

Antimicrobial bentonite by the addition of geranyl acetate for aflatoxin B1 adsorption

Bentonita antimicrobiana pela adição de acetato de geranila para adsorção de aflatoxina B1

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Received: 01/12/2022 | Reviewed: 01/16/2022 | Accept: 01/18/2022 | Published: 01/20/2022

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Abstract

A nanocomposite composed of organophilic bentonite (OB) with geranyl acetate (GA) was prepared on a shaker for 1 hours, 180 rpm and room temperature, being subsequently dried in an oven at $90 \pm 2 \text{ }^\circ\text{C}$ during 48 hours. This material was then characterized for its antibacterial against *Staphylococcus aureus* and *Escherichia coli* and antifungal activity against *Aspergillus flavus* and *Aspergillus niger*, respectively. Moreover, the adsorption capacity of OB and OB/GA was evaluated using Aflatoxin B1 in the concentration of $107 \mu\text{g}\cdot\text{L}^{-1}$. After 17 h, the composite removed a significant amount of mycotoxin, being below $20 \mu\text{g}\cdot\text{L}^{-1}$. The results showed that this composite has a good adsorption capacity, can be effective in the removal of mycotoxin in aqueous media and excellent antibacterial and antifungal activity.

Keywords: Bentonite; Geranyl acetate; Antimicrobial activity; Aflatoxin B1 adsorption.

Resumo

Um nanocompósito a base de bentonita organofílica (BO) e acetato de geranila (AG) foi preparado em shaker por 1 hora, 180 rpm e temperatura ambiente, sendo posteriormente seco em estufa a $90 \pm 2 \text{ }^\circ\text{C}$ por 48 horas. Este material foi então caracterizado por sua atividade antibacteriana contra *Staphylococcus aureus* e *Escherichia coli* e atividade antifúngica contra *Aspergillus flavus* e *Aspergillus niger*, respectivamente. Além disso, a capacidade de adsorção de BO e BO/AG foi avaliada utilizando Aflatoxina B1 na concentração de $107 \mu\text{g}\cdot\text{L}^{-1}$. Após 17 h, o compósito removeu uma quantidade significativa de micotoxina, ficando abaixo de $20 \mu\text{g}\cdot\text{L}^{-1}$. Os resultados mostraram que este compósito

possui boa capacidade de adsorção, pode ser eficaz na remoção de micotoxinas em meio aquoso e excelente atividade antibacteriana e antifúngica.

Palavras-chave: Bentonita; Acetato de geranila; Atividade antimicrobiana; Adsorção de Aflatoxina B1.

Resumen

Se preparó un nanocompuesto a base de bentonita organófila (BO) y acetato de geranilo (AG) en agitador durante 1 hora, 180 rpm y temperatura ambiente, siendo posteriormente secado en estufa a $90\text{ }^{\circ}\text{C}\pm 2\text{ }^{\circ}\text{C}$ durante 48 horas. Luego, este material se caracterizó por su actividad antibacteriana contra *Staphylococcus aureus* y *Escherichia coli* y antifúngica contra *Aspergillus flavus* y *Aspergillus niger*, respectivamente. Además, se evaluó la capacidad de adsorción de BO y BO/AG utilizando Aflatoxina B1 en la concentración de $107\text{ }\mu\text{g}\cdot\text{L}^{-1}$. Después de 17 h, el compuesto eliminó una cantidad significativa de micotoxinas, estando por debajo de $20\text{ }\mu\text{g}\cdot\text{L}^{-1}$. Los resultados mostraron que este compuesto tiene una buena capacidad de adsorción, puede ser eficaz en la eliminación de micotoxinas en medios acuosos y una excelente actividad antibacteriana y antifúngica.

Palabras clave: Bentonita; Acetato de geranilo; Actividad antimicrobiana; Adsorción de Aflatoxina B1.

1. Introduction

Animal nutrition usually includes a mix of foods that are listed in order to meet the nutritional needs of animals, also to provide what they need to maintain their health, well-being and production, and all this linked to the lowest possible cost (Pereira et al., 2019; Liu, et al., 2021). About 80% of the raw materials used to manufacture feed revolve around the use of corn, soybeans and their derivatives. However, due to climate change and other prevailing factors, many of these crops are subject to contamination by mycotoxins, among which stand out aflatoxins, produced by fungi of the genus *Aspergillus* that present an imminent risk to human and animal health and are related to several diseases and pathologies, in addition to causing great economic damage to the food industry (Zain, 2011; Nones et al., 2014; Raiola, et al., 2015; Oplatomska-Stachowiak, et al., 2016; Li, et al., 2018).

