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Phytochemical profile and antifungal action of *Anadenanthera colubrina* extract on the quality of maize seeds

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ABSTRACT

Maize (*Zea mays* L.) is among the most cultivated crops in the world and can be affected by several diseases, especially those transmitted by seeds. The study of alternatives to fungicides used for seed treatment has a promising field in essential oils. Thus, this study determined the phytochemical profile of the ethanolic extract from *Anadenanthera colubrina* (Vell.) Brenan and to evaluate its antifungal activity on the sanitary and physiological quality of maize seeds. The seeds used were of the Jaboatão cultivar, which were submitted to the following treatments: control (untreated seeds), commercial fungicide (dicarboximide) and *A. colubrina* extract at 200, 400, 600, 800, and 1,000 ppm. The seeds were subjected to sanitary and germination tests in a completely randomized experimental design. Phytochemical prospecting of *A. colubrina* extract indicated the presence of alkaloids, tannins, flavonoids and saponins, as well as the major compounds lupeol, gallic acid, ferulic acid, catechin and quercetin. The *A. colubrina* extract reduced the incidence of *Aspergillus* spp., including *Aspergillus niger*, *Alternaria* spp., *Curvularia* spp. and *Fusarium* spp. at all concentrations. The highest concentrations (800 and 1,000 ppm) of the *A. colubrina* extract reduced the incidence of *Penicillium* spp. and yielded an effective control of *Rhizoctonia* spp. The extract of *A. colubrina* did not present phytotoxic effect, guaranteeing the viability and vigor of maize seeds.

Keywords: secondary metabolites; seed pathology; alternative control.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most cultivated crops in the world, with wide adaptability to different edaphoclimatic conditions (DOMENE et al., 2016). In Brazil, in the 2016/2017 harvest, about 16,772 million hectares were sown and approximately 88,969 million tons of grain were produced (CONAB, 2017).

The health quality of seeds can be compromised by the association of fungi in all production stages. These pathogens are frequently responsible for the reduction in their physiological quality and can be disseminated over long distances through infected seeds and be transmitted via seed-seedlings (SALES et al., 2016).

Among the fungal diseases that affect the maize crop, those that cause seed rot and seedling damping-off in pre- and post-emergence are of great importance, as they are responsible for the reduction or total loss in yield, in addition to the significant increase in production costs (STEFANELLO et al., 2012). *Fusarium verticillioides*, *Stenocarpella maydis*, *Rhizoctonia* spp., *Penicillium oxalicum* and *Pythium* spp. are the main causal agents of this group of diseases, and these pathogens survive in the soil and inside seeds, that is, they are optional parasites (MACHADO et al., 2013).

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The exploration of the biological activity of secondary compounds, present in the plant crude extract or essential oils, can constitute, alongside biological control and resistance induction, in yet another potential form of alternative control (SOUZA et al., 2013). Several studies using plant extracts and essential oils have been conducted in the control of phytopathogens as an alternative to the use of synthetic pesticides (LORENZETTI et al., 2011; VENTUROSO et al., 2011); they have demonstrated efficiency in the control of rot and pathogens linked to seeds in maize (GURJAR et al., 2012).

The evaluation of the sanitary quality of seeds using plant extracts and/or essential oils has been carried out by several authors such as FLÁVIO et al. (2014), GOMES et al. (2016) and MEDEIROS et al. (2016), who concluded that these products, in addition to reducing the occurrence of fungal species, result in improvements in the germinability of the treated seeds.

The Caatinga vegetation has great botanical potential, but it is little explored regarding the knowledge of the biochemical constitution and biological control. Among the species of this biome, *Anadenanthera colubrina* (Vell.) Brenan, popularly known as angico, stands out for its wide distribution, abundance and use as a phytomedicine in popular medicine. This species contains compounds involved in chemical defense that include lectins, protease and amylase inhibitors, toxins and secondary metabolites of low molecular weight (RIEGELHAUPT; PAREYN, 2013).

In this context, aiming to explore the biochemical potential of Caatinga species and in view of the need for new alternatives to control fungi associated with seeds, this study aimed to determine the phytochemical profile of the ethanolic extract from *A. colubrina* and to evaluate its antifungal activity on sanitary and physiological quality of maize seeds.

MATERIAL AND METHODS

The experiment was conducted at the Semiarid Plant Health Laboratory (LAFISA), belonging to the Semiarid Sustainable Development Center (CDSA) of Universidade Federal de Campina Grande (UFCG), located in the municipality of Sumé, PB, Western Cariri micro region (07°40'18"S and 36°52'48"W).

