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Full Length Research Paper

Effect of auxin and cytokinin on phenolic content of *Baccharis myriocephala* DC. (Asteraceae) produced *in vitro*

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A protocol for large-scale *in vitro* production of *Baccharis myriocephala* was developed. Total phenolic content was analyzed in plants cultured on MS + 1.0 mg.L^{-1} of indoleacetic acid (IAA), benzyladenine (BA) or kinetin (KIN). Nodal segments obtained from seeds germinated *in vitro* on Murashige and Skoog basal medium (MS) were subcultured on MS basal medium, hormone-free or with different growth regulators added: IAA, BA or KIN. The total phenolic content was determined using the Folin-Denis spectrophotometric method, and the content of phenolics of extracts was expressed in terms of gallic acid-equivalent (µg GA/mg dry weight). The best growth and development of the plants was induced by MS + 1.0 mg.L^{-1} IAA, which stimulated the production of 23 new nodal segments from a single phytomer after 60 days of *in vitro* culture. However, MS + 1.0 mg.L^{-1} IAA accumulated the lowest phenolic content, and only 18.46 µg/mg dry weight. A significant negative correlation was noted, since the phenolic content increased when the dry weight decreased. This culture system is capable of supplying good-quality raw material in adequate quantity to meet the growing demand for medicinal plants. However, the accumulation of polyphenols is lower in cultures with higher dry weight.

Key words: Carqueja, micropropagation, growth regulators, phenolics accumulation.

INTRODUCTION

Baccharis myriocephala DC. (Asteraceae) is a three winged branched shrub; in Brazil, this and other species of the genus are known as *carqueja* (Simões-Pires et al., 2005; Abad, 2007). The use of medicinal plants such as *carqueja* generates an economic value that sometimes leads to cultivation of the species (Castro et al., 1999a) to meet the demands of industry and traditional users (Gupta and Birdi, 2017; Park et al., 2017). In natural

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populations, unregulated extraction contributes to a decline in numbers, consequent loss of genetic variability, and even extinction (Martinelli and Moraes, 2013; Leitão et al., 2014).

The allogamy of *Baccharis* species is the source of considerable genetic variability (Castro et al., 1999a, b). This causes difficulties in seedling production, generates plants with non-standard agronomic and phytochemical attributes, and consequently reduces the potential of a plant as raw material for medicine preparation.

In vitro culture techniques such as micropropagation have been widely used as an alternative to produce good-quality raw materials from the phenotype of interest (Cardoso and Teixeira da Silva, 2013; Ozarowski and Thiem, 2013; Ahmad et al., 2015). The micropropagation technique enables a plant to be produced when there are difficulties in the natural mechanisms of reproduction, preservation of endangered species, and maintenance of plant germplasm collections, and is the only tissue-culture technique that has been effective for large-scale production of plants of economic interest (Cardoso and Teixeira da Silva, 2013; Chen et al., 2016). Phenolic compounds, widespread in the plant kingdom, have beneficial effects on human health, such as reduction of blood glucose levels (Oliveira et al., 2014), antioxidant effects (Pádua et al., 2010) and many others (Hossen et al., 2017; Hu et al., 2017). Biotic and abiotic factors produce changes in the phytochemical profile of plants and may increase the production of chemicals of interest (Sartor et al., 2013; Victório et al., 2015).

The potential use of *carqueja* as raw material for a plant drug motivated the authors to develop a protocol for *in vitro* propagation of *B. myriocephala,* in order to provide an optimized alternative for large-scale production. The effects of different growth regulators on the development of *in vitro* plants and on the production of total phenolics were investigated.

MATERIALS AND METHODS

Plant material

Achenes of *B*. *myriocephala* were collected in Santa Rita de Jacutinga, Minas Gerais State, Brazil, and were identified by Gustavo Heiden from the Rio de Janeiro Botanical Garden Research Institute. A voucher specimen of this plant material was deposited in the Herbarium (RB 439.238) at the Institute.

Plant tissue culture

Achenes were pre treated by immersion in 2% commercial bleach solution for 20 min, and then rinsed thoroughly in running tap water inside the laminar air-flow chamber, the achenes' surfaces were sterilized in 70% ethanol for 20 min, followed by 15 min in a 20% commercial bleach solution containing 2 to 3 drops of Tween 20, and rinsed three times with sterile distilled water. The disinfected achenes were inoculated on MS (Murashige and Skoog, 1962) solid medium. Nodal segments were excised from plants emerged from achenes held in *vitro* for 10 days. Nodal segments were grown in sterile flasks with MS basal medium, hormone-free or supplemented with kinetin (KIN; 0.01, 0.1, 0.5 and 1.0 $mg.L^{-1}$), benzyladenine (BA; 0.01, 0.1, 0.5 and 1.0 $mg.L^{-1}$) or indoleacetic acid (IAA; 0.01, 0.1, 0.5 and 1.0 $mg.L^{-1}$). The effects of growth regulators on the organogenesis of shoots and roots were recorded after 60 days of culture.

In all media, the pH was adjusted to 5.8 ± 0.1 before addition of 7.5 $g.L^{-1}$ agar-agar. *In vitro* cultures were maintained in a growth room at 25 ± 2°C under a 16/8 h light/dark photoperiod (Silvania daylight fluorescent lamps, 23 mmol $m^{-2}.s^{-1}$).

Acclimatization

Seedlings cultured for 60 days on hormone-free MS basal medium were removed and their roots rinsed in running tap water to completely remove the culture medium, and then kept in bottles with water for 24 h. Then, the seedlings were transferred to plastic containers, 6 cm in diameter, containing soil conditioner enriched with humus. These plants were initially kept in an aquarium covered with transparent plastic, and generously irrigated to create a high-humidity environment. The plastic cover was removed gradually until day 30 when it was completely removed to transfer the plants to outdoor conditions.

