CHEMICAL COMPOSITION AND ANTIFUNGAL ACTIVITY OF PROPOLIS ON *Aspergillus flavus*

COMPOSIÇÃO QUÍMICA E ATIVIDADE ANTIFÚNGICA DE PRÓPOLIS SOBRE Aspergillus flavus

Alexandre LORINI[1]; Carmen WOBETO[2]*; Solange Maria BONALDO[2]; Sílvia de Carvalho Campos BOTELHO[3]; Adilson Paulo SINHORIN[2]

1. Doutorando em Ciência e Tecnologia de Alimentos, Universidade Federal de Pelotas, RS, Brasil; 2. Professor (a) Doutor (a), Universidade Federal de Mato Grosso, Campus Universitário de Sinop, MT, Brasil. *carmenwobeto2014@gmail.com;
3. Pesquisadora, Doutora, Empresa Brasileira de Pesquisa Agropecuária, Embrapa Agrossilvipastoril, Sinop, MT, Brasil

ABSTRACT: Research has indicated the antifungal activity of ethanol extracts from propolis (EEP) on fungi of the genera *Phakopsora*, *Colletotrichum,* and *Cercospora*. Here, chemical compositions and antioxidant activity of three EEP (propolis from *Scaptotrigona polysticta* stingless bee and two types produced by *Apis mellifera* - red and brown) were evaluated and their action against *Aspergillus flavus* was investigated. Ash, dry extract, total phenolic and total flavonoid contents were determined. Phenolic composition was evaluated by high performance liquid chromatography, with using the following reference substances: gallic acid, caffeic acid, *ρ*-coumaric acid, ferulic acid, quercetin, kaempferol, and apigenin. Tests on mycelial growth, sporulation, and germination of spores were performed to assess the biological activity of the EEP on *A. flavus*. All EEP showed low dry extract content (<11%) with adequate amounts of ash (<5%). The red EEP (*Apis mellifera*) showed the highest contents of total phenolic and total flavonoid (5.38 and 2.77 g 100 g^{-1}), while the highest recorded antioxidant activity was exhibited by brown EEP (92.9%). The EEP of *S. polysticta* presented higher levels of *ρ*-coumaric acid (10.99 mg g^{-1}), while red and brown extracts from *A. mellifera* stood out with the highest levels of quercetin (27.26 mg g^{-1}) and gallic acid (5.88 mg g^{-1}), respectively. No extract was effective in inhibiting mycelial growth and sporulation of *A. flavus*, but red EEP inhibited spore germination. On the basis of these results, it is suggested that the inhibitory effect of red EEP on spore germination may be associated with increased levels of flavonoids found in it as compared with the other extracts investigated here.

KEYWORDS: *Apis mellifera*. Phenolic. Fungitoxicity. *Scaptotrigona polysticta.*

INTRODUCTION

Fungal control is usually performed with the help of agrochemicals, i.e. synthetic xenobiotic products that generally contain toxic metals in their formulations, and thus, may harm the environment. The scientific community has been studying alternative controls, which do not cause environmental problems for post-harvest control of microorganisms (FERNANDES et al., 2007; MARINI et al., 2012; COSTA et al., 2013). An example of biological material researched and used for the control of microorganisms in foods (KAMEYAMA et al., 2008) or directly in humans (CUNHA et al., 2011) is ethanolic or aqueous extracts from propolis.

Propolis is a resinous material produced by bees from plant resins; its function is to ensure stability of the beehive and provide it protection against pathogens or invaders (INOUE et al., 2007). Propolis, as well as all other bee products, have their chemical composition dependent on climatic factors due to their seasonal and botanical origin. Propolis extracts from various Brazilian regions have been investigated, since it is a country of great biodiversity and climatic variability (BANKOVA et al., 2000; PARK et al., 2002; SALATINO et al., 2005; DAUGSCH et al., 2008).

Propolis has been reported to inhibit the growth and development of fungi that causes brown eye spot in coffee (*Cercospora coffeicola* Berk & Cooke (1881)) (PEREIRA et al., 2013), as well as fungi that cause anthracnose in bean (*Colletotrichum lindemuthianum* Briosi & Cavara, (1889)) (PEREIRA et al., 2014), rust, and leaf spot on vine (*Phakopsora euvitis* Ono (2000) and *Pseudocercospora vitis* Speg (1910)) (MARINI et al., 2012).