The maize seeds used were of the Jaboatão cultivar, from the municipality of São José dos Cordeiros/PB, belonging to the 2017 harvest. After the samples were taken to the LAFISA, the seeds were subjected to purity analysis, eliminating crop remains and deteriorated seeds.

Obtention of *Anadenanthera colubrina* ethanolic extract

For the obtention of *A. colubrina* extract, the cold extraction method was used, as proposed by MEDEIROS et al. (2016), in which the plant material (leaves) was dried in an oven at 40 °C for 72 h and, subsequently, crushed in a knife mill to obtain the plant powder; 150 g of plant powder were used, immersed in a beaker containing 0.5 L of absolute ethanol for 72 h at room temperature (25 ± 2 °C); the solution was filtered through filter paper. After the procedure, the solvent was left for 10 h in an oven with a ventilation system at 70 °C for the obtention of the crude ethanolic extract. The crude extract was diluted at the concentrations used, 200, 400, 600, 800, and 1,000 ppm.

Phytochemical prospecting of *Anadenanthera colubrina* extract

Anadenanthera colubrina extract was subjected to phytochemical characterization reactions, which were carried out according to the following methodologies:

a) Alkaloids: 25.0 mL of the ethanolic extract were evaporated, alkalinizing with 0.8 mL of 1.0% sodium hydroxide (NaOH). Subsequently, 6.0 mL of distilled water were added with 6.0 mL of chloroform ($CHCl_3$); the solution was then placed in a funnel for separation between the extract and the chloroform layer. 6.0 mL of hydrochloric acid (1.0% HCl) and 0.3 mL of Dragendorff's reagent were added to the chloroform phase (WU et al., 2005).

b) Tannins: were determined by the casein precipitation method, which consisted of adding 1.0 g of powdered casein and 6.0 mL of the extract diluted in 12.0 mL of distilled water in a 50.0-mL conical flask, kept under constant stirring for 3 h at room temperature (25 ± 2 °C). Subsequently, the sample was filtered on filter paper and the volume of the resulting filtrate was made up to 25.0 mL; 5.0-mL aliquots were removed from this solution and residual phenols were determined by the Folin–Ciocalteu method (PEIXOTO SOBRINHO et al., 2010).

c) Flavonoids: 15 mL of ethanolic extract were placed in a separatory funnel, and 15 mL of distilled water were added. The solution was then left to stand for 10 min, where 15.0 mL of $CHCl_3$ were added. After 5 min of the addition of chloroform, the layers were separated, disregarding the chloroform layer. The remaining extract was isolated and 3.0 mL of ethanol

were added, a 2.0-mL aliquot of this solution was collected in a test tube. In the tube, 0.5 mL of 10.0% HCl (hydrochloric acid) and 1.0 cm of magnesium tape were added, allowing the reaction until disappearance (CHUN et al., 2004).

d) Saponins: 0.25 mL of the ethanolic extract were placed in a test tube with distilled water and stirred well until foam was formed; 10 min were waited and it was observed if the foam remained or was not present. The presence of saponins indicates that the substance is highly soluble in water (VIEIRA et al., 2001).

Major secondary compounds were identified using gas chromatography (GC-MS).

Maize seed treatment and health testing

In the health test, 200 seeds were used per treatment, distributed in 10 replications of 20 seeds each. The seeds were subjected to asepsis with sodium hypochlorite (1.0%) for 3 min, immersed in 10.0 mL of the different concentrations of *A. colubrina* extract for 5 min and distributed in Petri dishes on a double layer of sterile filter and moistened with sterile distilled water. The dishes remained for seven days at a temperature of 25 ± 2 °C (ZAUZA et al., 2007).

The treatments applied to the seeds consisted of T_1: control (untreated seeds); T_2: fungicide dicarboximide, commercial formulation Captan (240 g i.a. ·100 kg^{-1} seeds); T_3: *A. colubrina* extract (EAc) at 200 ppm; T_4: 400 ppm EAc; T_5: 600 ppm EAc; T_6: 800 ppm EAc and T_7: 1,000 ppm EAc.

The fungi were detected and identified with the aid of an optical microscope and stereoscope, being compared to the descriptions in the literature (SEIFERT et al., 2011) and the results were expressed as a percentage of infected seeds for each identified fungus.