Determination of total phenolic content

The total phenolic content was determined using the spectrophotometric method of Folin-Denis reagent, as described by Waterman and Mole (1994). Samples with 200 mg of shoots from the culture medium containing 1.0 $mg.L^{-1}$ IAA, BA or KIN were lyophilized and milled, and then extracted four times with 5 mL of acetone : water (7:3, v/v) solution. The entire extract volume was combined with distilled water to make it up to 25 mL. A volume of 0.5 mL of Folin-Denis reagent was added to 0.5 mL plant extract plus 3 mL water. One milliliter of Na_2CO_3 saturated solution was added after 1 h of reaction, and then the absorbance of the extract was read in a spectrophotometer at 760 nm. The reference cuvette contained distilled water only. Samples were prepared in triplicate for each analysis, and the mean absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid to generate a calibration curve. Based on the measured absorbance, the concentration of phenolics was read (µg/mL) from the calibration curve, then the content of phenolics in the extracts was expressed in terms of gallic acid-equivalent (µg GA/mg dry weight).

Statistical analysis

The layout was totally randomized in all experiments. Tests with growth regulators were done in duplicate (n = 30 per treatment), and the rooting percentage was analyzed by the difference in the percentage (p_1 and p_2), at 5% significance. Total phenolic contents were analyzed in triplicate. The data were analyzed statistically with one-way ANOVA, using GraphPad Prism version 7.02. The significance of differences among mean values was assessed with Tukey's multiple comparison test at $p \leq 0.05$ or $p \leq 0.001$. The Pearson correlation coefficient was calculated using Microsoft Excel 2010.

RESULTS AND DISCUSSION

Effect of growth regulators on culture

Some easily measured characteristics were observed to establish the best combination of the basic salt medium

Table 1. Effects of plant growth regulators on *B. myriocephala* after 60 days of *in vitro* culture.

Culture medium ($mg.L^{-1}$) *	Number of buds x ± SD**	Number of nodal segments per bud	Multiplication rate	Plant elongation (cm) x ± SD**	Rooting (%)
MS	1.4±0.6^{f}	7.0^{a}	9.9^{b}	2.9±2.4^{d}	82.0^{c}
0.01 IAA	1.6±0.9^{f}	6.0^{b}	4.5^{c}	4.8±2.6^{b}	82.6^{c}
0.1 IAA	1.6±0.6^{f}	5.7^{b}	8.4^{b}	3.6±1.5^{c}	100.0^{a}
0.5 IAA	3.9±1.9^{d}	5.3^{c}	18.7^{a}	6.0±1.8^{a}	100.0^{a}
1.0 IAA	6.7±3.4^{b}	3.7^{d}	23.0^{a}	6.0±2.4^{a}	96.7^{b}
0.01 BA	1.6±0.7^{f}	5.9^{b}	8.6^{b}	2.0±1.0^{e}	66.0^{d}
0.1 BA	1.2±0.4^{f}	1.8^{e}	2.1^{d}	1.0±0.3^{f}	0
0.5 BA	***	***	***	***	0
1.0 BA	***	***	***	***	0
0.01 KIN	1.4±0.7^{f}	4.8^{c}	6.1^{c}	2.3±1.6^{e}	34.5^{e}
0.1 KIN	2.3±1.4^{e}	5.0^{c}	9.3^{b}	1.7±0.7^{e}	18.2^{f}
0.5 KIN	4.8±2.5^{c}	3.6^{d}	15.0^{a}	1.3±0.4^{f}	6.0^{g}
1.0 KIN	7.2±3.0^{a}	1.1^{e}	6.8^{c}	1.6±0.4^{e}	0

*Murashige and Skoog (1962) basal medium, hormone-free or supplemented with kinetin (KIN); benzyladenine (BA) or indoleacetic acid (IAA). **Mean (x) ± standard deviation (SD), n = 2 × 30 per treatment. Values in the same column followed by the same letter do not differ statistically at P ≤ 0.05. *** High proliferation of non-elongated shoots.

with the different growth regulators added to the medium, including the number of buds, nodal segments per bud, shoot length, and rooting produced in each culture medium. The ideal culture medium increased the multiplication rate in addition to producing elongated and rooted plants, to avoid the need for additional steps in the production of *in vitro* plants.

While increasing the IAA concentration favored organogenesis of shoots and rooting, making the culture more efficient, BA and KIN had the opposite effect because the increased concentration of these hormones reduced the multiplication rate and rooting or produced very short shoots, making it more difficult to isolate nodal segments for subculturing. The culture medium with 1.0 $mg.L^{-1}$ IAA allowed the production of 23 new nodal segments from a single phytomer inoculated. This result is statistically equivalent to that from 0.5 $mg.L^{-1}$ IAA which produced about 18 new nodal segments from a single phytomer inoculated. Both 0.5 and 1.0 $mg.L^{-1}$ IAA provided satisfactory rooting and elongation of plants (Figure 1 and Table 1).

Cytokinins, BA and KIN have been used in the induction of *in vitro* shoots of *Eclipta alba*, a member of Asteraceae of recognized medical value. Cytokinins promote shooting but often unsatisfactory shoot elongation or rooting, which for *E. alba* necessitated an additional step in the tissue culture (Dhaka and Kothari, 2005; Baskaran and Jayabalan, 2005; Ray and Bhattacharya, 2008).

The composition of the basic saline growth medium also affected the regeneration of new shoots in *E. alba* (Baskaran and Jayabalan, 2005) and *Baccharis tridentata* (Kajiki and Shepherd, 2006), where the MS medium gave the best results for shoot regeneration in both plants. Because many plants develop satisfactorily in MS medium, this was chosen for the *B*. *myriocephala in vitro* culture (Figure 1).

The results obtained in the tissue culture of *B. myriocephala* concord with the effects of auxins on rooting in *E. alba* (Dhaka and Kothari, 2005). Shoot elongation and multiplication combined with good rooting saves time and culture medium, by shortening the micropropagation process.

Acclimatization

A complete micropropagation protocol must include a well-established acclimatization process. Therefore, the transfer to outdoor conditions is still the greatest problem for the micropropagation of many plant species (Chandra et al., 2010). Because *B. myriocephala* cultured in hormone-free MS basal medium is highly sensitive to dehydration, it was necessary to provide a high-humidity environment for the first stage of acclimatization. With this strategy, the plants were well adapted to their new environment, with 100% survival. The plants did not show any variation in morphology. Previous rooting was not necessary for *B. myriocephala,* making the protocol more efficient and lower-cost. A prior rooting step was required for *Stevia rebaudiana* (Singh and Dwivedi, 2014; Ramírez-Mosqueda et al., 2016), *E. alba* (Singh et al., 2012) and *Gerbera jamesonii* (Cardoso and Teixeira da Silva, 2013).

