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Antifungal Evaluation of Brazil nut (*bertholletiaexcelsa*) Oil on the Growth of *a. parasiticus*

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Abstract

Aspergillus parasiticus is a specie incident in Brazil nut, and the ability to produce mycotoxins in food is a concern and a barrier to export. Samples of 5 different Brazil nut genotypes were collected at the Arapuã farm in the state of Amazonas. The fixed oils of each genotype were extracted in the Department of Chemistry of UFLA by a reflux system and then the fatty acids of the samples were characterized by gas chromatography.

The antimicrobial activity of Brazil nut oils (*Bertholletiaexcesa*) was studied on the growth of *Aspergillus parasiticus* using the Minimum Inhibitory Concentration (MIC) method. Oils 1, 3 and 5 presented similar profiles and Oils 2 and 4 presented profiles different from the others. The oil concentration influences on the ability of growth inhibit of *Aspergillus parasiticus*. The fatty acids obtained through the 5 different oils were efficient in the antifungal activity and theoilconcentrationinterferedinthegrowth.

Keywords: Brazil Nuts; Antifungal; CG; Fix oil

Introduction

Brazil is considered the second countryin the export of Brazil nut (*Bertholletiaexcelsa*), and its production is concentrated in the northern region, especially in the state of Amazonas [1]. The Brazil nut concentrates a high content of proteins, carbohydrates, lipids, vitamins and essential minerals [2,3].

Due to the growing demand for Brazil nut export, hygienic-sanitary aspects are increasingly demanded. The contamination process can occur from the collection, production, processing, storage, packaging, transportation, preparation, maintenance and consumption, both via toxic substances and microorganisms [4]. Mycotoxins are toxic secondary metabolites produced by some filamentous fungi and can prolifically affect global agriculture as the mycotoxins may be virtually ubiquitous at some low concentration in the human diet [5].

These substances are produced by species of the genus Aspergillus, and have highly toxigenic potential. There are a number of species within the Flavi section that have aflatoxigenic activity, but the major contaminants in nuts are *A. flavus, A. parasiticus, A.nomius* [6]. A. flavus has a higher capacity to produce aflatoxins B1 (AFB1) and B2 (AFB2), while *A. parasiticus* and *A. nomius* produce aflatoxins G1 (AFG1) and G2 (AFG2), as well as AFB1 and AFB2 [7].

Aflatoxin B1, and its metabolite precursor Sterigmatocystin, have been identified as carcinogenic by the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) [8], and considered a potent initiator of hepatocellular carcinomas [9]. There are synthetic chemicals, such as fungicides and preservatives, and natural compounds that have the function of reducing the losses caused by food contamination and deterioration by microrganisms, like filamentous fungi [10].The antifungal activity of some plant extracts and essential oils has been observed in several studies [11]. The possibility of using natural compounds as alternatives to synthetic fungicides to control growth and aflatoxin production has been increasingly explored by researchers [12]. By the diffusion of the search for new active substances for the control of microbial growth,the objective of this study was to characterize the profile of the fatty acids present in 5 different genotypes of Brazil nuts, and to evaluate the antifungal activity of the extracted oils on the growth of *Aspergillus parasiticus* in diferents concentrations.

Material and Methods

Sample collection and preparation

The samples were collected at theArapuã farmin the state of Amazonas. 1 kg of 5 different Brazil nut genotypes were collected in May 2015, being named A1606, A2609, A3Manoel Pedro I , A4 Manoel Pedro II and A5 Santa Fé. The Brazil nut samples were processed in the BromatologyLaboratory of the Department of Food Science (DCA), Federal University of Lavras (UFLA). The processing consisted of previous weighing of the samples and oven drying at 60°C for 7 days until reaching constant weight.

