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Phytochemical survey and antifungal activity of plant extracts in angico seeds (*Anadenanthera colubrina* Vell. Brenan)

Levantamento fitoquímico e atividade antifúngica dos extratos vegetais em sementes de angico (*Anadenanthera colubrina* Vell. Brenan)

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ABSTRACT
Plant extracts with antimicrobial properties are an ecological alternative to fungicides, and preliminary investigation of their chemical components provides information on their properties. The present study determined the phytochemical profiles of plant extracts from *Momordica charantia*, *Caesalpinia ferrea* and *Anadenanthera colubrina* and evaluated the effect of these extracts on microorganisms and on the physiology of A. *colubrina* seeds. Extracts were obtained by cold extraction. The phytochemical characteristics of the studied species were investigated by determining the presence of alkaloids, steroids, tannins, flavonoids and saponins. Major secondary metabolites were identified by gas chromatography/mass spectrometry. Seed health and germination tests were performed using plant extract concentrations of 500, 1000 and 1500 ppm, chemical treatments and a control. For the health test, 200 seeds per treatment were immersed in 20 mL of extract at the tested concentrations for five minutes. For the germination test, 200 seeds were germinated at 27 °C. A completely randomized design was used. A chemical survey of the extracts indicated the presence of alkaloids, steroids, tannins, flavonoids and saponins, as well as the absence of steroids in the *M. charantia* and *A. colubrina* extracts and of saponins in the *C. ferrea* extracts. All extracts and concentrations efficiently reduced *Periconia* sp., *Fusarium* sp. and *Macrophomina* sp. *C. ferrea* and *A. colubrina* extracts efficiently reduced *Botrytis* sp., *Alternaria* sp. and *Rhizoctonia* sp. at all concentrations. *C. ferrea* extract at 1000 and 1500 ppm and *A. colubrina* extract at all concentrations reduced *Colletotrichum* sp. *A. colubrina* extract negatively affected seed germination.

Keywords: Forest species, Seed pathology, Secondary metabolites.

RESUMO
Os extratos de plantas com propriedades antimicrobianas são alternativas ecológicas aos fungicidas, e uma investigação preliminar de seus componentes químicos fornecem informações sobre suas propriedades. O presente estudo determinou o perfil fitoquímico dos extratos vegetais de *Momordica charantia*, *Caesalpinia ferrea* e *Anadenanthera colubrina*, avaliando-se os efeitos desses extratos sobre os microrganismos e a fisiologia das sementes de *A. colubrina*. Os extratos foram obtidos por extração a frio. As características fitoquímicas das espécies estudadas foram investigadas através da determinação da presença de alcalóides, esteróides, taninos, flavonóides e saponinas. Os principais metabólitos secundários foram identificados por cromatografia gasosa/espectrometria de massa. Testes de sanidade e germinação das sementes foram realizadas utilizando-se concentrações dos extratos vegetais nas concetrações: 500, 1000 e 1500 ppm, tratamentos químicos e controle. Para o teste de sanidade, 200 sementes por tratamento foram imersas em 20 mL de nas concentrações dos extratos por cinco minutos. Para o teste de germinação, 200 sementes foram incubadas em B.O.D. a 27 °C. O delineamento experimental foi o inteiramente casualizado. O levantamento químico dos extratos indicou a presença de alcalóides, esteróides, taninos, flavonóides e saponinas, bem como a ausência de esteróides nos extratos de *M. charantia* e *A. colubrina* e de saponinas nos extratos de *C. ferrea*. Todos os extratos e concentrações reduziram eficientemente *Periconia* sp., *Fusarium* sp. e *Macrophomina* sp. Os extratos de *C. ferrea* e *A. colubrina* reduziram eficientemente *Botrytis* sp., *Alternaria* sp. e *Rhizoctonia* sp. em todas as concentrações. O extrato de *C. ferrea* a 1000 e 1500 ppm e o extrato de *A. colubrina* em todas as concentrações reduziram *Colletotrichum* sp. O extrato de *A. colubrina* afetou negativamente a germinação das sementes.

Palavras-chave: Espécies florestais, Patologia de sementes, Metabólitos secundários.