Germination test

For the germination test, the same treatments applied in the health test were used: 200 seeds were used, subdivided into four replications of 50 seeds per treatment, which were sown on previously sterilized germitest paper and moistened with distilled water in the proportion of 2.5 times its dry weight, and incubated in biochemical oxygen demand (BOD) at 27 °C and a 12-hour photoperiod. The counts of germinated and non germinated seeds were performed from the fourth to the seventh day after sowing and the evaluations were carried out according to the criteria established by the Rules for Seed Analysis (BRAZIL, 2009).

In the germination test, the germination percentages, dead seeds, hard seeds and the germination speed index (GSI) were evaluated. The GSI was evaluated together with the germination test, carrying out daily counts of normal seedlings according to the formula proposed by MAGUIRE (1962) (Eq. 1):

$$GSI = (G1 / N1) + (G2 / N2) + (G3 / N3) + ... + (Gn / Nn) \quad (1)$$

where, G1, G2, G3, and Gn = number of seedlings computed in the first, second, third, and last count; N1, N2, N3, and N_n = number of days from sowing to first, second, third, and last count.

Experimental design and statistical analysis

The experimental design used was completely randomized, totaling seven treatments. The health test consisted of ten replications of 20 seeds each, while the germination test was performed on four replications of 50 seeds per treatment. The data were submitted to analysis of variance by the F test, and the means were compared by the Tukey's test ($p \leq 0.05$), using the ASSISTAT statistical software.

RESULTS AND DISCUSSION

The phytochemical profile of the ethanolic extract of *A. colubrina* showed the presence of different groups of secondary metabolites, suggesting the availability of alkaloids, tannins, flavonoids and saponins. In general, the studied species suggests potential for antimicrobial activity due to the reported phytochemical constituents, which may be associated with antioxidant and antimicrobial activity (SOUZA et al., 2013).

The chemical constituents present in *A. colubrina* extract may account for the majority of its biological activity, although its form of action is normally combined with a certain bioactivity. Therefore, it is important to highlight its main property, for example, the ability to neutralize free radicals generated in the cell (BESSA et al., 2013).

The secondary compounds identified in the *A. colubrina* extract at considerable levels were lupeol, gallic acid, ferulic acid, catechin (polyphenol) and quercetin (flavonoid) (Table 1).

Table 1. Secondary compounds present in the ethanolic extract of *A. colubrina*, expressed in mg/10 g of crude extract.

Extract	Lupeol*	Gallic acid	Ferulic acid	Catechin	Quercetin
EAc**	11.2	2.2	0.9	7.6	12.0

* Identified through GC-MS. ** EAc: *Anadenanthera colubrina* extract.

According to JEFFREYS; NUNEZ (2016), lupeol is a triterpene that has an antimicrobial effect and its reactions are catalyzed by the enzyme lupeol synthase. Gallic acid is a phenolic compound of great importance in plant defense and a reference standard for the quantification of total phenolics; its antimicrobial activity has already been proven (MORAIS et al., 2016). Meanwhile, ferulic acid is related to cell wall resistance.

The determination of the chromatographic profile of the ethanolic extract from *A. colubrina* can be seen in Figure 1. Observing the retention times, the following majoritarian compounds were verified: palmitic acid (13.339 min); α-linolenic acid (14.893 min); ferulic acid (14.933 min); catechin (15.142 min); lupeol (15.441 min) and quercetin (18.357 min). Several complex molecules such as those mentioned above are synthesized by the secondary metabolism of plants and are of great importance in the plant-phytopathogen relationship.

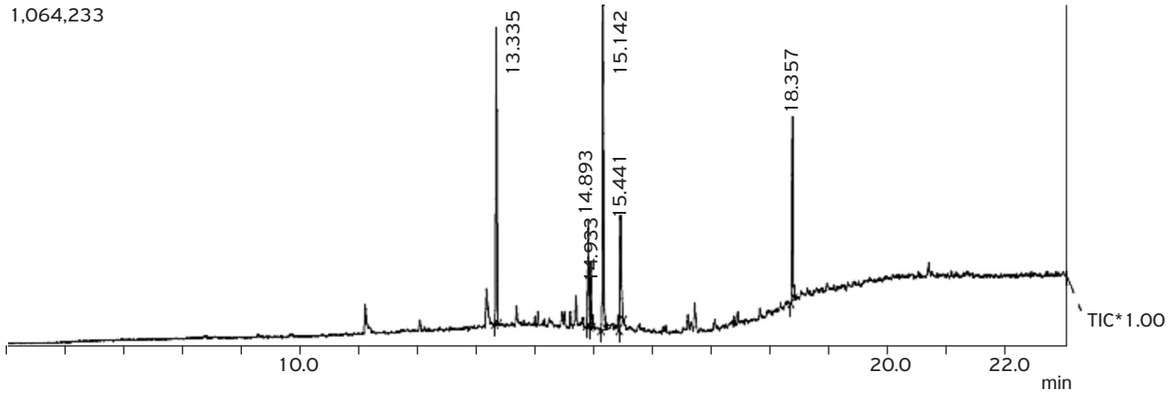

Figure 1. Chromatographic profile and retention time of major compounds in the ethanolic extract of *A. colubrina.*