Extraction of fixed oil

The extraction of the oils was carried out in the laboratory of Organic Chemistry of the Department of Chemistry (DQ) at UFLA. The method used was the reflux system [13], which was coupled to a 250 mL volumetric flask. Into this flask were placed, separately, 50g of macerated sample, along with 100 mL of hexane. The extraction time was 6 hours, from the moment of boiling. Flask contents were subjected to vacuum filtration and then rotoevaporated under 500 mmHg and 37°C. The filtrate was then stored in a sterile container, protected from light and wrapped in parafilm with small holes for total evaporation of the solvent. After complete evaporation of the solvent the oils were stored at -80°C.

Esterification of fatty acids and samples preparation

The esterification of oils to determine the fatty acid composition was conducted at the Department of Veterinary Medicine (DMV) at UFLA. Esterification was performed by saponification with sodium hydroxide solution in 0.5 M methanol, followed by methylation with ammonium chloride, methanol and sulfuric acid, according to methodology of [14]. After methylation the samples were submitted to gas chromatography.

Identification and quantification of fixed oil components

Analysis of the fatty acids was performed by gas chromatography on a Shimadzu GC 2010 chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA), equipped with a flame ionization detector, separation injection at a rate of 1:50 and Supelco SPTM-2560 capillary column, 100m x 0.25mm x 0.20m (Supelco Inc., Bellefonte, PA, USA). Chromatographic conditions were: initial column temperature 140°C/5 minutes; increasing 4°C/ minute to 240°C and held for 30 minutes, for a total of 60 minutes. The injector and detector temperature was 260°C and helium gas was used as transport. Fatty acids were identified by comparison with the retention times presented by the Supelco TM37 FAME standard mixture (Supelco Inc., Bellefonte, PA, USA) and expressed as a percentage (%) of the total fatty acids identified.

Antifungal activity

The evaluation of the antifungal activity of the fixed oils from Brazil nut (*Bertholletiaexcelsa*), was carried out in the Mycotoxin and Mycology Laboratory, DCA, UFLA. The fungal specie used in this experiment was isolated in a higher frequency of Brazil nuts in a previous study and this specie is deposited in the Culture Collection of Microorganisms of the Departamentof Food Science (*Aspergillus parasiticus* CCDCA-10445).

The sensitivity of the fungus to the fixed oils was determined using the disc diffusion test,after activation of the isolate in Malt Extract Agar culture medium (MEA, Sigma-Aldrich, USA). To evaluate the inhibitory effect on filamentous fungi, the disc diffusion test, accepted by the US Food and Drug Administration (FDA) and established by the National Clinical Laboratory Standards Committee [15], was used. A suspension of the spores in sterile distilled water containing 0.5% Tween 80 was prepared.

A Neubauer counting chamber was used to determine the final spore concentration (10^6 mL^{-1} [16,17]. This inoculum was transferred to a dish containing Malt Extract Yeast Agar (MEA, Sigma-Aldrich, USA), using the surface dispersion technique. Filter paper discs 6 mm in diameter were placed at equidistant points in the culture medium and were soaked with 10 µl of essential oils or standards diluted in DMSO at concentrations of 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 3.91 mL • mL-1. As a positive control, 10 µL of 2% hypochlorite (1 g • L-1), was used, whereas the same amount of DMSO was used as a negative control. The plates were incubated in BOD at 25°C for 72 hours and then the minimum inhibitory concentration (MIC) was defined as the lowest concentration of fixed oil at which the presence of an inhibition halo can be identified [18]. The analyzes were performed in three replicates.

Statistical analysis

Analysis of the principal components to group the genotypes with respect to the detected compounds and antimicrobial activity of the oils in various concentrations was tested by a negative binomial and chi-squared followed by the identification of possible differences among the microbial activity of the oils by means of the Tukey test ($p < 0.05$).