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1 INTRODUCTION

Plant extracts contain secondary metabolites with antifungal properties and are important sources of fungitoxic compounds; thus, interest has been generated in using plant extracts to control disease (SILVA *et al*., 2016). These properties depend on factors such as plant species, age, organ and vegetative stage, as well as pathogenic species, the type of disease to be controlled and the technological processes used to obtain and handle the plant extracts (MEDEIROS *et al*., 2016).

Several complex molecules are synthesized by plants' secondary metabolism and are important for ecological relationships (DINIZ *et al*., 2013; SAROJ *et al*., 2015). Terpenes, phenolics and alkaloids are among the most important plant secondary metabolites; these metabolites are derived from amino acids, the main components of proteins (GRANATO *et al*., 2013). These substances are commonly related to antioxidant activity, scavenging free radicals and inhibiting lipid peroxidation (KUMAWAT *et al*., 2012).

Caatinga vegetation has great botanical potential, but the chemical composition and therapeutic potential of its plants have not been thoroughly investigated. Angico (*Anadenanthera colubrina*), bitter melon (*Momordica charantia*) and pau-ferro (*Caesalpinia ferrea*) are abundant species in this biome and are used as herbal drugs in popular medicine. However, the high demand for wood and charcoal by local industry, as well as for domestic use, results in unsustainable and illegal extraction, as native plant species are exploited (RIEGELHAUPT *et al*., 2013).

Angico is widely distributed in the Caatinga biome; it reproduces vigorously, and its seeds have no dormancy and fast germination (RAMOS *et al.,* 2014). However, angico has several limitations, especially related to health, because of the many pathogens associated with its seeds, which result in seed deterioration (SALIB *et al*., 2012).

Fungi are among the most important microorganisms to infect seeds, spreading disease, seed rot in the soil, seed deterioration during storage and mycotoxin production (MARTINS *et al*., 2015).

The effectiveness of management strategies for disease control is clearly dependent on understanding the ecology of the pathogen and its population dynamics in seeds (GOMES *et al*., 2020). Therefore, the present study determined the phytochemical profiles of extracts from angico**,** bitter melon and pau-ferro and evaluated their effect on microorganisms and on angico seed physiology.

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2 MATERIALS AND METHODS

The present study was performed at the Laboratory of Natural Product Chemistry of the Natural Sciences Center and at the Laboratory of Phytopathology of the Agricultural Sciences Center, Federal University of Paraíba in Brazil.

To test seed health and germination, angico seeds were obtained from fruits collected directly from mother trees in the municipality of São João do Cariri, state of Paraiba (PB) (7°23′27″S, 36°32′7″W), Brazil. The fruits were placed in polyethylene trays to facilitate aeration until processing and then processed one day after harvest. Seeds were processed in sterilized benches. Seeds showing mechanical or pest damage were eliminated to obtain a visually homogeneous selection.

Plant extracts were obtained from A. *colubrina*, *M. charantia* and *C. ferrea* leaves collected in the municipality of Areia, PB (S 06°58'06" W 35º42'55"), Brazil. Leaves were dried in an oven at 40 ºC for 72 hours, ground to a powder using a knife mill, and extracted by cold extraction.

Leaf powder samples of 150 g were placed in a beaker containing 500 mL absolute ethanol for 72 hours at room temperature (25 ± 2 °C), and the solution was filtered through filter paper. The solvent was extracted in a rotary evaporator for approximately 2 hours at 78 ºC, and the crude ethanolic extract was obtained.

The resulting extracts were phytochemically characterized, and crude extracts were diluted to 500, 1000, 1500 and 2000 ppm.