Aspergillus flavus Link (1809) is related to many health problems, including carcinogenesis, owing to its tendency of producing mycotoxins, such as aflatoxins B1 and B2 molecules (GASPAROTTO et al., 2005). Aflatoxins cause a risk to human health due to their immediate toxic effects, including immunosuppressive, mutagenic, teratogenic, and carcinogenic effects (MAZIERO; BERSOT, 2010). Aly and Elewa (2007) found that besides inhibiting the growth of *Aspergillus versicolor* Tiravoschi (1908) in Ras cheese, propolis

Received: 29/11/17
Accepted: 15/06/18

can also inhibit toxin production by these microorganisms.

Thus, three propolis extracts were evaluated here for their physicochemical characteristics and antifungal action on *Aspergillus flavus* isolated from Brazil nuts.

CONTENTS

Three propolis types were collected: a red propolis from *Apis mellifera* from Paraiba state, Brazil; propolis from *Scaptotrigona polysticta* (stingless bee) and a brown propolis from *A. mellifera*, both from Mato Grosso state, Brazil. For the production of ethanolic extracts from propolis (EEP), the methodology described by Woisky & Salatino (1998) was employed after some modifications. Briefly, 20% propolis was kept in 70% ethanol for 15 days after maceration, while being shaken manually daily. However, as the propolis from *S. polysticta* did not provide satisfactory solubilization, another 100 mL of ethanol was added, which led to a concentration of 10% for the extract that was maintained for another 15 days of maceration. Afterwards, all EEP were filtered and transferred to amber glass bottles and stored at room temperature until further chemical analyses or biological assays.

Gravimetric methods were used to determine the dry extract and ash content (WOISKY; SALATINO, 1998). The contents of total phenolic and total flavonoid was determined by spectrophotometry using Folin–Ciocalteau reagent and aluminum chloride, respectively, with gallic acid and quercetin as reference compounds for total phenolic and total flavonoid, respectively (FUNARI; FERRO, 2006).

The antioxidant activity was determined by the scavenging of DPPH radicals, using the formula: Antioxidant Activity (%) = (Control Absorbance - Absorbance of the Sample × 100) / Control Absorbance (CARPES et al., 2009).

The phenolic and flavonoid analyses on EEP was conducted by High Performance Liquid Chromatography (HPLC) according to the methodology described by Barbari'c et al. (2011) with some modifications. A Varian Modular Analytical HPLC System was employed, which was equipped with UV detector (290 nm), C18 chromatography column (Agela - 4.6 mm × 250 mm, 5 µm particle diameter) was used , and two mobile phases (A and B): water/methanol/acetic acid (93:5:2) (A) and water/methanol/acetic acid (3:95:2) (B). Elution was performed at a flow rate of 1 mL/min, using the following gradient expression in time (min) as per the percentage of B (t/min, %B): (0, 20), (20, 40,), (30, 52), (50, 60), (70, 80), and (80, 20). The extracts were evaporated in a rotary evaporator and solubilized in HPLC grade methanol (10 mg mL^{-1}); from this, an aliquot of 20 µL was injected into the chromatographic system. For the identification and quantification of the phenolic and flavonoid compounds, standard curves were prepared using the following reference substances: gallic acid, caffeic acid, *ρ*-coumaric acid, ferulic acid, quercetin, kaempferol, and apigenin.

For determination of mycelial grown and sprorulation, EEP were employed at concentrations of 1.2 and 1.6 g 100 mL^{-1} for the initial bioassay and then at 2.5, 5.0, and 8.0 g 100 mL^{-1} in the second bioassay. Two controls were also prepared: one with 70% alcohol solution and one with a fungicide (Carbendazim 500 $g \cdot L^{-1}$). The extracts were filtered using a Millipore 45-mm diameter membrane and after sterilized.

The extracts being tested for treatments and controls were applied in amounts of 100 µL across the petri dish containing potato dextrose agar (PDA). A mycelial disc of 8-mm diameter was placed on the agar and a solution of fungus spores was added (*Aspergillus flavus*) and incubated at 25 ± 2°C. Growth was observed and recorded daily for 7 days by measuring the colonies along two perpendicular axes, and the mean diameters of the two were recorded as the radial colony diameter, while discounting the mycelium disk size (FRANZENER et al., 2007).