Several studies have shown the ability of adsorption of mycotoxins promoted by bentonites, recognized as a promising and effective food additive due to its cost-effectiveness and absence of significant side effects (Magnoli, et al., 2008; Carraro, et al., 2014; Gan, et al., 2019; Liu, et al., 2021). In order to maintain or enhance the adsorptive effect and still add other properties to bentonite, the insertion of organic compounds can be explored, which is still little explored, opening the way for the study of geranyl acetate, an important ester widely used in industries food, pharmaceutical and cosmetics (Gonçalves, et al., 2012; Gupta, et al., 2013; Zeferino, et al., 2021; Liu, et al., 2021).

The objective of this work was to obtain a composite with antimicrobial and adsorbent activity, using organophilized bentonite and geranyl acetate. By associating the adsorption properties of bentonite with the antifungal and antibacterial activity of geranyl acetate it is possible to obtain a compound capable of eliminating or controlling the growth of bacteria and fungi and, simultaneously, adsorb mycotoxins, which is not yet available on the market according to a bibliographic survey.

2. Methodology

2.1 Materials

The bentonite used in this work was from a Boane deposit in Mozambique, with cation exchange capacity (CEC) of $67\text{ mmol}\cdot 100\text{ g}^{-1}$ determined by Silva et al. (2010) and Massiga et al. (2010) (Macuvele, et al., 2017). The geranyl acetate was synthesized through heterogeneous catalysis using the ion exchange resin Lewatit®GF 101 from the geraniol esterification reaction with acetic anhydride, as described by Zeferino, et al. (2021). Intercalation with octadecylammonium cations was carried out using octadecylamine (ODA), $\text{CH}_3-(\text{CH}_2)_{17}-\text{NH}_2$ (Merck, 90%), protonated by treating it in situ with hydrochloric acid (Êxodo Científica). The culture media used in the microbiological tests were Plate Count Agar (PCA, Merck) and Sabourand with chloramphenicol (Kasvi).

2.2 Obtaining the composite

The reaction mixtures were stirred vigorously at constant temperature for 1 hour. Synthesis conditions were performed, considering the temperature of 60 ± 5 °C and concentration of surfactant the $85\text{ mmol}\cdot 100\text{ g}^{-1}$. After intercalation, all ODA-bentonite samples were washed, and separated by filtration, all samples were dried in an oven at 80 °C for 24 h. The organophilization step was based on the methodology suggested by according to Macuvele, et al. (2017). After organophilization, a composite was prepared in the following proportions: 2.5 g organophilic bentonite: 40 mL acetone: 0.75 g geranyl acetate (Bent/ODA/0.75 GA). The mixture was kept under agitation for 24 h using a shaker at 180 rpm, at room temperature. After this mixing period, the samples were dried in an oven at 50 ± 2 °C for 24 h.

2.3 Antimicrobial Testing

2.3.1 Antimicrobial analysis for bacteria

The antimicrobial activity was evaluated for gram-positive bacteria *Staphylococcus aureus* (ATCC 6538) and gram-negative bacteria *Escherichia coli* (ATCC 35218), according to methodologies previously described by Clinical and Laboratory Standards Institute (CLSI, 2012). For inoculum preparation, some bacterial colonies were selected and transferred to a sterile 0.9% saline. The solution turbidity was adjusted using a Spectrophotometer with a wavelength reading of 619 nm, yielding the equivalent concentration of about $10^4\text{ UFC}\cdot\text{mL}^{-1}$.

In this technique, we seeded the microorganism of interest in Petri dishes with a culture medium Plate Count Agar (PCA) using a swab. Three equidistant holes were made in each plate, with 8.0 mm diameter approximately. In each hole was deposited a composite and incubated at 36 ± 1 °C for 24 h. This antimicrobial analysis was performed in triplicate and the diameters of the inhibition halos were measured and associated with the antimicrobial actions according Eq. 1, in methodology proposed by Fiori, et al. (2009), the inhibition halos diameter measurements were subjected to the Tukey test in order to assess the significant differences existence at the 5% significance level ($p < 0.05$).

$$D_{\text{bac}} = D_{\text{ex}} - D_{\text{in}} \quad (1)$$

where D_{bac} is the sum of the bactericide diameters and represents the bactericide action of the materials, D_{ex} is the inhibition halo diameter of the microorganism and the D_{in} is the diameter occupied by the composite.