Results and Discussion

The results of the profile of the fatty acids found in each genotype are demonstrate in Table 1. A similarity can be observed in the composition of Nut oils 1, 3 and 5, which present similar amounts of eicosanoic acid, linolenic acid and henoecosainoic acid. However, linolelaidic acid was found in Brazil nut oil 1and 4, and linoleic acid in nut oils 3 and 5. Brazil nut oil 2 presented the highest amount of different fatty acids and was the only one where tridecanoic acid was found. In the characterization of Oil 4, linoleic acid was determined with a higher percentage than the other oils, and it was the only one that presented traces of palmitic acid in its composition. In a study of the in vitro activity of Brazil nut oil on aflatoxigenic strains of Aspergillus parasiticus conducted by [10], linolenic, linoleic, oleic, palmitic and stearic acids were determined in the composition of the Brazil nut oil [19,10,20]. However, there are few studies that evaluate the antifungal activity of the oils found in nuts in the control of aflatoxigenic fungi.

The Figure 1 presents the results found in the Principal Component Analysis of the fatty acids profile characterized from the Brazil nut oil samples. The biplot presented explains 98.73% of the effects. Principal Component 2 (PC2) explaining 4.93% and Principal Component 1 (PC1) 93.80%. Therefore, it is possible to verify the formation of 3 different groups. The first formed by the Brazil nut oil 2, which concentrates the highest proportion of tridecanoic acid and this dispersed it to the others. Brazil nut oils 3 and 5 form the second group. They present a similar fatty acid profile, with emphasis on the concentration of the henoecoisanoic and linoleic oils. On the other group, the Brazil nut oils 1 and 4, showing a similar profile, emphasizing the linolenic and linolelaidic oils.

In Figure 2 presents the groupings via dendogram. It corroborates effects clearly observed in Figure 1, where the Brazil nut oils 1 and 4 and 3 and 5 was grouped by similarity, and finally Brazil nut oil 2 was separated. The difference in fatty acid profile found in this study may be related to the different genotypes of the evaluated nuts and these genotypes were classified in relation to the number of fruits and mass of the fruit seeds. The age of the plants can explain the differences between the production and the composition of the fruits of native populations [21,22].

The in vitro activity of Brazilnut oil, as a function of the concentrations evaluated, are shown in Figure 3. There was no interaction between concentration and genotype.

Thus, it can be said that all Brazilnut oils had aninhibitory effect on the growth of *Aspergillusparasiticus.* A significant difference was observed among the concentrations studied, with the 1:1 concentration presenting the smallest inhibition halo and the 0.02 concentration obtaining the largest inhibition halo diameters. However, the 0.02 concentration did not differ from the 0.01; 0.03; 0.25 and 0.5 concentrations regarding microbial activity inhibition. Considering this it is possible to observe that *Aspergillusparasiticus* is sensitive to Brazilnut oil, especially in low concentrations.

The result so btained [10] showed that the effect of Brazilnut oil on the growth of the Aspergillusparasiticus was time and concentration dependent.

The fattyacids, in general, have possible antimicrobialactivity [23]. Palmitic, linoleic, oleic, linolenic and stearicacids are known for antifungal potential [24]. The ability of fatty acids to act on bacterial activity is associated with the ability to cause celllysis [10].

Accordingto[25], the lipoprotein structure of the fungal membrane is an effective barrier to many types of molecules, which cross by active diffusion or transport. The lipid component of the fungusis called ergosterol, apolar sterol. Chemically classified as highly lipophilicandany action of oils in this molecule can trigger an imbalance in the fluidity of the fungal plasma membrane, leading to changes in intracellular homeostasis [26]. Its absencemay cause alterations in plasma permeability and growth inhibition and this process can be favored by the nature of the constintuintes of the oil used. Thus, the presence of a polar constituents favor the interaction of the oil with the fungalmembrane [27].

The hydrophobicity of the oil sand their constituents, has the capacity to interact with the lipidlayer of the cellmembranes, which can generate alterations in its structures, and may cause extravasation of cellular content [28], preventing fungal growth.

