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TOXICITY OF *Rosmarinus officinalis* ESSENCIAL OIL IN THE CONTROL OF THE FUNGUS *Aspergillus brasiliensis*

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ABSTRACT

Fungi are the primarily mainly responsible for food spoilage, resulting in great losses, in addition to having the ability to produce toxins, which can be harmful to human and animal health. These fungi have been controlled using pesticides, which are harmful contaminants to the environment and human health. In addition, these microorganisms develop resistance to the action of these substances, which become inefficient. These factors have prompted the search for alternative methods of controlling these microorganisms. In this scenario, essential oils are raised as a possible alternative to fungicides. The objective of this study was to identify the main components and to evaluate the effectiveness of rosemary essential oil (*Rosmarinus officinalis*) for controlling the fungus *Aspergillus brasiliensis*. The main components of the essential oil identified by gas chromatography in decreasing order of concentration were: 1.8-cineole (48%), camphor (12%), and β-pinene (8%). The effect of the essential oils on the fungal mycelial growth was tested *in vitro* with doses ranging from 0.8 to 25 μL/mL, the latest providing the greatest fungal control. Based on the results of the *in vitro* tests, serial microdilution was performed in microplates and found the minimum inhibitory concentration at 25 μL/mL. The results showed that the essential oil has fungicidal potential against *A. brasiliensis*.

Keywords: Rosemary, Alternative control; Natural products, Fungicidal properties, Fungal pathogens.

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RESUMO

Toxicidade do óleo essencial de *Rosmarinus officinalis* no controle do fungo *Aspergillus brasiliensis*. Os fungos são os maiores responsáveis por deteriorar alimentos, acarretando em grandes perdas, além de apresentarem a capacidade de produzir toxinas, que podem ser prejudiciais à saúde humana e animal. O controle desses fungos ocorre através da utilização de agrotóxicos, que são contaminantes nocivos ao ambiente e a saúde, além do fato desses microrganismos se tornarem resistentes à ação dessas substâncias tornando-as ineficiente. Devido a esses fatores iniciou-se a busca por métodos alternativos do controle desses microrganismos. Neste cenário, os óleos essenciais mostram-se como possível alternativa. O objetivo deste estudo foi identificar os principais componentes e avaliar a eficácia do óleo essencial de alecrim (*Rosmarinus officinalis*) para controle do fungo *Aspergillus brasiliensis*. Os principais componentes do óleo essencial, identificados por cromatografia gasosa, em ordem de concentração, foram: 1,8-cineol (48%), cânfora (12%) e o β-pineno (8%). Inicialmente verificou-se o efeito dos óleos essenciais sobre o crescimento micelial do fungo em testes *in vitro* entre as doses de: 0,8 a 25 μL/mL, sendo esta a dose de maior controle fúngico. Baseando-se nos resultados dos testes *in vitro*, foi realizada a microdiluição seriada em microplacas para encontrar concentração mínima inibitória, a qual foi de 25 μL/mL Os resultados demonstraram que o óleo essencial apresentou potencial fungicida sobre o *A. brasiliensis*.

Palavras-chave: Alecrim; Controle Alternativo; Produto Natural; Propriedades Fungicidas; Patógeno Fungo.

INTRODUTION

Fungal contamination causes considerable economic losses to the food industry. Fungal spoilage is associated with reduced nutritional value, altered taste, as well as the commercial unfeasibility due to mycotoxin production, which affects both human and animal health. Fungi of the genus *Aspergillus* are known to be the major cause of damage to grain post-harvest in the world (Yu, 2010; Alves et al., 2014).

In a study on *Aspergillus*, Parenicová et al. (2001), observed that *Aspergillus brasiliensis* had distinct characteristics from *A. niger* and *A. tubingensis*, suggesting the classification of a new species, which was later described by Varga et al. (2007). The strains were isolated from Brazilian soils and subsequently found in the soils of Australia, the United States and the Netherlands, including stored grains (Oliveira et al., 2020).

Fungicides are responsible for environmental contamination and are harmful to human health. The harms caused by these synthetic products led to the search for alternative control methods such as substances derived from plant extracts and oils (Kasper et al., 2018).