The mycelial index growth speed (MIGS) was calculated using the formula: MIGS = [∑(D-Da)]/N. Where, D is the current average colony diameter, Da is mean diameter on the previous day, and N is the number of days after incubation (OLIVEIRA, 1991).

For the determination of sporulation, a spore suspension was prepared by adding 10 mL of distilled water in Petri dishes at the end of the seventh day, followed by rasping and filtering it through cheesecloth and counting the number of spores using a Neubauer chamber in an optical microscope (FRANZENER et al., 2007).

The germination of spores was performed in the presence of 100 µL of EEP being tested and 500 µL of spore suspension at a concentration of 1.0×10^5 spores mL^{-1}. These materials were spread on the surface of Petri dishes containing agar-water (AW). The plates were then incubated at 25 ± 2°C, and their evaluation was started 24 hours after the initiation of incubation by counting 100 spores per repeat, i.e., a total of 800 spores per treatment, using

an optical microscope. Germinated spores were considered those that developed a germ tube (REGENTE et al., 1997).

The results of physicochemical analyses are represented as averages and standard deviations of 3 replicates. Bioassays for mycelial growth and sporulation were performed on 5 replicates, while those for spore germination were performed on 8 replicates. The results for all bioassays were compared by the Scott–Knott test ($p<0.05$) using the Assistant software (SILVA; AZEVEDO, 2009).

The values for physical and chemical parameters of propolis are shown in Table 1. It was found that according to the current legislation (BRASIL, 2001), all propolis *A. mellifera* extracts tested here showed adequate levels of ash (≤ 5 g /100 g^{-1}). However, none of the EEP carried the dry extract content required by law (≥ 11 g /100 mL^{-1}).

Table 1. Chemical composition of propolis produced by *Scaptotrigona polysticta* (stingless bee), as well as the brown and red propolis from *Apis mellifera*

Propolis	Ash[1] (g 100 g^{-1})	Dry extract[2] (g 100 mL^{-1})	Total flavonoids[2] (g 100 g^{-1})	Total phenolic compounds[2] (g 100 g^{-1})	Antioxidant activity[2] (%)
Stingless	3.75 ± 0.33	2.55 ± 0.389	0.08 ± 0.07	0.54 ± 0.20	67.9
Red	1.99 ± 0.01	8.65 ± 0.849	2.77 ± 0,90	5.38 ± 1.17	88.7
Brown	0.8 ± 0.06	5.63 ± 0.247	0.46 ± 0.66	0.87 ± 0.36	92.9
LAW[3]	≤ 5	≥ 11	> 0.25	> 0.5	-

[1]Crude propolis; [2]Ethanol extracts from propolis; [3]Quality standard for propolis from *Apis mellifera* (BRASIL, 2001)

For the EEP from *A. mellifera*, both the red and brown extracts showed the contents of total flavonoids (>0.25 g /100 g^{-1}) and total phenolic compounds (> 0.5 g /100 g^{-1}) to be within the legislation standard (BRASIL, 2001). Other studies have shown contents to vary from 0.033 to 8.13 g 100 g^{-1} for total phenolic compounds and from 0.002 to 2.11 g 100 g^{-1} for total flavonoids (SILVA et al., 2006; SOUSA et al., 2007; CABRAL et al., 2009; CHAILLOU; NAZARENO, 2009; RIGHI et al., 2011; SILVA et al., 2011).

There are no quality standards stipulated by law for the composition of propolis from *S. polysticta*; in addition, there are no studies that have characterized the propolis from this species of stingless bee. However, there are studies on geopropolis from *Melipona fasciculata,* where the levels of flavonoid content (0.17–2.67%) was shown to be lower than those for phenolic compounds (7.36–37.04%) (DUTRA et al,. 2008; CUNHA et al., 2009); these findings are in concordance with our results.

The minimum established level for dry extract by the Ministry of Agriculture, Livestock, and Supply is 11% (BRASIL, 2001), but it is noticeable that none of the samples tested here reached this recommended percentage. Bastos et al. (2011) tested propolis from *A. mellifera* collected in the Andean region of Colombia and whose extract preparation was carried out with 30% of propolis in 70% ethanol; their study did not present the required minimum levels for dry extracts either.