In the sanitary evaluation of maize seeds, storage fungi *Aspergillus* spp., especially *Aspergillus niger*, and *Penicillium* spp. were detected (Table 2). Regarding the incidence of *A. niger* and *Aspergillus* spp., it was observed that all treatments were efficient in reducing the occurrence of these fungi in relation to the control (T_1). A similar effect was observed by DOMENE et al. (2016), who verified a reduction in the incidence of fungi of the genera *Penicillium*, *Fusarium* and *Aspergillus* in maize seeds when treated with eucalyptus essential oil (*Eucalyptus camaldulensis*), equivalent to the commercial fungicide Captan 500.

As for the occurrence of *Penicillium* spp., it was observed that seeds treated with commercial fungicide (T_2) and *A. colubrina* extract at a concentration of 1,000 ppm (T_7) had a significant reduction in the incidence of this fungus, when compared to the other treatments (Table 2).

Table 2. Incidence of storage fungi associated with maize seeds submitted to treatments with the ethanolic extract of *A. colubrina.*

Treatments	*Aspergillus niger* (%)	*Aspergillus* spp. (%)	*Penicillium* spp. (%)
T_1 - Control	33.0 a*	64.0 a	35.0 a
T_2 - Commercial fungicide	0.0 cd	2.0 d	0.0 d
T_3 - EAc 200 ppm	18.0 b	21.0 b	31.0 a
T_4 - EAc 400 ppm	5.0 c	17.0 bc	30.0 a
T_5 - EAc 600 ppm	0.0 cd	2.0 d	26.0 a
T_6 - EAc 800 ppm	0.0 cd	5.0 d	19.0 ab
T_7 - EAc 1,000 ppm	0.0 cd	5.0 d	12.0 c
CV (%)	41.3	34.5	21.2

*Means followed by the same letter in the column do not differ by the Tukey test at 5% probability. EAc: *A. colubrina* extract.

The antifungal activity of *A. colubrina* extract may be related to the presence of terpenes, such as lupeol (Table 2), whose mechanisms of action of this class of compounds involve the rupture of the plasma membrane and the accumulation of reactive oxygen species (ROS) induced by mitochondrial dysfunction, with consequent cell death (LAGROUH et al., 2017).

According to MACHINSKI JUNIOR et al. (2001), the mycotoxins that can be found in maize grains are produced mainly by *Penicillium* spp. (ochratoxin) and *Aspergillus* spp. (aflatoxins and ochratoxin), which can cause risks to human and animal health. The occurrence of these fungi immediately after harvest was also reported by STEFANELLO et al. (2012); however, its incidence can show wide variations, as a function of genotype or climatic conditions.

The percentage of fungal incidence commonly related to diseases of maize shoots, represented in this study by *Alternaria* spp., *Colletotrichum* spp. and *Curvularia* spp., are shown in Table 3. For *Alternaria* spp. and *Curvularia* spp., it was found that all treatments with extract were efficient in reducing the occurrence of these fungi, being equivalent to the commercial fungicide (T_2) and differing statistically from the control (T_1). Regarding the incidence of *Colletotrichum* spp. there was no significant difference between the treatments applied.

Table 3. Incidence of disease-causing fungi detected in maize seeds submitted to treatments with the ethanolic extract of *A. colubrina*.

Treatments	*Alternaria* spp. (%)	*Colletotrichum* spp. (%)	*Curvularia* spp. (%)
T_1 - Control	13.0 a*	4.0 a	17.0 a
T_2 - Commercial fungicide	0.0 c	0.0 a	1.0 bc
T_3 - EAc 200 ppm	6.0 b	0.0 a	5.0 b
T_4 - EAc 400 ppm	7.0 b	0.0 a	1.0 bc
T_5 - EAc 600 ppm	1.0 c	0.0 a	0.0 c
T_6 - EAc 800 ppm	0.0 c	0.0 a	0.0 c
T_7 - EAc 1,000 ppm	0.0 c	0.0 a	0.0 c
CV (%)	12.6	18.2	20.4

*Means followed by the same letter in the column do not differ by the Tukey's test at 5% probability. EAc: *A. colubrina* extract.