Total phenolic content

Environmental factors lead to variations in the phenolic

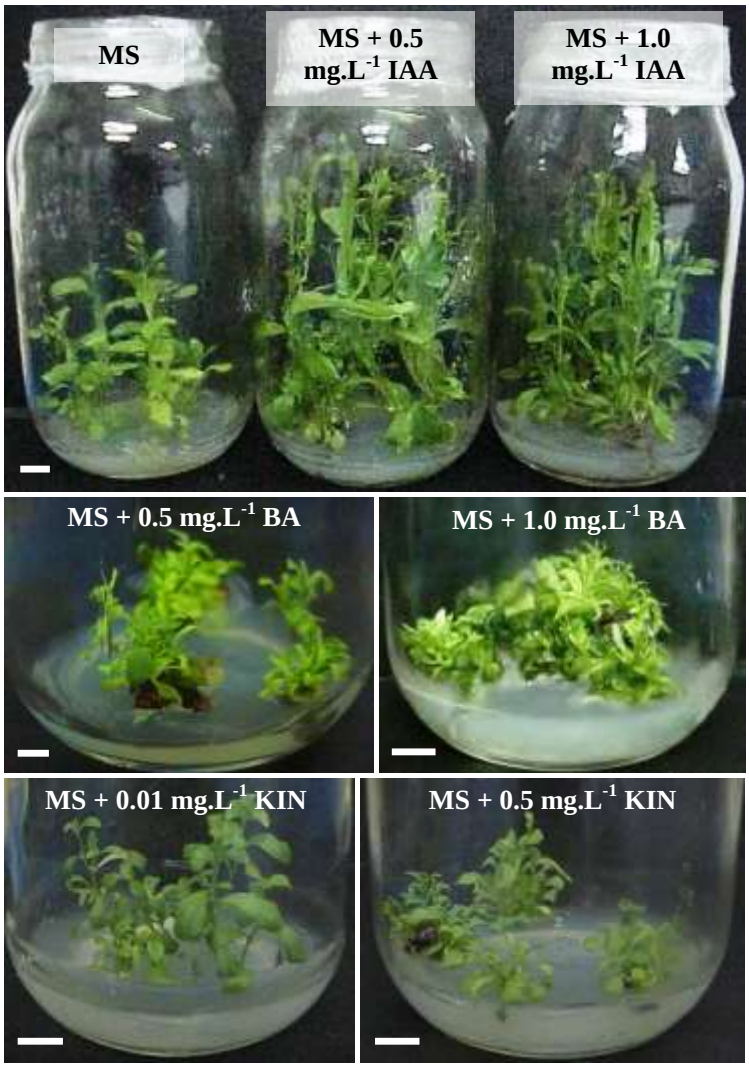

Figure 1. Effects of growth regulators on *B. myriocephala* after 60 days of culture. MS, Murashige and Skoog basal medium; IAA, Indoleacetic acid; BA, benzyladenine; KIN, kinetin. Bar = 1.0 cm.

content. The carbon fixed by the plant is managed between the primary and secondary metabolisms, and therefore in situations or seasons of lower growth of the plant body, the production of phenolics is higher (Sartor et al., 2013).

The environmental conditions of the *in vitro* culture are controlled, stable and predictable, and it is possible to relate the amount of polyphenol produced to the effect of the culture-medium composition on the development of the plant. The polyphenol versus biomass accumulation profile presented corroborates the observation in other studies, that plants with smaller growth and development accumulate more polyphenols (Grzegorczyk-Karolak et al., 2015; Ahmad et al., 2016; Kousalya and Bai, 2016). Nevertheless, the polyphenol accumulation can be influenced by the plant growth regulator utilized, regardless of biomass accumulation, or the two may show a positive relationship (Szopa et al., 2013; Kousalya and Bai, 2016).

Thus, the culture medium MS + 1.0 mg.L^{-1} IAA induced the best growth and development of the plants but led to the lowest accumulation of polyphenols; only 18.46 µg/mg dry weight (Table 2). The hormone-free MS and the MS + 1.0 mg.L^{-1} BA culture media produced similar amounts of polyphenols (Table 2), and these plants grew reasonably well, although plants cultured on the BA-containing medium showed high shoot proliferation, so that *in vitro* development parameters could not be analyzed (Table 1). Budding with low growth and a lower rate of multiplication were observed in the medium with MS + 1.0 mg.L^{-1} KIN, but the plants cultured on this medium had the highest polyphenol content; 27.16 µg/mg dry weight (Table 2). The Pearson correlation coefficient between the variables: phenolic accumulation (x) and dry

Table 2. Total phenolics content of *Baccharis myriocephala* cultured on MS basal medium, hormone-free or supplemented with 1 mg L IAA, BA or KIN (mean ± SD).

Culture medium (mg.L^{-1})*	Total phenolics (µg/mg dry weight) **
MS	23.81 ± 0.029^{b}
1.0 IAA	18.46 ± 0.026^{d}
1.0 BA	23.14 ± 0.036^{c}
1.0 KIN	27.16 ± 0.105^{a}

*Murashige and Skoog (1962) basal medium, hormone-free or supplemented with with kinetin (KIN); benzyladenine (BA) or indoleacetic acid (IAA). ** Different letters in a column indicate a significant difference (P ≤ 0.001, Tukey's multiple comparisons test).