2.3.2 Antimicrobial analysis for fungi

The antimicrobial activity was evaluated for fungi *Aspergillus niger* (ATCC 6275) and *Aspergillus flavus* (ATCC 9643), according methodologies described previously by American Society for Testing and Materials (ASTM) G21-15, with some modifications. For the tests, the fungus concentration was adjusted in sterile saline solution (0.9%) to a concentration of $10^5\text{ spores}\cdot\text{mL}^{-1}$, being counted by the Neubauer chamber method under an optical microscope.

An amount of $0.200\pm 0.05\text{ g}$ of organophilized bentonite and Bent/ODA/0.75 GA were weighed into a sterile test tube and 80 μL of the fungal suspension was inoculated. The test tubes were incubated in a bacteriological incubator at 28 ± 2 °C for 7 days, this assay being performed in duplicate. After 7 days of incubation, 10 mL of 0.9% saline solution was added to each test tube, homogenized by vortexing for approximately 1min and a 1mL aliquot was removed and seeded in depth along with approximately 20 mL of Sabourand agar with chloramphenicol. The plates were homogenized and incubated at 28 ± 2 °C in a bacteriological incubator for 7 days, after which a visual analysis of the plates was performed, indicating whether there was growth, reduction or non-growth of fungi, as shown in ASTM G21-15 standard.

2.4 Adsorption Experiments

Batch adsorption experiments were performed according methodology proposed by Vila-Donat, et al. (2019), with some modifications. Using a homogenized suspension of 5.00 ± 0.01 mg of organo-bentonites and Bent/ODA/0.75 g GA and 25 mL of an aflatoxin B1 solution at a concentration of approximately $100 \mu\text{g}\cdot\text{L}^{-1}$. The adsorption experiments were conducted in a thermostat shaker at 180 rpm and 37°C for 17 h. Post-adsorption reaction mixtures were centrifuged in centrifuge with a rotation of 3600 rpm for 20 min and filtered through 0.22 mm syringe. Filtered samples were then analyzed by Liquid Chromatography-Mass Spectrometry (LCMS) regulations.

For the construction of the calibration curve, standard solutions of AFB1 were prepared in ultrapure water at concentrations of 0.02, 0.10, 0.20, 1.00 and $2.00 \text{ mg}\cdot\text{L}^{-1}$, and the calibration curve for AFB1 was determined based on the concentrations versus the peak area. Analyzes were performed using an Agilent chromatograph coupled to a mass detector. A 3.0×100 mm 2.7 Poroshell Sb C18 column (Agilent, PN 685975-302) was used. The mobile phase used was composed of methanol:ultrapure water with 0.1% formic acid (50:50), which was pumped without a ramp in isocratic mode, with a flow of $0.850 \text{ mL}\cdot\text{min}^{-1}$, with an oven temperature 40°C .

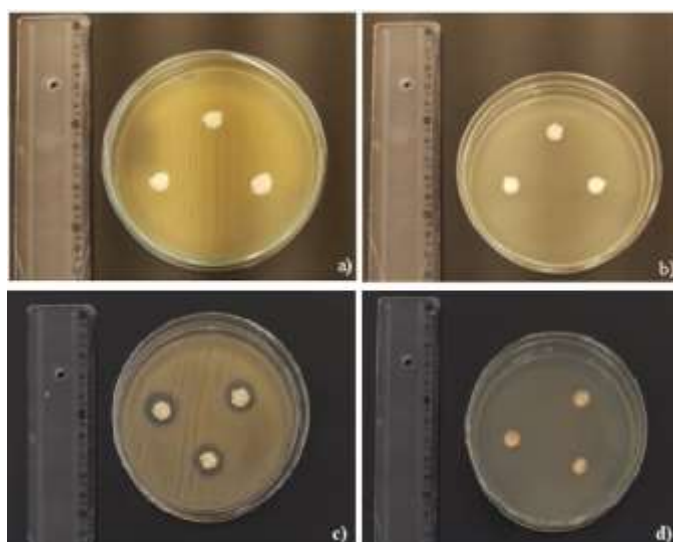
3. Results and Discussion

3.1 Antimicrobial tests

3.1.1 Antibacterial activity

To evaluate the organo-bentonites and Bent/ODA/0.75 g GA, diffusion tests were carried out in solid medium. Figure 1 show tests performed images with the bacteria *Staphylococcus aureus* and *Escherichia coli*, respectively. The inhibition halos diameter values are shown in Table 1.