Extracts were characterized using the following methods:

a) Alkaloids: Twenty-five microliters of ethanolic extract was evaporated and alkalinized with 0.8 mL of 1% sodium hydroxide (NaOH). Subsequently, 6 mL of distilled water and 6 mL of chloroform ($CHCl_3$) were added, and the solution was placed in a funnel to separate the extract from the chloroform layer. Six milliliters of 1% HCl and 0.30 mL of Dragendorff's reagent were added to the chloroform phase (WU *et al.,* 2005).

b) Steroids: Ten microliters of ethanolic extract was evaporated, 2.5 mL of $CHCl_3$ was added until completely homogenized, and a 0.5-mL aliquot was collected. Subsequently, 2 mL of $CHCl_3$ and 1 mL of acetic anhydride were added and carefully stirred, and then, 2 mL of H_2SO_4 was added (KUJALA *et al.,* 2000).

c) Tannins: Tannins were determined using the casein precipitation method, in which 1 g of powdered casein and 6-mL aliquots of the extracts diluted in 12 mL of distilled water were placed into a 50-mL Erlenmeyer flask and stirred constantly for three hours at room temperature (25 ± 2 ºC). The samples were then filtered through filter paper, and the resulting filtrate was diluted to 25

mL. Residual phenols were determined in 5-mL aliquots using the Folin-Ciocalteu method (SOBRINHO *et al.,* 2010).

d) Flavonoids: Fifteen microliters of ethanolic extract was placed in a separation funnel and 15 mL of distilled water was added. The solution was left to stand for 10 minutes, and 15 mL of $CHCl_3$ was added. Layers were separated 5 minutes after adding chloroform, and the chloroform layer was discarded. The remaining extracts were separated, and 3 mL of ethanol was added. A 2-mL aliquot of the resulting solution was placed in a test tube, and 0.5 mL of 10% HCl and a 1-cm magnesium ribbon were added and left to react until the ribbon disappeared (CHUN *et al.,* 2004).

e) Saponins: Ethanolic extracts (0.25 mL) were placed in test tubes containing water and vigorously stirred to form foam. Foam persistence or absence was recorded after 10 minutes. Saponin presence indicated that the substance was highly water soluble (VIEIRA *et al.,* 2001).

Major secondary metabolites were identified by gas chromatography-mass spectrometry (GC-MS).

Fungal incidence in the angico seeds was visually evaluated using the filter paper method (BRASIL, 2009). Seeds were sterilized with 1% sodium hypochlorite for 3 minutes; immersed in 10 mL of angico, bitter melon or pau-ferro extract at the tested concentrations for five minutes; and placed in petri dishes lined with a double layer of sterile filter paper moistened with sterile distilled water. The petri dishes were kept at 25 ± 2 °C for seven days.

The following seed treatments were tested: T_1 - control (untreated seeds); T_2 - dicarboximide fungicide (240 g/100 kg); T_3 - bitter melon extract (BME, 500 ppm); T_4 - BME (1000 ppm); T_5 - BME (1500 ppm); T_6 - pau ferro extract (PFE, 500 ppm); T_7 - PFE (1000 ppm); T_8 - PFE (1500 ppm); T_9 - angico extract (ANE, 500 ppm); T_{10} - ANE (1000 ppm); and T_{11} - ANE (1500 ppm).

Fungi were detected and identified using a light microscope and a stereoscope and compared to descriptions in the literature (SEIFERT et al., 2011).

Two hundred seeds were used for the germination tests and were divided into four replicates of 50 seeds per treatment. The seeds were sown in sterile germitest paper moistened with distilled water at 2.5 times its dry weight and incubated in a biological oxygen demand (BOD) incubator at 27 ºC under a 12-h light:12-h dark photoperiod. The same treatments were tested as those for the health test.

Germinated and ungerminated seeds were counted from day 4 to day 10 after sowing and evaluated per the Rules for Seed Analysis (BRASIL, 2009). The following parameters were evaluated for the germination test: first germination count (FGC), germination (GER), dead seeds

(DS), hard seeds (HS), shoot length (SL), root length (RL), plant length (PL) and emergence speed index (ESI). A completely randomized design was used.

Fourteen treatments were tested, with 10 replicates of 20 seeds per treatment for the health test and four replicates of 50 seeds per treatment for the germination test. Means were compared using the Scott-Knott test at the 1% significance level, using SISVAR® Statistical Software (FERREIRA, 2011).