In the search for natural alternatives to combat the harmful effects of fungi on food, essential oils have been important target of research, since they prove to be useful in phytosanitary control and as insect repellent (Knaak and Fiuza, 2010; Desai and Parikh, 2014; Bedini et al., 2020). Studies such as those by Vardar-Unlu et al. (2003) attested the antioxidant activity and demonstrated the biological effect of essential oils on several microorganisms, including phytopathogens. Other studies such as Guimarães et al. (2011) showed the antifungal and fungitoxic action of essential oils in the conservation of grains.

The species *Rosmarinus officinalis,* commonly known as rosemary, of the family Lamiaceae, is a small, aromatic plant, native to the Mediterranean region, but cultivated in several temperate countries,

is used as a condiment in culinary and in traditional medicine. The essential oil consists of a mixture of volatile components which characterize the typical aroma, including mainly cineol, α-pinene and camphor (Lorenzi, 2008; Araújo et al., 2013). Studies such as Lee et al. (2020), Álvarez et al. (2023), Boy et al. (2023), Baiotto et al. (2023), showed the effectiveness of rosemary essential oil against microorganisms.

In this context, the use of essential oils has been a promising methodology for the control of phytopathogenic fungi among the natural alternatives that do not cause harmful effects or environmental damage. The objective of this study was to identify the main components and evaluate the fungicidal potential of the essential oil of rosemary (*R. officinalis*) on the fungus *A. brasiliensis*.

MATERIAL AND METHODS

Aspergillus brasiliensis strains identified as CCCD AA002 were purchased from the commercial supplier Didática Scientific Eireli. Cultures were grown in PDA medium (potato, dextrose and agar) in Petri dishes at 30 º C for seven days. For spore collection, the plates were flooded with 15 mL sterile distilled water and conidia were harvested with a pipette. The spore suspension was adjusted with sterile distilled water to give the final concentration of 4.5×10^6 spores mL^{-1} using a Neubauer chamber. The suspension was stored at 4 ºC until use.

The essential oil of *Rosmarinus officinalis* was purchased from a cosmetics industry and trade company. Analysis of essential oil constituents was performed by gas chromatography-mass spectrometry (GC/MS). The compounds were separated in a fused-silica capillary column with DB-5 stationary phase (30 m long x 0.25 mm internal diameter x 0.25 uM inner film thickness). Helium was used as carrier gas at a flow rate of 1.0 mL min^{-1}. The temperature of the injector was hold at 220 °C and the detector at 240 °C. The initial oven temperature was maintained at 60 °C for 2 min and programmed with a heating rate of 3 °C min^{-1} to 240 °C and held for 30 min, in a total analysis time of 91 minutes. The split ratio was 1:20 and the solvent cut-off time was 5 minutes. The sample injection volume was 1 μL, at a concentration of 10,000 ppm, using hexane as solvent. Compounds were identified by comparing the mass spectra obtained with those of the apparatus database and by the Kovats Retention Index (IK) of each component (Lanças, 1993). The quantitative analysis of the main components of the essential oil, expressed as a percentage, was performed by the peak area integration normalization method, as described by Zhang et al. (2006).

For the *in vitro* antifungal assay, the following doses of rosemary essential oil were tested: 0.8; 1.6; 3.2; 6.4; 12.8; 15; 17.5; 20; 22.5 and 25 μL/mL. Plates were prepared by pouring 20 mL of PDA culture medium into Petri dishes previously sterilized at 121 °C for fifteen minutes in autoclave, containing the *R. officinalis* essential oil concentrations diluted in 1% DMSO (dimethyl sulfoxide). Petri dishes were incubated with 7 mm mycelial discs of both species in the center of the plate. Four replicates were used for each treatment. Two control treatments without essential oil were performed: one with fungus growing on PDA medium only; and the other with fungus growing in PDA medium added with DMSO to evaluate the influence of the surfactant on fungal growth. The plates were incubated in B.O.D (Biological Oxygen Demand) at 30 °C until the mycelial growth in the control treatments covered the entire Petri dish, with 92 mm diameter, which was considered the end of the incubation time, which was between 7 and 10 days.