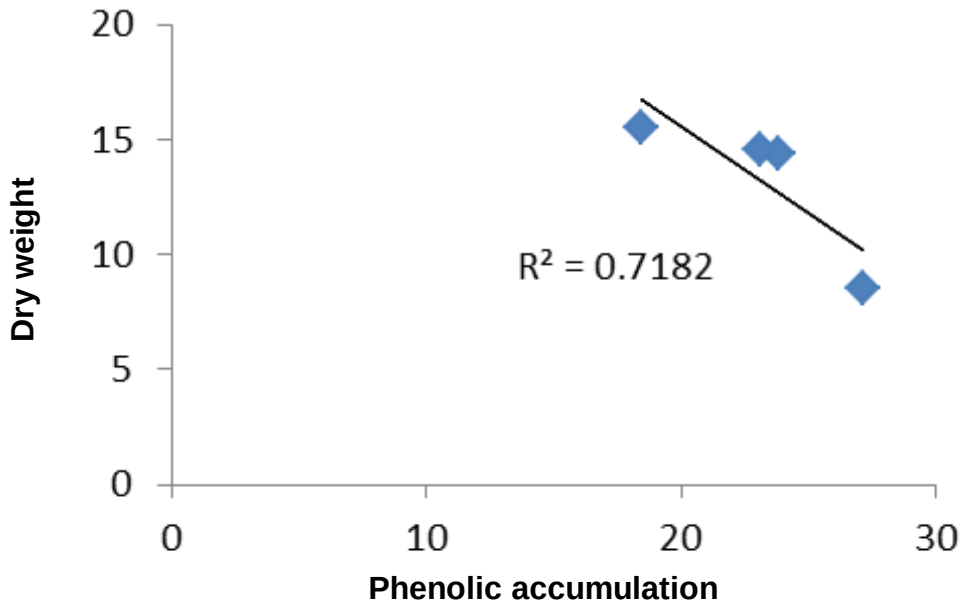

Figure 2. Correlation between phenolic accumulation and dry weight for plants cultured on MS basal medium, hormone-free, MS + 1 mg.L^{-1} IAA, MS + 1 mg.L^{-1} BA or MS + 1 mg.L^{-1} KIN, at 60 days of culture.

weight (y) was r = - 0.847447, showing a strong negative correlation between variables, since the phenolics accumulate when the dry weight decreases. In addition, 71% of the dry weight variation was due to variation in the accumulation of polyphenols (r^2 = 0.7182) (Figure 2).

Conclusion

The present results demonstrated that the MS medium is suitable for *in vitro* culture of *B. myriocephala.* Supplementation of MS medium with 1.0 mg.L^{-1} IAA maximized the multiplication rate to 23 new nodal segments from a single phytomer inoculated. In addition, IAA provided satisfactory rooting and elongation of plants, making the micropropagation process faster and more cost-effective. The success of acclimatization depends on an environment with initially high humidity that is gradually lost. Plants cultured on MS + 1.0 mg.L^{-1} KIN accumulated the highest content of polyphenols.

The efficient *in vitro* production system developed in this study provided sterile and consistent tissues for large-scale production of *B. myriocephala*, or for investigation of phytochemicals and germplasm conservation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Development of a rich fraction in phenolic compounds with high antioxidant and antimicrobial activity in *Amburana cearensis* seeds extracts

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***Amburana cearensis* is a medicinal plant widely used in folk medicine. The purpose of this study was to identify the more appropriate extraction solvent for maximum antioxidant and antimicrobial effect. The extraction of *A. cearensis* seeds were carried out gradually to obtain the highest yields and constituents of the extracts using as solvent hexane, methanol, 80% alcohol and water in this sequence. Phytochemical screening showed phenolic compounds and thin layer chromatography (TLC) showed the flavonoid morin. Antioxidant activity was also evaluated by the method of scavenging the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH); rutin and ascorbic acid was used as standard; the hydro-alcoholic extract (EEA) showed an IC_{50} value of 17.95 µg/ml, similarly to the standards rutin and ascorbic acid, indicating high antioxidant action. The microdilution assay (MIC) showed antibacterial activity against the bacteria concentrations, values ≥0.25 mg/mL for EEA and aqueous (AEA) extracts against *S. aureus, P. aeruginosa* and *S. flexneri*. High performance liquid chromatography (HPLC) analysis of *A. cearenses* seed extracts revealed a high content of flavonoids and tannin compounds corroborating with TLC analysis. The extraction by exhaustion was very effective in exposing the bioactive principles of *A. cearenses* seeds showing their best compounds mainly in EEA extract.**

Key words: *Amburana cearensis*, exhaustion extraction, seed, phytochemical profile, antioxidant, antimicrobial activity.

INTRODUCTION

Amburana cearensis, known as umburana or cumaru is a leguminous plant of Fabaceae family, used in perfumery and for pharmaceutical purposes; it can be observed in practically all of America, from Peru to Argentina (Canuto

and Silveira, 2006). Many parts of this plant are used in traditional medicine, stem bark and leaves contain phenolic compounds with anti-inflammatory and antioxidant effects (Leal et al., 2003), they are also useful in conducting several physiological disorders such as diabetes mellitus, hypertension, vascular fragility and improvement of the health of gastrointestinal tract (Scalbert et al., 2005). The seeds are used for stomach and liver diseases (Leal et al., 2008). Besides, some peptides from *A. cearensis* have antifungal (dos Santos et al., 2010) and antimicrobial activity (Sá et al., 2011).

The antimicrobial activity of phenolics and flavonoids are also well documented (Erdemoglu et al., 2007; Xia et al., 2011). The phenolics compounds can affect the growth and metabolism of bacteria, activating or inhibiting the microbial growth according to their constitution and concentration (Alberto et al., 2006; Nazzaro et al., 2009).

The need to find new antimicrobial substances against microorganisms presents a challenge in the treatment of infectious disease (Gonçalves and Santana, 2010).

Currently, many bacterial strains are resistant to almost all antimicrobials. The search for antibacterial properties of plant extracts has been encouraged and intensified, substances derived from plants constitute approximately 25% of medically prescribed agents in industrialized countries (Sá et al., 2011).

Several studies have demonstrated the presence of secondary metabolites and antimicrobial activity in leaves and stem bark of the *A. cearensis*, however, the seeds do not present similar results (Lima et al., 2013), this is already expected because the main constituents of most seeds are carbohydrates, lipids and proteins, followed by other constituents in smaller amounts such as vitamins, minerals and water (Vaclavik and Christian, 2006). Furthermore, the solubility of secondary compounds depends on the type of solvent used, degree of polymerization, as well as interaction with other food constituents and formation of insoluble complexes (Naczk and Shahidi, 2004), indicating the need for further studies with this part of the plant.

Due to the widespread use of *A. cearensis* for medicinal purposes and the scarcity of phytochemical reports of its seeds in the literature, it has become essential to carry out its study, to discover the possible response for the therapeutic properties of *A. cearensis*. The aim of this study was to determine the antioxidant capacity and antimicrobial activity of *A. cearensis* extracts against bacteria strains.

MATERIALS AND METHODS

This study was done in Natural Products Research Laboratory of Federal University of Tocantins (UFT), Palmas Campus, Tocantins, Brazil.

