and the total content of phenolic compounds, as well as the total flavonoid content, which reinforces the idea that the flavonoids found in *A. mangium* Willd. match the antioxidant activity exhibited in the evaluation by the ABTS method.

Chelating activity has been used to determine the capacity of the components present in plant extracts for the sequestration of free metal ions. These metal ions are important catalysts for the generation of highly reactive hydroxyl radicals via Fenton reaction *in vivo* and *in vitr*o systems [28].

The low observed activity can be explained as suggested by [29] that metal quelation is related to the structures of the substances present in the medium, in which the bonding efficiency of the phenolic compound with metal ions may be dependent on the position in which the electron donor group is in the polyphenol. Thus, the results of this analysis suggest that the compounds with this activity are in low concentrations in the extract of the studied plant and, therefore, it can be inferred that this is not the main mechanism involved in the antioxidant action of the substances extracted by the solvent used in this experiment with *A. mangium* Willd.

The antioxidation of β-carotene/linoleic acid is a method that differs from the others tested in the present study, because it has an emulsion as a reaction matrix. In emulsions, greater protective efficacy of lipophilic antioxidants is reported [31]. Phenolic compounds have different polarities due to several factors, such as the chemical nature of these substances, interaction with other nutrients present in the sample and the polarity of the solvent used for extraction [32]. In your work [33] observed that plants with higher levels of phenolic compounds presented higher antioxidant potential by the ABTS method and by the β-carotene/linoleic acid method, corroborating the results of this study.

In evaluation of the anticancer activity of the methanol extract of *Acacia macrostachya* in KB tumor cells [35] reported a significant cytotoxic effect of the extract, with inhibition of up to 95% of the cells. They reinforce that the presence of triterpenes, steroids and polyphenols (flavonoids and tannins) may justify the anticancer activity of this plant. The contribution of bioactive metabolites of species extracts of the genus *Acacia* to the inhibition of tumor

cell viability is already reported being, that in these cases, flavonoids were indicated as metabolites with little or no cytotoxic effect on healthy cells while they are cytotoxic against several human cancer cells [27, 36].

The leaf ethanol extract of *A. mangium* Willd. showed high rates of flavonoids (483.70 ± 3.08 mg.g^{-1}) and tannins (116.75 ± 9.24 mg.g^{-1}) and, following experiments, exhibited an important antioxidant action, both in the verification by the ABTS method and by the β-carotene method, showing better than the standards. However, this activity was not observed in the ferro^{2+} quelation system. These results reinforce the thesis, already defended by other authors, that flavonoids and tannins are probably the holders of the antioxidant action of this species, which is reinforced by studies with other plants of the genus Acacia and other groups of vegetables. The results of antiproliferative activity assays showed that the extract was effective in inhibiting the in vitro proliferation of Sarcoma 180 cells, which is an unprecedented data regarding the species *A. mangium* Willd. The studied species can be considered an alternative source of anti-oxidants.

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Conflict of Interests

The authors declare that does not exist an interest conflict.

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