Other studies have shown that 70% ethanol is an appropriate solvent for the production of EEP from *A. mellifera*, but it is recommended to increase the maceration time up to 30 days; manual shaking was employed in this study because this is the process most commonly used by beekeepers. Park et al. (1998) conducted tests to determine the optimal concentration of solvent for production of extracts and found that ethanol concentrations between 60 and 80% helped achieve the best results for the extraction of flavonoids and for effective biological activity. Cunha et al. (2004) found that, when using ethanol as solvent, extraction is stabilized after reaching a concentration of 70%, which is the minimum concentration recommended by the authors; they also observed an increase in dry extracts when maceration times were between 10 and 30 days.

It was found that there is no a relationship between the antioxidant activity (AA) and total flavonoid and phenolic contents. Furthermore, AA was higher in brown propolis (92.9%) as compared to in the red one (88.7%), where the latter showed the highest levels of these compounds (Table 1). Funari and Ferro (2006) reported that the antioxidant and other possible biological activities of propolis are not only linked to the content of total flavonoids and phenolic compounds but also to the entire set of existing compounds in the extracts (aromatic acids, terpenoids, aldehydes, alcohols, aliphatic acids, esters, amino acids, steroids, sugars, etc.).

For the standards used in this study, only phenolic acids were found in brown propolis, whereas for the red propolis only flavonoids were identified. As seen in Table 2, the predominant compound in brown propolis was gallic acid (5.88 mg g^{-1}), *ρ*-coumaric acid in *S. polysticta* (10.99 mg g^{-1}), and quercetin in red propolis (27.26 mg g^{-1}). The flavonoids queretin and apigenin have also been identified in *A. mellifera* red propolis in other studies (DAUGSCH et al., 2007; ALENCAR et al., 2007; HATANO et al., 2012).

Table 2. Average levels of flavonoids and phenolic acids (mg g^{-1}) in the EEP from *Scaptotrigona polysticta* (Stingless bee), and the brown and red extracts from *Apis mellifera*

	Etanolic extract from propolis (EEP)		
Componds	Stingless	Brown	Red
Gallic acid	1.87 ± 0.04	5.88 ± 0.22	ND*
Caffeic acid	2.92 ± 0.38	2.00 ± 0.16	ND
p-coumaric acid	10.99 ± 0.05	ND	ND
Ferulic acid	ND	3.35 ± 0.43	ND
Quercetin	ND	ND	27.26 ± 0.79
Apigenin	ND	ND	25.97 ± 0.27
Kaempferol	ND	ND	10.50 ± 1.49

*ND = not detected

Contents of Gallic acid, caffeic acid, *p*-coumaric acid, ferrulic acid, quercetin, apigenin and kaempferol found in *A. meliffera* propolis investigated here are in accordance with the levels reported in other studies (CHAILLOU; NAZARENO, 2009; GUO et al., 2011; Hatano et al., 2012; PAPOTTI et al., 2012). However, there are no research that have characterized the phenolic composition of própolis from *Scaptotrigona polysticta*.

There was no effect of the EEP on the mycelial growth (Figure 1) and the MIGS of Aspergillus flavus (Figure 2).