Plant

The seeds of *A. cearensis* were obtained from popular commerce in the city of Palmas, Tocantins, Brazil, and their authenticity recognized by EMBRAPA Herbarium, voucher (CPAP 5948). The seeds were dried in a stove at 45°C and ground using knives crusher (Start FT 50 - Fortinox). The ground seeds were stored in bottles and kept at room temperature and covered from light and humidity (Al-Marby et al., 2016).

Seed extract preparation

To prepare the extracts, 15 g of powdered seeds were extracted for 4 h using the Soxhlet apparatus (Mabiki et al., 2013). First, an extraction was performed with 80% ethanol at the boiling temperature of the solvent, resulting in the crude extract (CEA). Subsequently, another extraction was carried out using different solvents, *n*-hexane, methanol, 80% ethanol and water, from the lowest to the highest polarity to extract the maximum seed components.

Each solvent was used after being dried in a stove at 40°C for 24 h. The solvents were removed on a rotary evaporator at -600 mm Hg (Fisaton 804) at 45°C. They were dried in the exhaust hood and stored in an amber bottle and kept at 4°C. The hexanic, methanol, 80% ethanol, and water extracts were respectively named as, hexanic extract (HEA), methanol extract (MEA), ethanol extract (EEA), and aqueous extrac (AEA).

Phytochemical screening

To elucidate which compounds were mainly present in CEA extract, previous phytochemical analysis was achieved according to previously described methods (Matos, 2009), based on coloration/precipitation tests.

This analysis was complemented by thin layer chromatography (TLC). The test was fulfilled using benzene-ethyl acetate-formic acid-methanol (60/30/10/5, v/v/v/v) as the mobile phase.

Then, dried plates have been sprayed with a 1% solution of 2-aminoethyl diphenylborinate in methanol and spray with a 0.5% ethanolic solution of polyethylene glycol (NEU/PEG reagent). Next, the plates were visualized using a darkroom viewing cabinet (SOLAB®, model SL 204) UV light at 365 nm. The retention factor (Rf) values were calculated measuring the distance reached by the extract divided by the distance of the mobile phase and compared with Rf of standards rutin, quercetin, and morin.

Determination of phenolic compounds and total flavonoid content

Total phenolic content was quantified using the Folin-Ciocalteu method as described by Amorim et al. (2008) and determined by interpolation of the absorbance of the samples and the calibration curve constructed with standard, gallic acid in methanol (y = 1.3631x + 0.0213, adjusted r^2 = 0.962). The result was exposed as mg gallic acid equivalents (GAE) per gram of *A. cearensis* extract

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(mg GAE/g).

Total flavonoid content was performed using the method described by Soares et al. (2014) with few modifications and determined by using the absorbance of the samples against a calibration curve (y = 1.3336x + 0.0078, adjusted r^2 = 0.9999) constructed with the rutin standard and expressed as milligrams of rutin equivalents (RE) per gram of dry extract (mg RE/g).

Evaluation of antioxidant activity

The antioxidant activity was performed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich®) assay, described by Peixoto-Sobrinho et al. (2011). Standards were used as rutin and ascorbic acid (Sigma-Aldrich®). The percentage scavenging values were calculated following the equation:

$$AA\ (\%) = [(A_0 - (A_S - A_{blank}))/A_0] \times 100$$

where AA is the antioxidant activity, A_S is the absorbance of the sample, A_{blank} is the absorbance of the blank, and A_0 is the absorbance of DPPH without sample.

The IC_{50} value calculated indicates the concentration of a sample required to decrease the absorbance at 517 nm by 50%. The IC_{50} was expressed in µg/mL.

HPLC analysis

The *A. cearensis* extracts were analyzed by high-performance liquid chromatography (HPLC) at Scientific Instrumentation Laboratory (LABIC) UFT-Palmas, Tocantins, Brazil. The HPLC system (Shimadzu, Tokyo, Japan) consisted of a chromatograph (LC-10 Avp series) with a pump (LC-10 AD), a degasser (DGU-14A) to pump the mobile phase, rheodyne manual injector (20 µL loop) and class integrator (LC-10A), a UV-Vis (SPD - 10A) detector and a column oven (CTO 10A). The extracts and standards were prepared with methanol and filtered with Millipore membrane (0.22 µm pore size). The separation was achieved by a gradient system, using a reverse-phase Phenomenex Luna 5 mm C18 (2) (250 4.6 mm^2) column with direct-connect C18 Phenomenex Security Guard Cartridges (4 3.0 mm^2) filled with similar material as the main column. Mobile Phase A was 0.1% phosphoric acid in Milli-Q water and mobile phase B was 0.1% phosphoric acid in Milli-Q water/acetonitrile/methanol (54:35:11). Flow rate: 1 ml/min, temperature: 22 1C. UV detection was done at 280 nm. The standards used were gallic acid, rutin, ellagic acid, naringin, myricetin, morin, quercetin, naringenin, and kaempferol (Sigma®). The compounds were expressed in micrograms per milligram of extract (µg/mg) by correlating the area of the analyte with the calibration curve of standards built in concentrations of 4.5 to 18 µg/mL.

Bacterial strains

The American Type Culture Collection (ATCC) bacterial strains used were *Staphylococcus aureus* (29213), *Listeria monocytogenes* (35152), *Escherichia coli* (25922), *Aeromonas* species (7966), *Pseudomonas aeruginosa* (27853), and *Shigella flexneri* (700930). The strains were stored at -20°C, and sub-cultured two days before the assays.

Antimicrobial activity in broth

The minimum inhibitory concentration was determined by the microplate dilution technique (96 holes) according to the methodology described according to the M7-A6 standard of the Manual 38 Clinical and Laboratory Standards Institute (CLSI, 2006), the most recommended method for this determination (Benfatti et al., 2010). The microplate wells were filled with 100 µL of Muller-Hinton broth medium (MHB-Micro Med), then 100 µL of extract solutions were added and a serial dilution was performed: 1000, 500, 250, 125, 62.5, 31.2, 15.6, and 7.8 µg/mL. In addition, 20 µL of the microorganism suspensions were inoculated into each well of the microplates. As a positive control, chloramphenicol was used at the same dilutions as extracts. Control of the culture medium, bacterial growth control and negative control (solvents) were also performed. The microplates were incubated in an incubator at 37°C for 24 h, all tests were performed in triplicate.