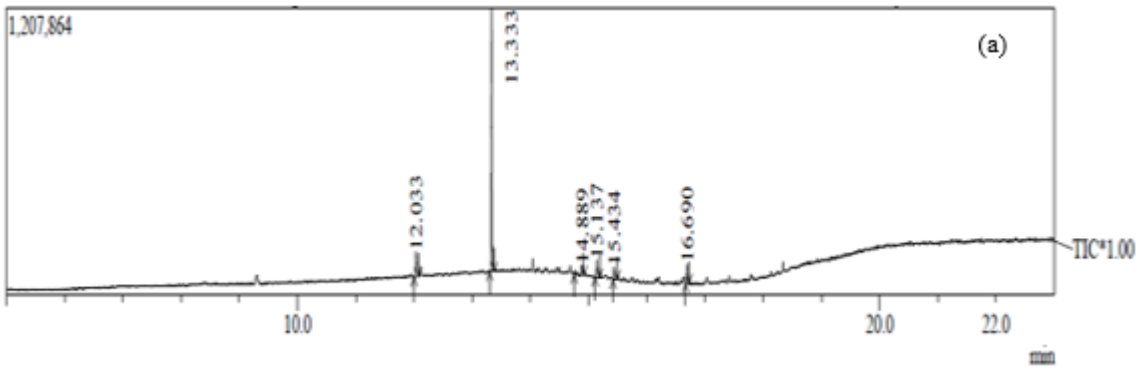
3 RESULTS AND DISCUSSION

Bitter melon, pau-ferro and angico ethanol extracts presented different secondary metabolite groups, suggesting the presence of alkaloids, steroids, tannins, flavonoids and saponins (Table 1).

Table 1 Phytochemical survey for detecting secondary metabolites in *Momordica charantia*, *Caesalpinia ferrea* and *Anadenanthera colubrina* ethanolic extracts.

Secondary metabolites	Ethanolic extracts			Indicator
	M. charantia	*C. ferrea*	*A. colubrina*	
Alkaloids	Positive	Positive	Positive	Dragendorff
Steroids	Negative	Positive	Negative	Digestion
Tannins	Positive	Positive	Positive	Gelatin
Flavonoids	Positive	Positive	Positive	Mg ribbon
Saponins	Positive	Negative	Positive	Foam

The chromatographic profiles of the *M. charantia*, *C. ferrea* and *A. colubrina* extracts are presented in Fig. 1. Based on the retention times, the major compounds detected in the *M. charantia* extract (Fig. 1a) were myristic acid (12.033 min), palmitic acid (13.333 min), linoleic acid (14.889 min), stearic acid (15.137 min), α-linolenic acid (15.434 min) and lupeol (16.690 min).