The colony diameter was recorded daily with a digital caliper. The percentage of colony inhibition (PI) was calculated with the following equation (Billerbeck et al., 2001; Tatsadjieu et al., 2010).

$$PI = \frac{\varnothing_0 - \varnothing_T}{\varnothing_0} 100 \quad \text{(Equation 01)}$$

Where:

$\varnothing_0$ = diameter of colonies without treatment;

$\varnothing_T$= diameter of colonies treated with essential oil.

The minimum inhibitory concentration (MIC) of the essential oil (EO) on the fungi studied was determined by serial microdilution in microplate. The doses tested were defined from the results of the *in vitro* test and the following EO doses were tested: 50; 40; 36; 30; 25; 20; 18; 15; 12,5; 12; 10; 9; 7.5; 6.25; 6.5; 4,5; 3.75; and 3 μL/mL . Each dose tested had four replicates in PD medium (potato and dextrose) with the solution containing essential oil, DMSO, and spore suspension (10^7). A control treatment was used without the essential oil. The plates were kept in a B.O.D chamber at 35 ºC for 72 h. After the incubation time, the results were analyzed visually. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of essential oil in which no fungal growth occurred (Pandey et al., 2003; DellaValle et al., 2011).

The experiment was arranged in a completely randomized design. The results were analyzed by analysis of variance and means compared by the Scott-Knott test at 5% significance level. Data analysis was performed using SISVAR® (Ferreira, 2014).

RESULTS

Figure 1 shows the chromatogram obtained in the identification of the components of the essential oil of rosemary used in the experiment.

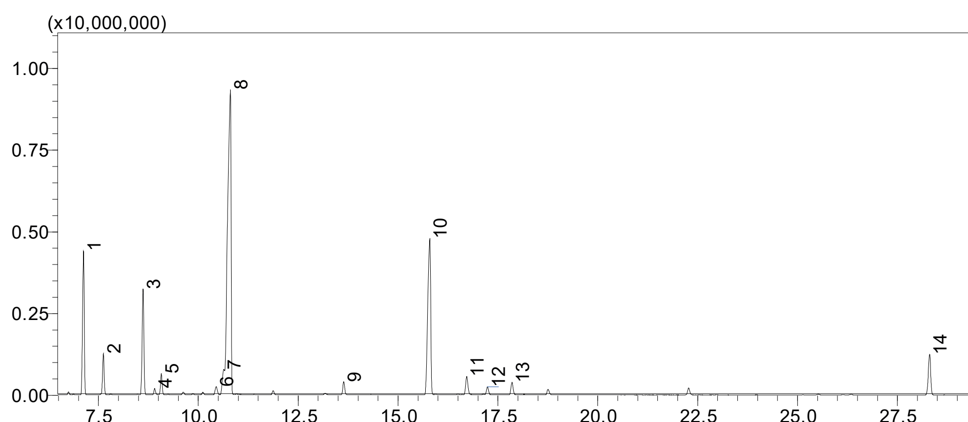


Figure 1. GC/MS chromatogram of the *Rosmarinus officinalis* essential oil.

Table 1 shows the average retention time and Kovats index of the components identified by the chromatogram shown in Figure 1, some peaks were not identified. The major components were: 1,8-cineole 48%, camphor 12% and β-pinene 8%.

Table 1. Main components of *Rosmarinus officinalis* essential oil determined by GC- MS.

Peak	Component	Retention time (min)	Kovats Index*	Adams	Other authors**
01	α-pinene[1]	7.127	963	-	-
03	β-pinene	8.616	989	980	990[2]/981[3]
05	Myrcene	9.073	996	991	994[2]
08	1,8-cineole	10.806	1036	1033	1039[2]
09	Linalool	13.638	1093	1098	-
10	Camphor	15.796	1143	1143	-
11	Borneol	16.719	1164	1165	-
12	Terpinen-4-ol	17.236	1175	1177	1178[3]

*Column DB-5. [1]Adams (2007), **[2]Hognadottir and Rouseff (2003), [3]Choi (2003).

The *in vitro* inhibitory effect of the different doses of essential oil in relation to the control treatment (dose 0) can be seen in Figure 2, which shows the growth of the diameter of the colonies as a function of the incubation period.