The MIC was expressed as the lowest concentration that inhibited growth, which was judged by the well. The turbidity in each well was then measured at 450 nm by using a spectrophotometer (GT - 722G). The MIC values equal to or less than 1000 µg/mL were considered active (Kuete, 2010).

Statistical analysis

The experiment was totally randomized, with three replicates per treatment. The ANOVA test was obtained using the ASSISTAT 7.6 beta program.

RESULTS AND DISCUSSION

Phytochemical screening

Many studies have demonstrated the profuse presence of coumarin, flavonoids and tannins in stem bark and leaves of *A. cearensis* as responsible for pharmacological activities of this species according to the effects observed in tests pure substances (Marinho et al., 2004; Canuto, 2006); however, these phenolic compounds have not yet been well documented in seeds, this can occur because secondary metabolites link competitively for some solvents during the extraction process (Naczk and Shahidi, 2004). In this work, the sequential extraction process eliminated this interference, favoring the extraction of phenolic compounds. A similar extraction methodology was performed by Zhu et al. (2010) in a study with *Portulaca oleracea* L, obtaining such results. Seed extraction from *A. cearensis* provided 1.315 g (8.73%) hexane extract, 3.877 g (25.91%) methanolic extract, 1.009 g (6.66%) hydroethanolic extract and 0.876 g (5.33%) aqueous extract.

Analysis by thin layer chromatography (TLC)

The evaluation by TLC showed, in EEA *A. cearensis* seed extracts the presence of flavonoids, as evidenced by emergence of fluorescence in Rf stains EEA (Rf=75), MEA (Rf=73) and AEA (Rf=80). Characteristic for reference standards (Rutin Rf=31; Quercetin Rf=81; Morin Rf=75) (Figure 1).

Table 1 shows the total phenol, flavonoids and the evaluation of total antioxidant activity of *A. cearensis* seed extracts. The EEA extract presented higher phenolic

Table 1. Total phenolic content, total flavonoids content and antioxidant activity (DPPH IC_{50}) of seed extracts obtained by sequential extraction.

Extract	Total phenolic (mg GAE/g)	Flavonoids (mg RE/g)	DPPH (IC_{50}) (µg/mL)
CEA	66.29 ± 1.12	7.75 ± 0.04	68.97 ± 1.60
HEA	6.62 ± 1.84	ND	83.54 ± 0.32
MEA	68.25 ± 2.77	5.48 ± 0.11	150.05 ± 0.35
EEA	426.67 ± 5.86	13.10 ± 0.13	17.95 ± 0.43
AEA	71.43 ± 1.84	5.05 ± 0.04	137.04 ± 2.08

ND: Non-detectable value.

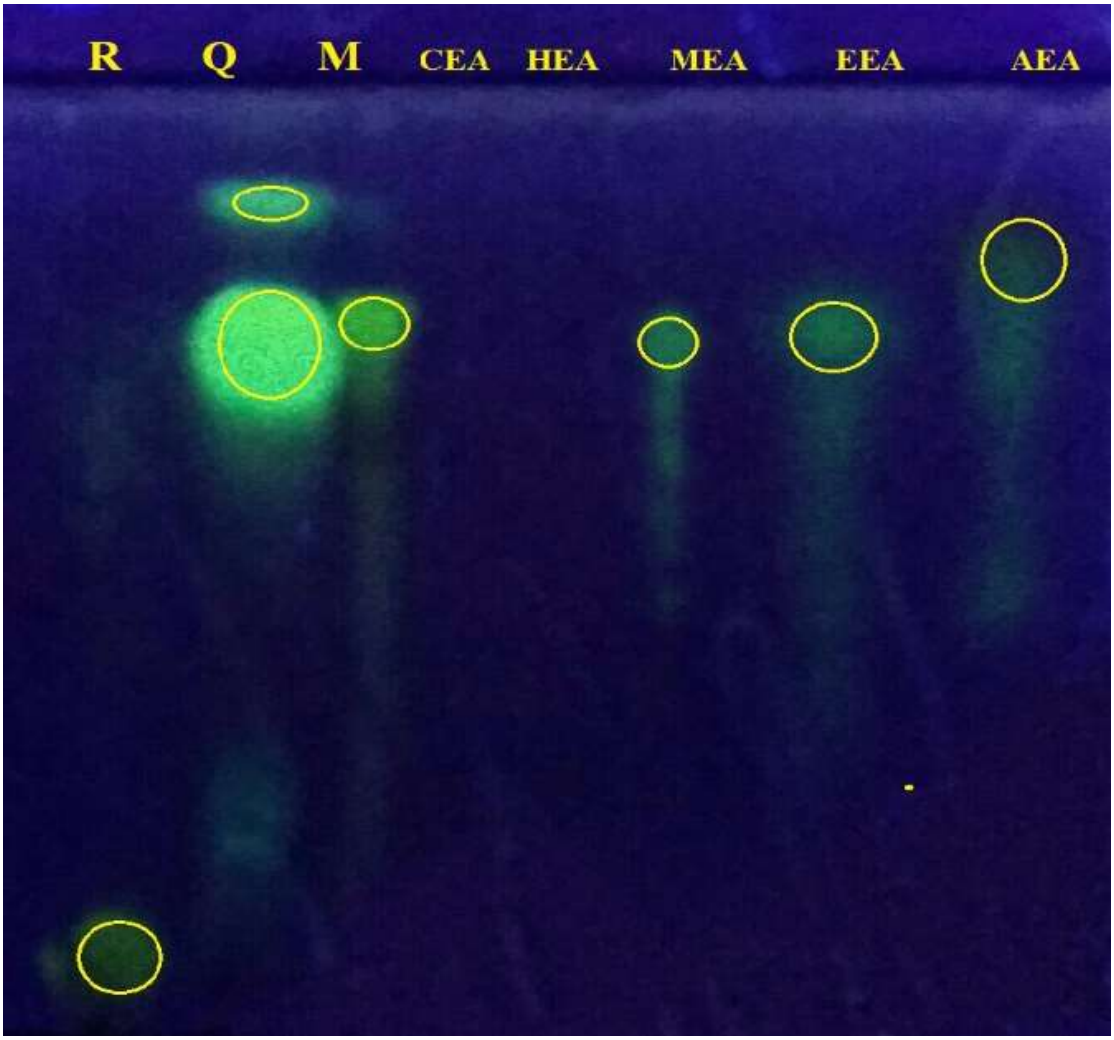

Figure 1. TLC: Standards Rutin (R), Quercetin (Q), and Morin (M), followed by extracts CEA, HEA, MEA, EEA and AEA.

and flavonoid content, this high antioxidant activity is probably due to the type of extraction performed, since the EEA is an extract practically free of interferents as fatty compounds, moreover, this extract was the only one which presented rutin in HPLC analysis (Figure 3).