In a study using ginger extract, the increase in inhibition halo occurred in proportion to the product concentration. As the product concentration increased, there was an increase in the inhibition halo [29]. Many essential oils, fixedoils, plant extract sand their compounds present biological activity on them ostdiversemicroorganisms, but little is known about their action mechanisms [30,31].

FattyAcids	Oils				
	1	2	3	4	5
Myristic	0.050	-	0.032	0.055	-
Stearic	0.058	0.001	-	0.183	-
Oleic	0.289	0.003	0.340	0.434	0.192
Linolelaidic	14.841	-	-	13.103	-
Arachidic	0.292	0.003	-	1.026	0.603
Y- linolenic	0.085	0.005	-	-	-
Eicosanoic	11.329	0.096	9.956	10.344	11.907
Linolenic	34.667	0.308	35.675	48.066	33.508
Henecosanoic	38.111	0.388	39.692	26.474	38.240
cis, 11 eicosanoisc	0.182	-	-	-	-
cis 11,14,17 - eicosa-trienoic	0.096	0.001	-	0.303	0.113
Tridecanoic	-	99.058	-	-	-
Linoleic	-	0.136	14.305	-	15.350
cis 11, 14, eicosanoic	-	0.001	-	-	-
Palmitic	-	-	-	0.012	-
Behenic	-	-	-	-	0.052
Eurucic	-	-	-	-	0.034