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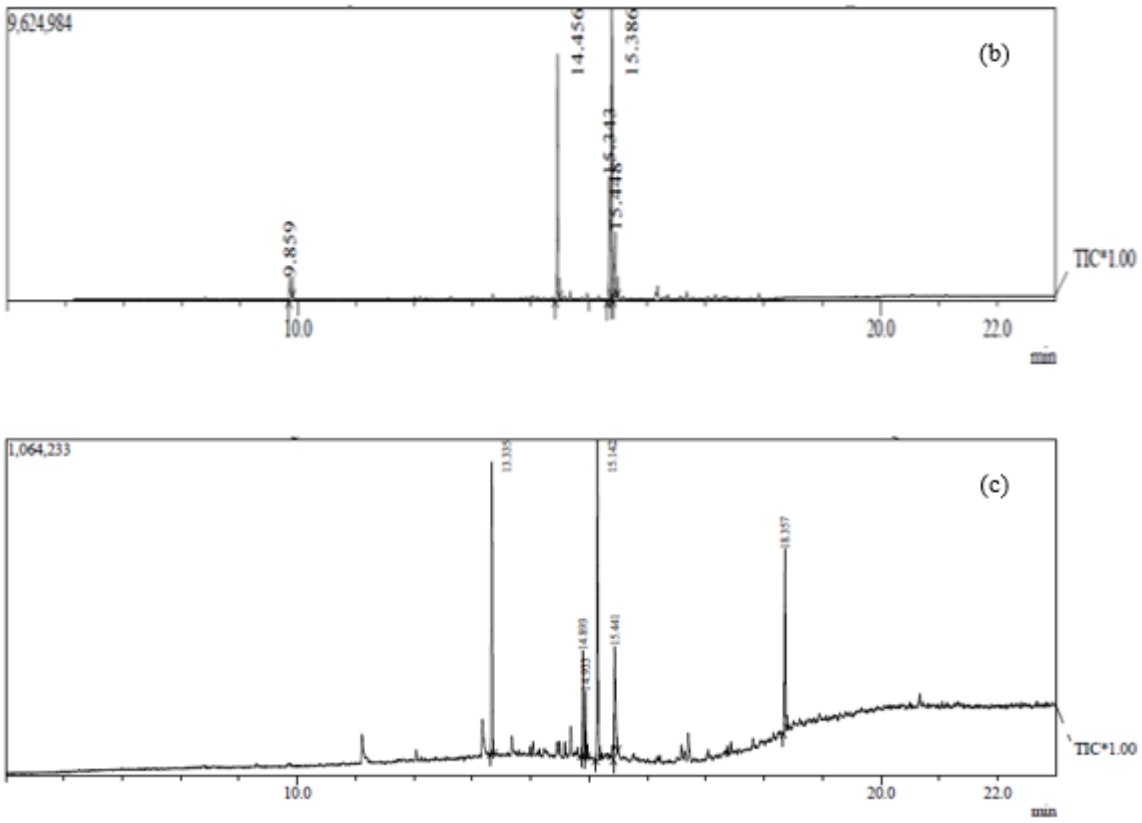

Figure. 1 Chromatographic profile of the ethanolic extracts of *Momordica charantia* (a), *Caesalpinia ferrea* (b), and *Anadenanthera colubrina* (c) and the retention times of the major compounds

The compounds detected in the *C. ferrea* extract (Fig. 1b) were gallic acid (9.859 min), galloyl shikimic acid (14.456 min), spinasterol (15.346 min), β-sitosterol (15.386 min) and lupeol (15.448 min). The compounds detected in the *A. colubrina* extract (Fig. 1c) were 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 in the plants secondary metabolism and play important roles in plant-pathogen relationships.

The secondary metabolite contents identified in the *M. charantia*, *C. ferrea* and *A. colubrina* extracts are presented in Table 2. Pau-ferro extract presented the highest contents of lupeol (15.4 mg/10 g), gallic acid (9.8 mg/10 g), myristic acid (1.7 mg/10 g) and α-spinasterol (3.4 mg/10 g).

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Table 2 Secondary compounds in *Momordica charantia*, *Caesalpinia ferrea* and *Anadenanthera colubrine* extracts (mg/10 g crude extract).
*

Species	Lupeol	Gallic Acid	Myristic Acid	α- Spinasterol	Ferulic Acid	Catechin	Quercetin
BME**	6.6	2.1	0	0	1.4	4.5	2.0
PFE	15.4	9.8	1.7	3.4	0	0	7.1
ANE	11.2	2.2	0	0	0.9	7.6	12.0

Identified by CG-MS. ** BME = *Momordica charantia* extract; PFE = *Caesalpinia ferrea* extract; ANE = *Anadenanthera colubrina* extract

M. charantia extract presented the highest ferulic acid content (1.4 mg/10 g). This compound is related to cell wall resistance (HOSON; WAKABAYASHI, 2015). *A. colubrina* extract presented the highest catechin (polyphenol) and quercetin (flavonoid) contents, at 7.6 and 12.0 mg/10 g, respectively.

Fungal incidences in angico seeds treated with *A. colubrina*, *C. ferrea* and *M. charantia* extracts are presented in Table 3. Compared with the control, angico extract at all concentrations and pau-ferro extract at 1000 and 1500 ppm efficiently reduced *Colletotrichum* sp. Compared with the control, pau-ferro and angico extracts at all concentrations efficiently reduced *Botrytis* sp., and bitter melon, pau-ferro and angico extracts at all concentrations reduced *Periconia* sp.