According to SALES et al. (2016), the inhibitory effect of plant extracts on fungal reduction is related to the presence of natural bioactive compounds present in their composition, such as those identified in the *A. colubrina* extract (Table 1), highlighting lupeol and gallic acid, for having antimicrobial properties. VENTUROSO et al. (2011) found that the aqueous extract of clove (*Syzygium aromaticum* L.) completely inhibited the *in vitro* development of all studied phytopathogens (*Aspergillus* sp., *Penicillium* sp., *Colletotrichum* sp., *Fusarium solani*, *Cercospora kikuchii* and *Phomopsis* sp.). This antifungal action is attributed to the presence of eugenol, a major component of clove (LORENZETTI et al., 2011).

Associated with maize seeds, fungi considered to be soil fungi, such as *Fusarium* spp., *Rhizoctonia* spp. and *Pythium* spp., were also identified (Table 4). All treatments tested differed from the control (T_1) in reducing the percentage of *Fusarium* spp. incidence, highlighting those applied with the highest extract concentrations (800 and 1,000 ppm), which yielded an effective control of this fungus. This reduction in the occurrence of *Fusarium* spp. was also observed by GOMES et al. (2016) in fava bean seeds treated with essential oils of diesel tree (*Copaifera langsdorffii*) and basil (*Ocimum basilicum*), both at a concentration of 2 $ml.L^{-1}$.

Table 4. Incidence of soil fungi detected in maize seeds submitted to treatments with the ethanolic extract of *A. colubrina*.

Treatments	*Fusarium* (%)	*Rhizoctonia* spp. (%)	*Pythium* spp. (%)
T_1 - Control	24.0 a*	12.0 a	4.0 a
T_2 - Commercial fungicide	0.0 d	0.0 c	0.0 a
T_3 - EAc 200 ppm	13.0 b	8.0 b	0.0 a
T_4 - EAc 400 ppm	6.0 c	10.0 ab	0.0 a
T_5 - EAc 600 ppm	1.0 d	3.0 c	0.0 a
T_6 - EAc 800 ppm	0.0 d	0.0 c	0.0 a
T_7 - EAc 1,000 ppm	0.0 d	0.0 c	0.0 a
CV (%)	19.0	24.0	14.3

*Means followed by the same letter in the column do not differ by the Tukey's test at 5% probability. EAc: *A. colubrina* extract.

The seeds treated with commercial fungicide (T_2) and *A. colubrina* extract at 600 (T_5), 800 (T_6) and 1,000 (T_7) ppm showed a lower occurrence and control of the fungus *Rhizoctonia* spp., when compared to the other treatments, differing

significantly from the control (T_1). These results demonstrate the effectiveness of the antifungal action of *A. colubrina* extract on the development of these phytopathogens. For the development of *Pythium* spp., there was no significant difference between the analyzed treatments (Table 4).

The use of plant extracts and essential oils as potent natural fungicides has shown promising results in the control of several phytopathogens, such as the reduction in the incidence of fungi of the genera *Curvularia* and *Fusarium* in sorghum seeds treated with aqueous cinnamon extract (*Cinnamomum zeylanicum*) (FLÁVIO et al., 2014). According to SALES et al. (2016), several complex molecules are synthesized by the secondary metabolism of plants and are of great importance in the control of plant diseases. Among the most important metabolites, alkaloids, quinones, flavonoids, glycosides, saponins, tannins and terpenoids are particularly noteworthy, some of which are present in *A. colubrina* extract.

Fusarium fungi can survive in the soil through resistance structures and also in internal seed structures, such as the embryo, in addition to being able to produce a variety of mycotoxins, among them, fusaric acid (MACHADO et al., 2013). The genus *Rhizoctonia* comprises a group of fungi that survive saprophytically in the soil in the form of mycelium and sclerotia, which can be transmitted to seedlings via seeds, causing root problems and seedling damping-off (LAZAROTTO et al., 2012).

Regarding the results for the germination test, it was found that maize seeds treated with *A. colubrina* extract at 200 (T_3), 400 (T_4) and 600 (T_5) ppm showed a significant increase in the percentage of germination, with an increase of 8.0% in relation to the control (T_1), although without differing from other treatments. Treatments with *A. colubrina* extract, regardless of concentration, led to a significant reduction in the percentage of dead seeds, when compared to the control (Table 5).