Table 1: Characterization fatty acids in Brazil nut oil

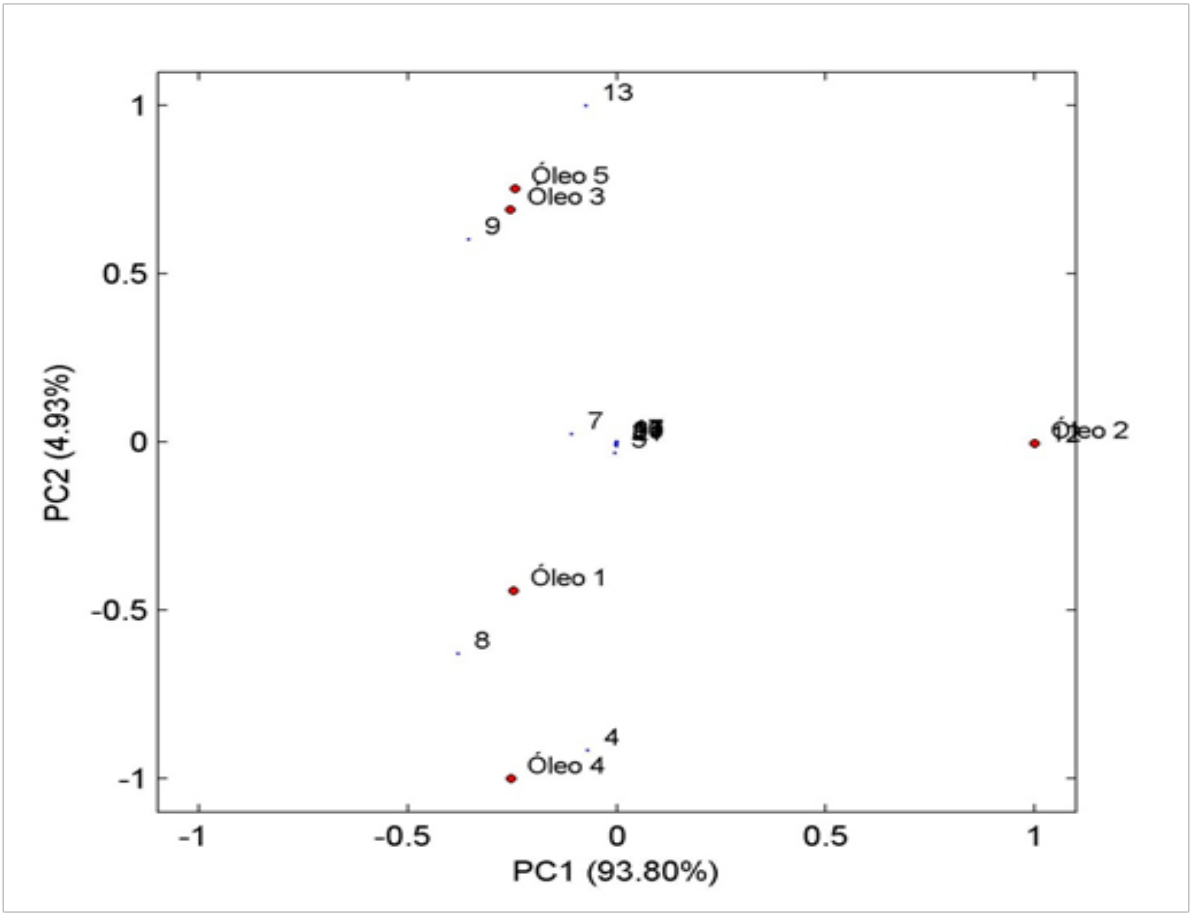

Figure 1. Principal component analysis of the lipid profile of Brazil nut oils.