Values below the IC_{50} reflect high antioxidant activity. EEA extract values were very close to the ascorbic acid and rutin patterns (Figure 2).

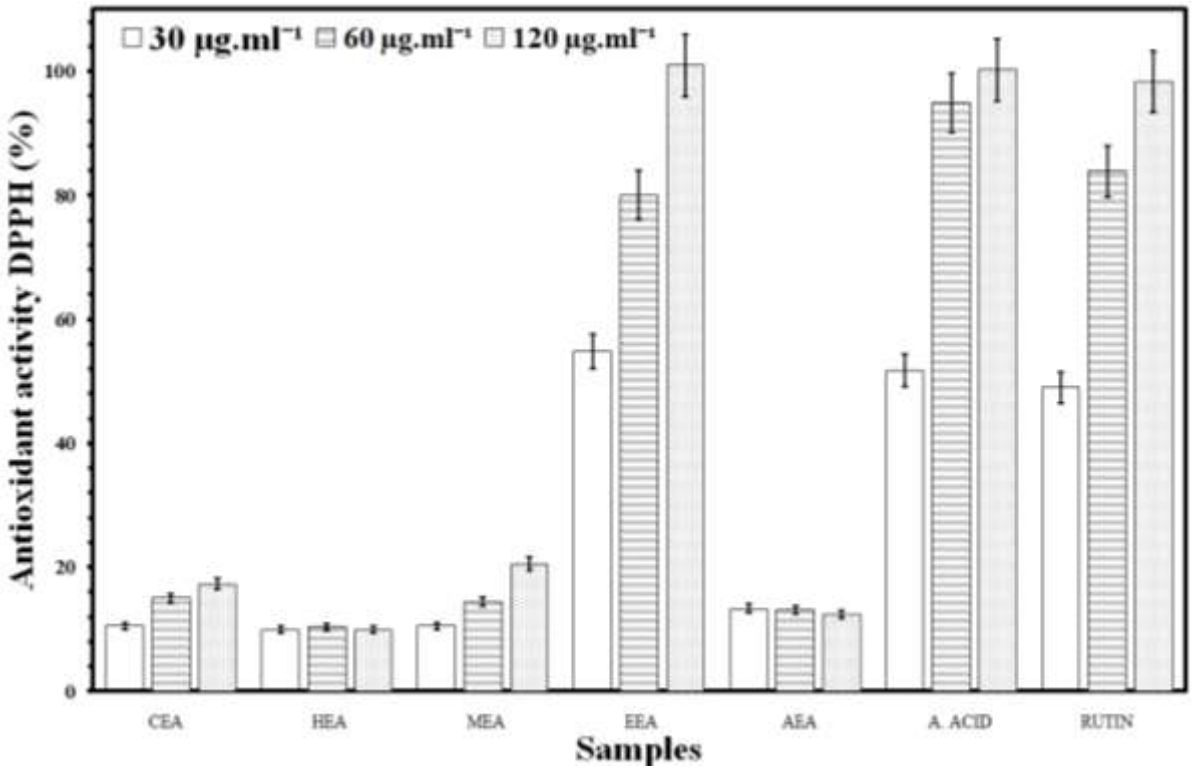

Figure 2. Antioxidant activity of the extracts in three different concentrations (30, 60 and 120 µg.mL^{-1}) measured in DPPH assay. Values represent percentage radical scavenging. CEA: Crude extract; HEA: Hexanic extract; MEA: Methanol extract; EEA: Ethanol extract; AEA: Aqueous extract; A. Acid: Ascorbic acid; Rutin.

HPLC analysis

The fingerprinting of *A. cearensis* extracts obtained by HPLC is as shown in Figure 3. The retention time of samples and the standards made possible the identification of various phenol compounds, such as: gallic acid (time 16.5 min), catechin (time 23.7 min), rutin (time 40.6 min), ellagic acid (time 41.5 min), naringin (time 43.4 min), myricetin (time 46.8 min), and morin (time 49.5 min). The substances detected in extracts are shown in Table 2.

Rutin is a powerful free radical scavenger that has a significant therapeutic potential against cancer; morin possess antibacterial activity (Pereira et al., 2015). Catechin is considered antifungal (Anand and Rai, 2016) and has shown activity against many bacteria (Shahid et al., 2016).

The HPLC data made evident that the *A. cearensis* seeds extracts obtained are plentiful in phenolic compounds of pharmacological importance and antioxidant significance. Different phenolic compounds appear in the different extracts, demonstrating the extraction method used was effective to elucidate the potential stored in *A. cearensis* seeds. EEA extract has the highest number of phenolic compounds (Table 2) corroborating with results of DPPH and TLC tests. The not identified peaks shown in the chromatograms (Figure 3) can be phytocomponents with important biological activity and different potential. Hence, more research with *A. cearensis* seeds could improve the pharmacological knowledge about this plant.