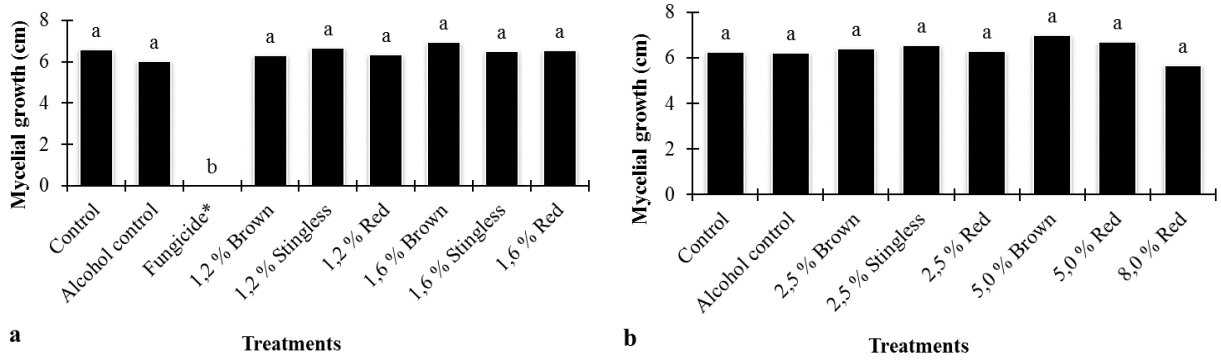

Figure 1. Mycelial growth (cm) of *Aspergillus flavus* under different EEP types (brown and red propolis from *Apis mellifera* and EEP from *Scaptotrigona polysticta, a* stingless bee) at varying concentrations and controls after a 7-day incubation. (a) Initial bioassay and (b) second bioassay. Means followed by the same letter on the same chart are not statistically different as per the Scott–Knott test ($p<0.05$). CV: 8.39%. *Carbendazim 500 $g \cdot L^{-1}$

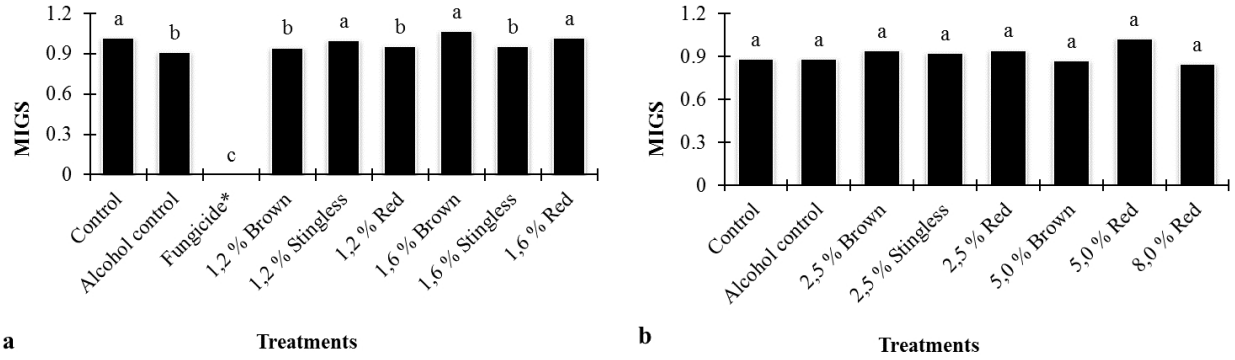

Figure 2. Mycelial growth index speed of *Aspergillus flavus* under different EEP types (brown and red propolis from *Apis mellifera* and EEP from *Scaptotrigona polysticta, a* stingless bee) at varying concentrations and controls after a 7-day incubation. (a) Initial bioassay and (b) Second bioassay. Means followed by the same letter on the same chart are not statistically different as per the Scott–Knott test (p<0.05). CV: 10.56%. *Carbendazim 500 g·L^{-1}

Vieira and Andrade (2009) evaluated the effect of EEP on the incidence and severity of powdery mildew (*Sphaerotheca fuliginea*) on cucumber leaves, when applied at concentrations of 0.8 and 1.6%, to observe that the extracts showed inhibitory effect to some extent on the Nikkei and Podium cultivars only. From these results, it was concluded that the concentration of 1.6% showed antifungal activity against powdery mildew.

The MIGS represent the speed of fungal growth in comparison to the control. Smaller values of MIGS represent higher percentages of growth inhibition. It was found that there were no significant differences in the MIGS tests (Figure 2b), which revealed that mycelial growth was independent of concentration and type of extract, i.e., the extracts were not effective in inhibiting the development of *A. flavus* isolated from Brazil nuts. In Figure 2a, it can be seen that there were differences between the control that had only distilled water and the tests, but they did not differ from the control with 70% alcohol.

In case of sporulation, no significant differences were observed between any of the treatments except for the treatment with fungicide, where the presence of any spores was not observed (Figure 3).