Bitter melon extract at 1000 and 1500 ppm and pau-ferro and angico extracts at all concentrations efficiently reduced *Alternaria* sp. Extract efficiency in reducing fungal incidence is related to the secondary metabolites present and their effects on plant pathogens.

Several authors have studied the effects of plant extracts on seed health. Leite *et al.* (2011) and Medeiros *et al.* (2015), reported that extract application reduced microorganisms and promoted seed germination.

All tested extracts at all concentrations, compared with the control, efficiently reduced *Fusarium* sp. All tested extracts at all concentrations, except bitter melon at 500 ppm, reduced *Rhizoctonia* sp. (Table 3).

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Table 3 Fungal incidence and efficiency of *Anadenanthera colubrina*, *Caesalpinia ferrea and Momordica charantia* extracts for reducing fungal incidence in *Anadenanthera colubrina* seeds.

	Fungal incidence (%)					
Treatments	*Colletotrichum* sp.	*Botrytis* sp.	*Periconia* sp.	*Alternaria* sp.	*Fusarium sp.*	*Rhizoctonia sp.*
T1 - Control	25.0 a	16.0 a	20.0 a	10.0 a	11.0 a	7.0 a
T2 - Dicarboximide	0.0 d	0.0 f	1.0 f	0.0 c	1.0 c	0.0 c
T3 - BME 500 ppm	23.0 a	14.0 a	10.0 d	8.0 a	7.0 b	8.0 a
T4 - BME 1000 ppm	23.0 a	17.0 a	9.0 d	4.0 b	7.0 b	4.0 b
T5 - BME 1500 ppm	24.0 a	14.0 a	12.0 c	2.0 c	8.0 b	0.0 c
T6 - PFE 500 ppm	23.0 a	11.0 b	12.0 c	5.0 b	6.0 b	1.0 c
T7 - PFE 1000 ppm	17.0 b	8.0 c	17.0 b	1.0 c	2.0 c	0.0 c
T8 - PFE 1500 ppm	15.0 b	7.0 c	10.0 d	0.0 c	0.0 c	0.0 c
T9 - ANE 500 ppm	12.0 c	7.0 c	7.0 e	0.0 c	1.0 c	0.0 c
T10 - ANE 1000 ppm	10.0 c	4.0 d	7.0 e	0.0 c	0.0 c	0.0 c
T11 - ANE 1500 ppm	10.0 c	5.0 e	4.0 f	0.0 c	0.0 c	0.0 c
CV (%)	22.0	17.0	16.0	19.0	26.0	18.0
S.S.D.	1.70	1.22	1.30	0.91	0.89	1.12

Means followed by the same letter within the same column did not significantly differ per the Scott-Knott test at $p \leq 0.01$. CV = coefficient of variation; S.S.D. = significant standard deviation; BME = *Momordica charantia* extract; PFE = *Caesalpinia ferrea* extract; ANE = *Anadenanthera colubrina* extract

Regarding the physiological quality of the angico seeds, the highest first germination count (85%) was observed for the treatment with bitter melon extract at 1000 ppm. Germination was highest for the treatments with bitter melon extract at 1000 ppm (96%) and 1500 ppm (94%) and with pau-ferro extract at 500 ppm (96%). The fewest dead seeds were observed for the same treatments. No significant differences were observed in hard seeds, shoot length, root length, plant length or emergence speed index (Table 4).

Table 4 Mean first germination count (FGC), germination (GER), dead seeds (DS), hard seeds (HS), shoot length (SL), root length (RL), plant length (PL) and emergence speed index (ESI) for *Anadenanthera colubrina* seeds.