Table 5. Average values of germination, dead seeds, hard seeds and germination speed index (GSI) of maize seeds submitted to treatments with the ethanolic extract of *A. colubrina*.

Treatments	Germination (%)	Dead seeds (%)	Hard seeds (%)	GSI (%)
T_1 - Control	92.0 b*	8.0 a	0.0 a	6.6 a
T_2 - Commercial fungicide	98.0 ab	2.0 b	0.0 a	8.8 a
T_3 - EAc 200 ppm	100.0 a	0.0 b	0.0 a	9.1 a
T_4 - EAc 400 ppm	100.0 a	0.0 b	0.0 a	10.2 a
T_5 - EAc 600 ppm	100.0 a	0.0 b	0.0 a	9.3 a
T_6 - EAc 800 ppm	98.0 ab	2.0 b	0.0 a	8.4 a
T_7 - EAc 1,000 ppm	98.0 ab	2.0 b	0.0 a	7.8 a
CV (%)	15.92	12.85	0.0	6.72

*Means followed by the same letter in the column do not differ by the Tukey's test at 5% probability. EAc: *A. colubrina* extract.

These results prove the potential of the antifungal activity of *A. colubrina* extract determined in the sanitary analysis of the seeds (Tables 2, 3 and 4), due to the fact that the efficient control of mycoflora associated with maize seeds results in an increase in their germination capacity. Different results were found by FLÁVIO et al. (2014), who concluded that the treatments with aqueous cinnamon extract (*C. zeylanicum*) and clove basil essential oil (*Ocimum gratissimum*) presented a phytotoxic effect, reducing the viability and vigor of sorghum seeds.

For the variables percentage of hard seeds and germination speed index (GSI), there were no significant differences between the treatments evaluated (Table 5), which indicates that germinability and germination speed were not affected by the *A. colubrina* extract. This is possibly due to the fact that this treatment does not have an allelopathic effect on maize germination. Similarly, GOMES et al. (2016) did not verify the influence of treatments with essential oils of diesel tree (*C. langsdorffii*), clove (*Caryophyllus aromaticus*) and basil (*O. basilicum*) on the germination and germination speed of fava beans.

In general, through the obtained results, it can be inferred that the ethanolic extract of *A. colubrina* is a viable alternative in the control of mycoflora associated with maize seeds, favoring their germinability, due to the presence of substances with fungicidal properties in its composition. For MEDEIROS et al. (2016), seeds predisposed to the action of microorganisms, when treated, reduce the ability of phytopathogens to survive and enhance seed longevity, their germinative power and the vigor of future plants.

CONCLUSIONS

The phytochemical profile of the ethanolic extract from *A. colubrina* indicated the presence of alkaloids, tannins, flavonoids and saponins, in addition to the major compounds lupeol, gallic acid, ferulic acid, catechin and quercetin.

Anadenanthera colubrina extract reduced the incidence of *Aspergillus* spp., including *Aspergillus niger*, *Alternaria* spp., *Curvularia* spp. and *Fusarium* spp. at all concentrations.

The highest concentrations (800 and 1,000 ppm) of *A. colubrina* extract reduced the incidence of *Penicillium* spp. and yielded an effective control of *Rhizoctonia* spp.

Anadenanthera colubrina extract had no phytotoxic effect, guaranteeing the germinability of maize seeds.

AUTHORS' CONTRIBUTIONS

Conceptualization: Medeiros, J.G.F.; Silva, J.V.B. **Data curation:** Medeiros, J.G.F.; Araujo Neto, A.C. **Formal analysis:** Araujo Neto, A.C.; Demartelaere, A.C.F. **Investigation:** Silva, E.C.; Rodrigues, R.M.; Silva, J.V.B. **Project administration:** Medeiros, J.G.F. **Supervision:** Medeiros, J.G.F. **Validation:** Araujo Neto, A.C.; Demartelaere, A.C.F., **Writing – original draft:** Araujo Neto, A.C.; Demartelaere, A.C.F.; Medeiros, J.G.F. **Writing – review & editing:** Araujo Neto, A.C.; Silva, J.V.B.

AVAILABILITY OF DATA AND MATERIAL

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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CONFLICTS OF INTEREST

All authors declare that they have no conflict of interest.

ETHICAL APPROVAL

Not applicable.

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Not applicable.

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