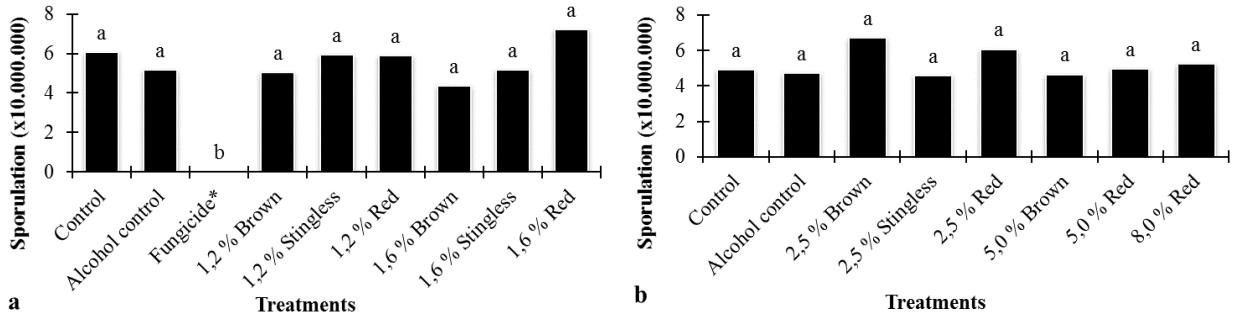

Figure 3. Sporulation of *Aspergillus flavus* under different EEP types (brown and red propolis from *Apis mellifera* and EEP from *Scaptotrigona polysticta, a* stingless bee) at varying concentrations and controls after a 7-day incubation. (a) Initial bioassay and (b) Second bioassay. Means followed by the same letter on the same graph are not statistically different as per the Scott–Knott test (p<0.05). CV: 30.27%. *Carbendazim 500 g·L^{-1}

Oliveira et al. (2012) used the propolis extracts at concentrations of 5, 10, 15, 20, and 30% on various microorganisms (*Staphylococcus aureus*, *Candida albicans*, *Escherichia coli, Salmonella typhimurium*) to find out that the extracts, regardless of their concentrations, did not inhibit the growth of any of the tested pathogens.

In studies on the inhibition of sporulation of *Elsinoe ampelina,* Marini et al. (2009) observed that alcoholic propolis extracts had an inhibitory effect on sporulation; however, the results were not statistically different among the treatments containing alcohol only, which revealed that the

group's results were not specific for the inhibitory potential of propolis.

The spore germination test showed significant differences between the investigated treatments. While the fungicide inhibited the germination of 100% of the spores, red EEP (*A. mellifera*) at a concentration of 1.6 g 100 mL^{-1} had the best effect (90.13%) as compared to the other EEP investigated here (Figure 4). However, the red EEP, at concentrations of 2.5 and 8.0 g 100 mL^{-1} showed significantly weaker effect than at 1.6 g 100 mL^{-1} with 79.13 and 82% percent reduction of sporulation, respectively.

The inhibition of spore germination is probably related to the presence of quercetin, apigenin, and kaempferol in the red EEP because these flavonoids have not been identified in other EEP types investigated in this study.

The concentration of 1.6 g mL^{-1} was the most effective in inhibiting germination, while higher concentrations had a weaker effect. This observation is attributable to the EEP chemical complexity, which allows it to have a synergy among its different components, i.e., while some can present an inhibitory effect on spore germination, others have a stimulatory effect.

Baptista and Siqueira (1994) tested the effect of synthetic flavonoids on the germination of mycorrhizal fungi spores (*Gigaspora gigantea*) and to report that quercetin was a strong inhibitor, while apigenin did not exert any effect on spore germination.