Treatments	FGC	GER	DS	HS	SL	RL	PL	ESI
	..... (%)				..... (cm).....			
T1 - Control	58 d	84 b	15 c	1 a	6.0 a	3.8 a	9.8 a	3.9 a
T2 - Dicarboximide	65 c	96 a	4 d	0 a	5.9 a	4.6 a	10.5 a	4.2 a
T3 - BME 500 ppm	68 c	83 b	17 c	0 a	5.6 a	3.6 a	9.2 a	3.7 a
T4 - BME 1000 ppm	85 a	96 a	4 d	0 a	5.4 a	3.8 a	9.2 a	4.3 a

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T5 - BME 1500 ppm	76 b	94 a	6 d	0 a	5.5 a	4.1 a	9.6 a	4.6 a
T6 - PFE 500 ppm	77 b	96 a	4 d	1 a	5.2 a	3.6 a	8.8 a	4.4 a
T7 - PFE 1000 ppm	54 d	82 b	18 c	0 a	5.3 a	3.8 a	9.1 a	3.8 a
T8 - PFE 1500 ppm	62 c	66 d	34 a	0 a	5.1 a	3.6 a	8.7 a	3.9 a
T9 - ANE 500 ppm	54 d	74 c	26 b	0 a	5.9 a	5.0 a	10.9 a	3.8 a
T10 - ANE 1000 ppm	54 d	69 d	31a	0 a	4.7 a	4.0 a	8.7 a	3.4 a
T11 - ANE 1500 ppm	52 d	69 d	31 a	0a	4.7 a	4.5 a	9.2 a	3.1 a
CV (%)	10.34	9.22	15.21	13.10	15.36	16.50	12.44	8.49
A.S.D.	1.26	0.75	1.41	2.15	0.58	1.36	1.14	1.10

Means followed by the same letter within the same column did not significantly differ per the Scott-Knott test at $p \leq 0.01$. CV = coefficient of variation; S.S.D. = significant standard deviation; BME = *Momordica charantia* extract; PFE = *Caesalpinia ferrea* extract; ANE = *Anadenanthera colubrina* extract

Overall, the studied species presented phytochemical components with antimicrobial potential, likely related to their antioxidant and antimicrobial activities (SOUZA *et al.*, 2013).

M. charantia, *C. ferrea* and *A. colubrina* extracts contain compounds that may be the main factors causing the extract's biological activity, although their mechanisms of action are usually associated with specific bioactivities. Their main properties should therefore be highlighted, such as their ability to neutralize free radicals produced by cells (BESSA *et al.,* 2013).

Lupeol is a triterpene with antimicrobial action, and its reactions are catalyzed by lupeol synthase (JEFFREYS *et al.*, 2016). Gallic acid is an important phenol for plant defense, with proven antimicrobial activity, and is the reference standard for quantifying phenol (MORAIS *et al.,* 2016). Myristic acid is a fatty acid that acts as a metabolic energy reserve (LOPES *et al.,* 2010) α-Spinasterol has powerful antioxidant effects against several reactive oxygen species (TREVISAN *et al.,* 2012).

Fungi of the genus *Fusarium* can survive in the soil through resistance structures and in seeds' internal structures, such as the embryo, producing various mycotoxins such as fusaric acid (SHI *et al.,* 2016). Fungi of the genus *Rhizoctonia* survive saprophytically in the soil as mycelia and sclerotia (OKUBARA *et al.,* 2014) and can be transmitted to seedlings through seeds, causing root problems and seedling lodging (LAZAROTTO *et al.,* 2012). Fantiniel *et al.* (2013), evaluated fungal incidence and transmissibility in golden trumpet tree seeds (*Handroanthus chrysotrichus*) and observed a low percentage of *Rhizoctonia* sp. (0.70% for a seed lot from Santa Cruz do Sul and 0.00% for a seed lot from Venâncio Aires, state of Rio Grande do Sul).

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4 CONCLUSIONS

The phytochemical surveys of *Momordica charantia*, *Caesalpinia ferrea* and *Anadenanthera colubrina* extracts indicated that alkaloids, steroids, tannins, flavonoids and saponins were present.

All tested extracts and extract concentrations efficiently reduced the incidence of *Periconia* sp., *Fusarium* sp. and *Macrophomina* sp.

C. ferrea and *A. colubrina* extracts at all concentrations efficiently reduced *Botrytis* sp., *Alternaria* sp. and *Rhizoctonia* sp. *C. ferrea* extract at 1000 and 1500 ppm and *A. colubrina* extract at all concentrations reduced *Colletotrichum* sp. And *A. colubrina* extract negatively affected angico seed germination.

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