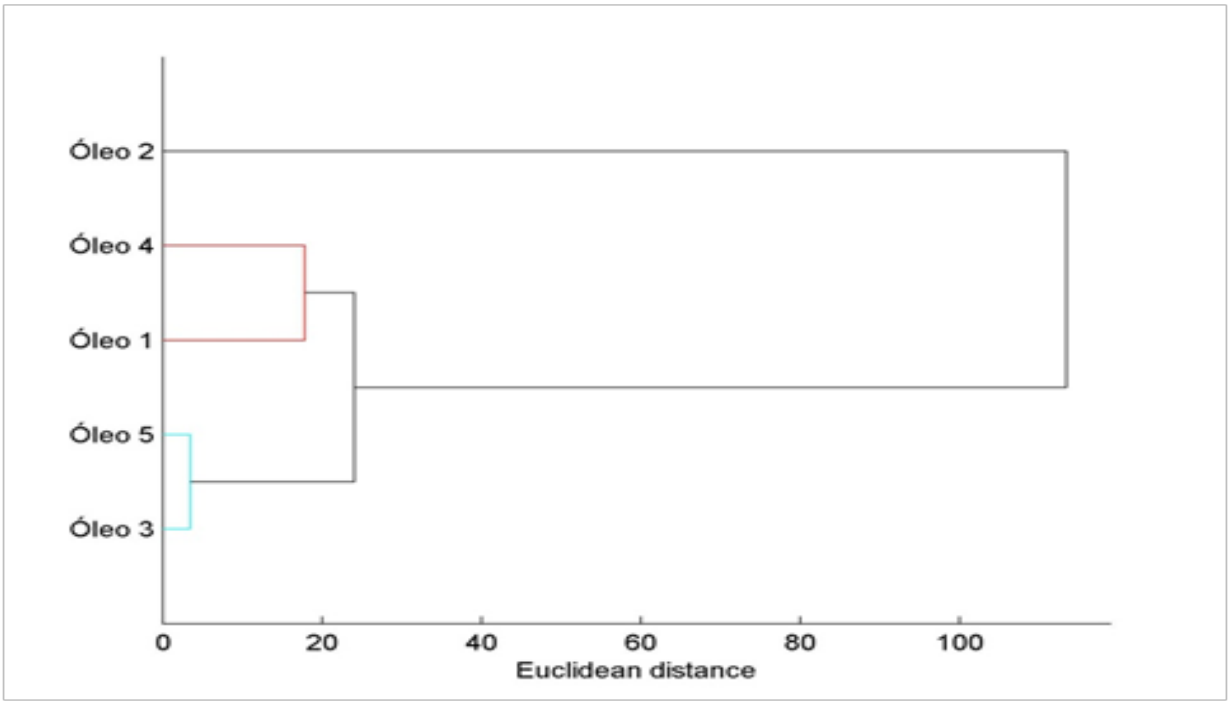

Figure 2: Grouping of Brazil nut oils according to their lipid profile

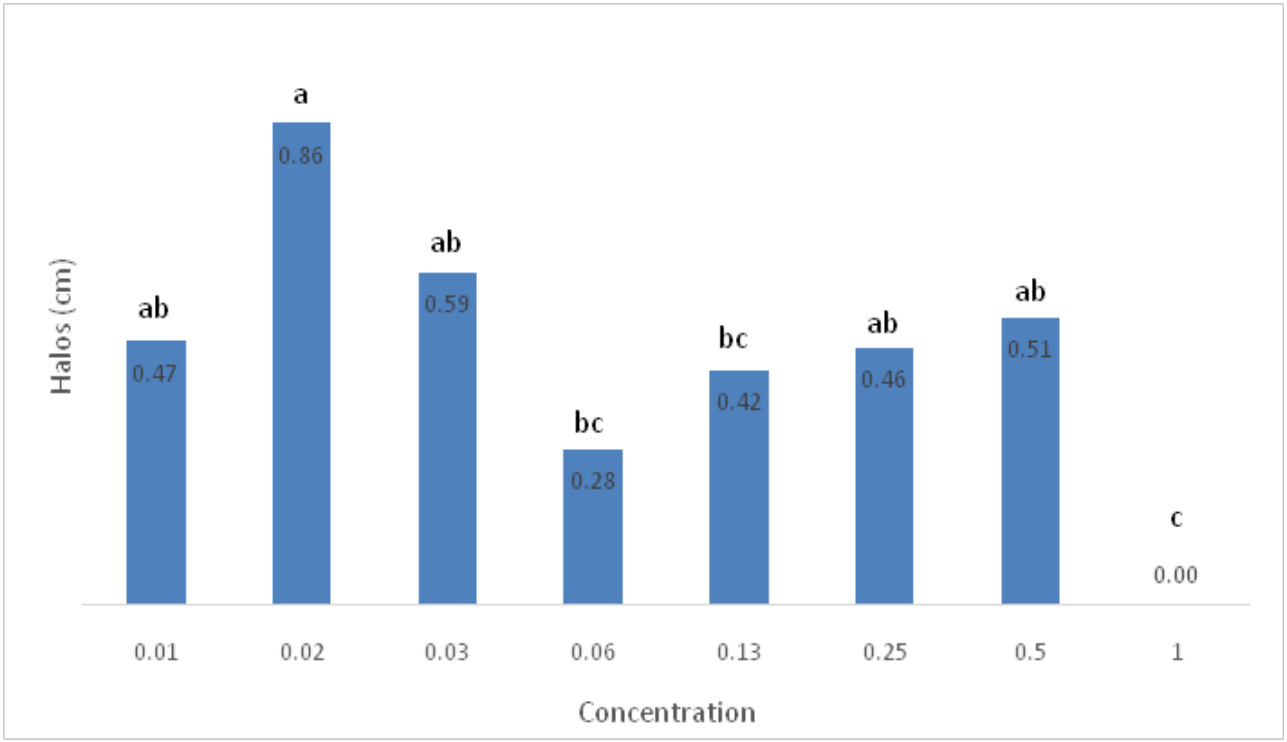

Figure 3. Diameter of inhibition halos under Brazil nut oil concentrations
*Averages followed by the same letter do not differ from one another by the Tukey test ($p<0.05$).

Conclusion

There is difference among the genotypes with respect to the fatty acid profile of each sample evaluated.

All oil sex tracted from different genotypes have the ability to inhibit the growth of A. parasiticus.

Further studies are needed with other fungi species, fungiostatic and fungicidal effects to determine thes pecificpotentialofbrazilnutoil.

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