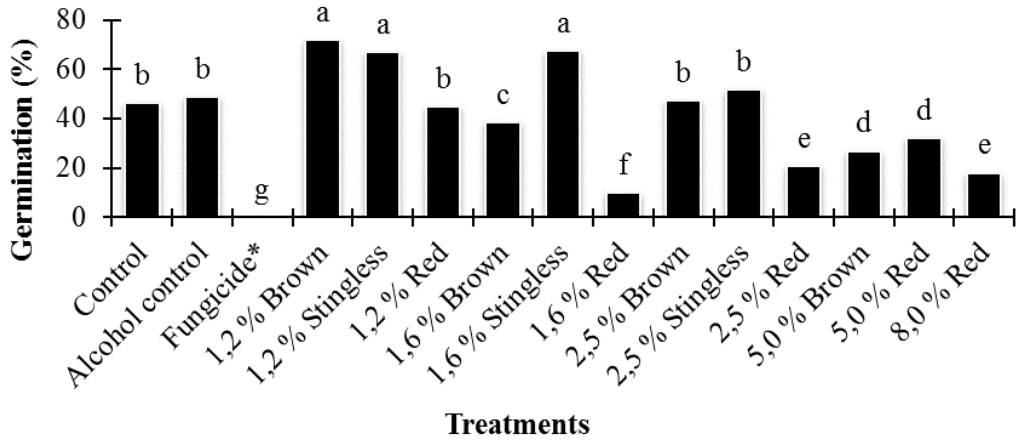

Figure 4. Spore germination by *Aspergillus flavus* under different EEP types at varying concentrations and controls after a 7-day incubation. Means followed by the same letter do not differ significantly as per the Scott–Knott test ($p<0.05$). CV: 16.56%. *Carbendazim 500 g·L^{-1}

Cabral et al. (2009) fractionated the red propolis extract using hexane and chloroform; they further observed that the activities of this extract were not due to a synergistic effect of the various components in the crude extract; instead, the authors verified that the chloroform fraction was more effective at bacterial inhibition, while the hexane fraction was more effective in scavenging the DPPH radical (antioxidant activity). It is, thus, advisable that further studies are conducted on this phenomenon so that additional discussions and reliable conclusions can be generated.

Future work may be performed to investigate the fractionation of EEP to isolate compounds with antifungal activities on *A. flavus*.

In conclusion, the physicochemical composition of propolis extracts is within the quality standard, with the exception of dry extract levels. The EEP tested here have no inhibitory effect on the mycelial growth and sporulation of *A. flavus* isolated from Brazil nuts. The best inhibitory effect on spore germination may be related to the quantities of flavonoids found in red EEP from *Apis mellifera* (quercetin, apigenin, and kaempferol), because these components were not detected in brown EEP from *A. mellifera* and the EEP from *S. polysticta*.

RESUMO: Outros estudos apontaram a ação antifúngica dos extratos etanólicos de própolis (EEP) sobre fungos do gênero *Phakopsora*, *Colletotrichum* e *Cercospora*. Neste estudo, avaliou-se a composição química e a atividade antioxidante de três EEP (própolis produzido pela abelha sem ferrão *Scaptotrigona polysticta* e dois tipos de própolis produzida pela *Apis melífera*: marrom e vermelha) e sua ação contra o *Aspergillus flavus*. Foram determinados os teores de

cinzas, extrato seco, fenólicos e flavonoides totais nos EEP. A composição fenólica foi determinada por cromatografia líquida de alta eficiência, empregando-se as seguintes substâncias de referência: ácido gálico, ácido *p*-cumárico, ácido ferrúlico, quercitina, kaempferol e apigenina. Para avaliar a atividade biológica dos EEP sobre *A. flavus* foram realizados os testes de crescimento micelial, esporulação e germinação de esporos. Todos os EEP apresentaram baixos teores de extrato seco (< 11%), porém quantidades de cinzas adequadas (< 5%). O EEP vermelha apresentou os maiores teores de fenólicos e flavonoides totais (5,38 e 2,77 g $100g^{-1}$), enquanto que a maior atividade antioxidante registrada foi do EEP marrom (92,9%). O EEP de *S. polysticta* apresentou maiores teores de ácido p-cumárico (10,99 mg g^{-1}), enquanto que os de *A. mellifera*, vermelha e marrom, destacaram-se com os maiores níveis de quercetina (27,26 mg g^{-1}) e de ácido gálico (5,88 mg g^{-1}), respectivamente. Nenhum extrato foi eficaz na inibição do crescimento micelial e esporulação de *A. flavus*, porém observou-se inibição na germinação de esporos pelo EEP vermelha. Sugere-se que o efeito inibitório na germinação de esporos pode estar relacionado com os maiores níveis de flavonoides encontrados na própolis vermelha, quando comparado com os outros extratos investigados.

PALAVRAS-CHAVE: *Apis mellifera*. Fenólicos. Fungitoxicidade. *Scaptotrigona polysticta*.

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