Antibacterial assay

The bioassays with extracts of *A. cearensis* showed that EEA and AEA extratcs had activity against *E. coli*, *P. aeruginosa*, *S. flexneri*, *L. monocytogenes* and *S. aureus*, with MIC values ranging from 250 to 1000 µg/mL. CEA, HEA and MEA extracts had no significant effect against these microorganisms (Table 3). There is no report of antibacterial activity in *A. cearensis* seeds extracts prior to this work. Santos et al. (2010) have shown activity of peptide from *A. cearensis* seeds only against phytopathogenic fungi and yeasts and Lima et al. (2013) showed no antibacterial activity against strains of *S. aureus*, *E. coli* and *P. aeruginosa* with their *A. cearensis* extract. Catechin and naringin is present in EEA extract (Table 2), it is known that these phenolic compounds act against microorganisms (Anand and Rai, 2016), then it explains the antimicrobial activity of this extract. The standard antibiotic for this assay, chloramphenicol,

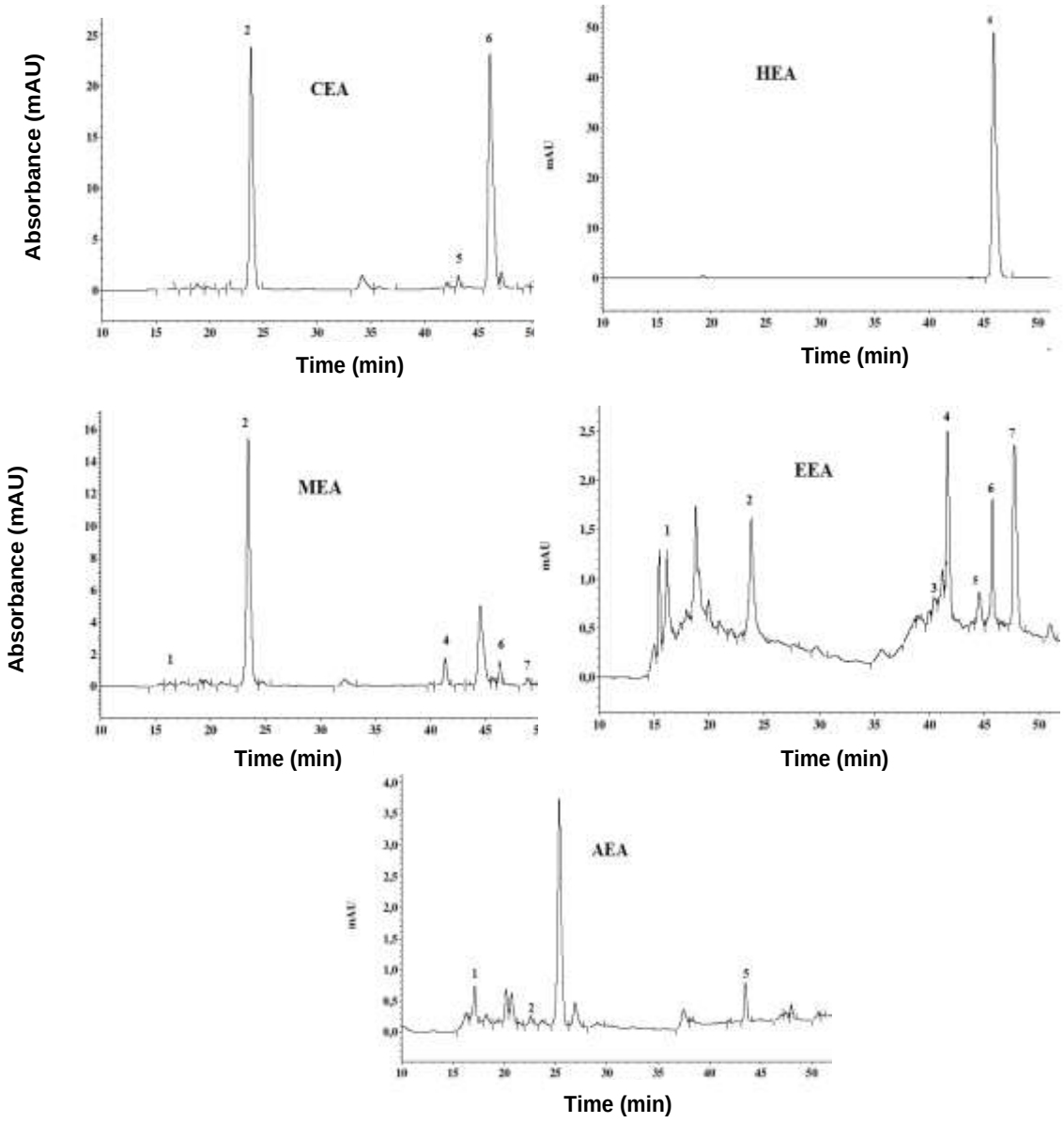

Figure 3. HPLC analysis profile of the *A. cearensis* seeds from different extracts detected at 280 nm. Peak 1: Gallic acid; peak 2: Catechin; peak 3: Rutin; peak 4: Ellagic acid; peak 5: Naringin; peak 6: Myricetin; peak 7: Morin.

Table 2. Phenol compounds identified by HPLC in *A. cearensis* seeds extracts.

Phenol compound	Extract concentration (µg/mg extract)				
	CEA	HEA	MEA	EEA	AEA
Gallic acid	-	-	1.18	3.16	1.42
Catechin	221.70	-	151.72	14.94	3.73
Rutin	-	-	-	11.42	-
Ellagic acid	-	-	9.11	9.64	-
Naringin	3.45	-	-	1.94	1.99
Myricetin	138.05	274.46	5.45	1.02	-
Morin	-	-	0.44	2.46	-

-: Not detected.

Table 3. Antibacterial activity of *A. cearensis* seed extracts on selected bacterial strains.

Microorganism extract	Extract					Chloramphenicol
	CEA	HEA	MEA	EEA	AEA	
Gram-negative bacteria						
E. coli	-	-	-	1000	1000	7.8
Aeromonas spp.	-	-	-	1000	1000	7.8
P. aeruginosa	-	-	-	250	500	7.8
S. flexneri	-	-	-	250	500	7.8
Gram-positive bacteira						7.8
S. aureus	-	-	-	250	1000	7.8
L. monocytogenes	-	-	-	500	500	7.8

Minimal inhibitory concentration (MIC) in $\mu g.mL^{-1}$. *Inactive.

showed a broad spectrum of antibacterial activity with MIC values ranging from 7.8 to 1000 µg/mL. The MIC values for the extracts and the positive control are shown in Table 3.

Conclusion

This work has demonstrated that exhaustion extraction of *A. cearensis* seeds was effective in obtaining extracts with high quantity of phenolic compounds, separating the interferents of these compounds as lipid components and revealing high antioxidant action compared to rutin standard. The result of the antioxidant activity in this work showed that *A. cearensis* seeds are abundant in substances capable of scavenging free radicals and HPLC analysis exposed that these seeds are a source of compounds with pharmacological features. Moreover, the antimicrobial activity justifies its use in folk medicine for the treatment of many diseases. Hence, this data indicates *A. cearensis* as a new source of potential antioxidant and antimicrobial agents.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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