Figure 2. Effect of concentrations of *Rosmarinus officinalis* essential oil on the mycelial growth of the fungus *Aspergillus brasiliensis*.

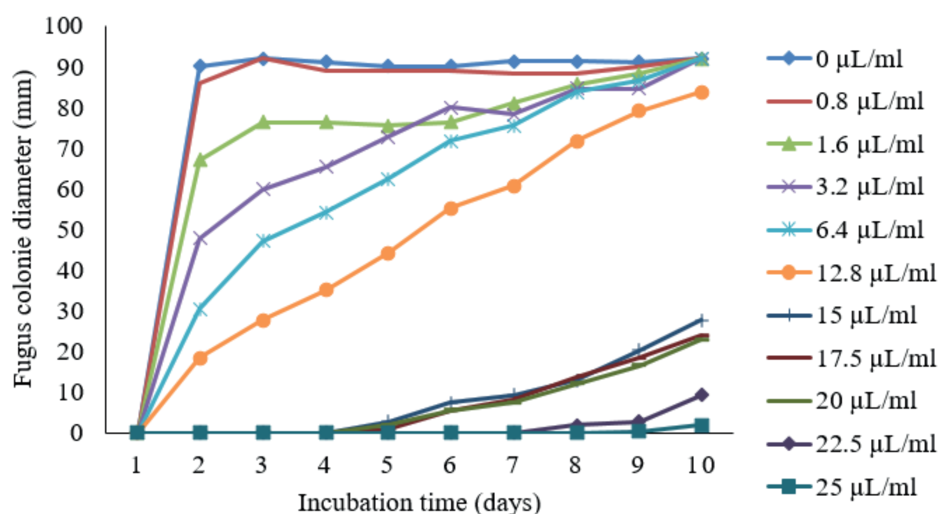
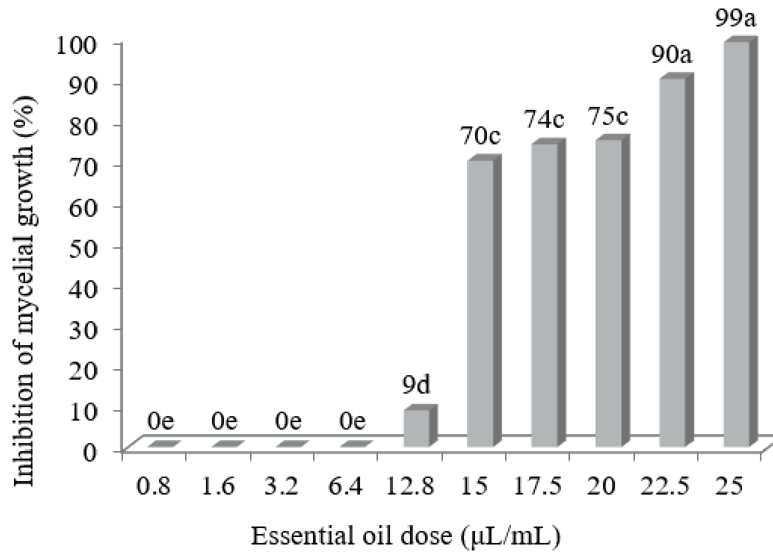


Figure 02 illustrates the sharp decrease in the radial growth of the fungus from the dose of 15 µL/mL. The doses 20 and 25 µL/mL showed the least growth from ten days of incubation.

Analysis of variance of the effect of the rosemary essential oil (*R. officinalis*) and the incubation time on the mycelial growth of the fungus *A. brasiliensis* demonstrated that there was a significant effect of the different doses of rosemary essential oil, and the incubation time, as well as of their interaction, indicating that the inhibition of the growth of the fungus by the essential oil depends on the interaction between the oil dose and the incubation time.

Figure 3 indicates the percentage of inhibition of mycelial growth by the fungus *Aspergillus brasiliensis* at the different doses tested at the end of the incubation period, showing that 25 μL/mL was the most efficient dose for the control of the microorganism.

Figure 3. Percentage inhibition of mycelial growth of *Aspergillus brasiliensis* at different doses (μL/mL) of *Rosmarinus officinalis* essential oil from ten days of incubation.



According to Figure 03, the percentage of inhibition was less than 9% for doses below 12.8 μL/mL, revealing low antifungal activity. At doses higher than 15 μL/mL, the percentage of inhibition was above 70%, in which the doses of 15, 17.5, and 20 μL/mL were not significantly different, and the doses 22.5 and 25 μL/mL showed the highest percentage of inhibition, above 90%, and were significantly different.

Serial microdilution in microtiter plate was performed to determine the minimum inhibitory concentration (MIC) of the essential oil to inhibit fungal growth. The results are presented in Table 04. The concentrations tested were based on the results of the *in vitro* tests. The MIC was determined as 25 μL/mL, showing no visible fungal growth.

Table 4. Indication of fungal growth* in serial microdilution of *Aspergillus brasiliensis* at different concentrations (µL/mL) of rosemary (*Rosmarinus officinalis*) essential oil.

Dose (µL/mL)	Aspergillus brasiliensis	Dose (µL/mL)	*Aspergillus brasiliensis*
50	-	**12**	+
40	-	**10**	+
36	-	**9**	+
30	-	**7.5**	+
25	-	**6.25**	+
24	+	**6**	+
20	+	**5**	+
18	+	**4.5**	+
15	+	**3.75**	+
12.5	+	**3**	+

* + indicates fungal growth; - no growth.

DISCUSSION

The main components of the rosemary essential oil used in this study in comparison with the published literature were: α-*pinene,* β-*pinene, myrcene, 1.8 cineol, linalool, camphor, borneol, terpinen-4-ol* (Mekonnen et al., 2016; Satyal et al., 2017; Hendel et al., 2019; Bedini et al., 2020).

The essential oil of rosemary showed antifungal activity against *A. brasiliensis* at the doses tested, showing 99% total inhibition of fungal growth at the highest concentration tested (25 µL/mL). The radial growth of the fungus at doses above 15 µL/mL was inhibited until the fourth day of incubation, but from the sixth day, only the doses 22.5 and 25 µL/mL continued to inhibit the growth of the fungus without significant difference, proving to be the most efficient.

In this study, the major components of rosemary essential oil were: 1,8-cineole (48%), camphor (12%) and β-pinene (8%). Chao et al. (2000) tested essential oil containing α-1,8-cineole (43.6%), camphor (12.3%), β-pinene (5%), and pinene (7.4%) against *Aspergillus niger* and obtained no control, which may be associated with the different chemical composition of the essential oil, whereas in his work, Baratta et al. (1998) managed to control the same fungal species with 1,8-cineole (46.6%) and α-pinene (11.8%) in the oil composition. Bomfim et al. (2020) controlled the growth of the species *A. flavus* with - 1,8 cineole (52.2%), camphor (15.2%), and α-pinene (12.4%).

The MIC was 25 µL/mL according to the most efficient control dose in the *in vitro* test. Variations in MIC values have also been found in other studies, using *Rosmarinus officinalis* such as in Camiletti et al. (2014) major components β-Myrcene 20.18%, Eucaliptol 24.34% and Camphor 13.99% for *A. flavus* >

500 µL/mL and Baghloul et al. (2017) 1.8 cineole, major compound with 65.63% for *A. niger* 500 µL/mL.

The composition of the essential oil may vary according to the location of the plant, the stage of development of the plant, the climate and soil conditions, harvest time, form of extraction, among others, and consequently influences the control of different species according to the quantity of its main components.

The development of an essential oil-based product for the control of spoilage food fungi such as the genus *Aspergillus* requires the study of the effect of each component separately and their necessary quantity for the formulation of a fungicide with antifungal compounds.

CONCLUSION

The essential oil of *Rosmarinus officinalis* showed fungicidal effect against the fungus *Aspergillus brasiliensis* in the *in vitro* test, with greatest inhibition (99%) at the dose 25 µL/mL, which was regarded as the minimum inhibitory concentration (MIC). The major component of the essential oil under study was 1,8-cineole, accounting for 48% of its chemical composition, followed by camphor 12%, and ***β***-pinene 8%. From the foregoing, therefore, it is evident that the use of natural products can be an alternative for the control of the genus *Aspergillus*.

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