Figure 1. Diffusion in solid medium for organo-bentonites against (a) *Staphylococcus aureus* and (b) *Escherichia coli* bacteria and Bent/ODA/0.75 g GA against (c) *Staphylococcus aureus* and (c) *Escherichia coli* bacteria with inoculum $10^4 \text{ CFU}\cdot\text{mL}^{-1}$



Source: Authors.

Table 1. Inhibition zone mean values generated by the organo-bentonites and Bent/ODA/0.75 g GA from the diffusion test in solid medium against the bacteria *Staphylococcus aureus* and *Escherichia coli*.

Samples	Inhibition zone average diameter (mm)	
	<i>S. aureus</i>	<i>E. coli</i>
Bent/ODA	0,0±0,0 ^{Aa}	0,0±0,0 ^{Aa}
Bent/ODA/0.75 g de AG	8,0±0,0 ^{Bb}	0,0±0,0 ^{Aa}

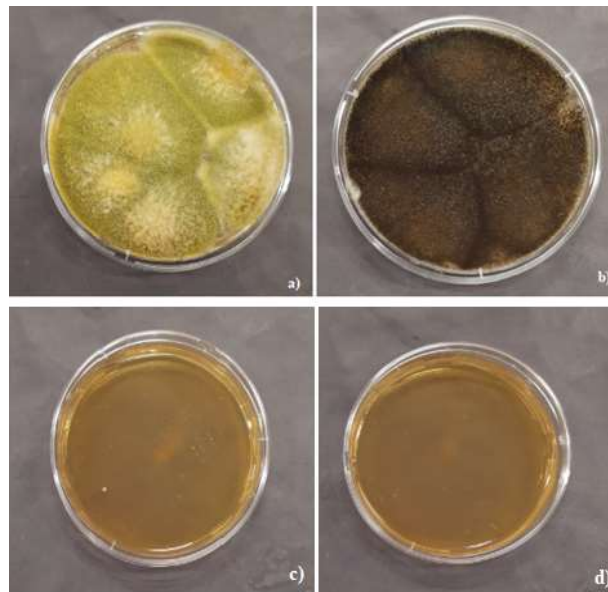
* Equal lowercase letters represent that there are no significant differences between rows ($p < 0.05$) and equal uppercase letters represent that there are no significant differences between columns ($p < 0.05$). Source: Authors.

It is possible to observe through the Figures above that for the organophilized bentonite there was no formation of an inhibition halo for both tested bacteria, which once again reinforces that the bentonite does not present antimicrobial activity by itself and that the organophilization process does not changed this feature. After the insertion of geranyl acetate to the organophilized bentonite, it was verified the formation of an inhibition halo, only for the gram-positive bacteria *Staphylococcus aureus*, this difference is due to the fact that the Gram-positive bacterial cells have only one outer layer as a plasma membrane, which facilitates the penetration of antimicrobial compounds and the interaction with the bacterial cytoplasm. When the structure of Gram-negative bacteria is evaluated, they have an additional membrane, forming a more resistant phospholipid bilayer structure that increases the cytoplasmic protection of antimicrobial agents (Muñoz-Bonilla & Fernández-García, 2012).

3.1.2 Antifungal activity

After organophilization and insertion of geranyl acetate, the composite was tested for its antifungal activity. Figure 2 shows the antifungal activity of organophilized bentonite and Bent/ODA/0.75 g of GA against fungi producing Aflatoxin B1, *Aspergillus flavus* and *Aspergillus niger* at a concentration of 10^5 spores·mL⁻¹.

Figure 2. Antifungal activity of organophilized Bentonite against (a) *Aspergillus flavus* and (b) *Aspergillus niger* and Bent/ODA/0.75 g of GA against (c) *Aspergillus flavus* and (d) *Aspergillus niger*



Source: Authors.

Figure 2 shows the results obtained for the antifungal analysis for organophilized bentonite and Bent/ODA/0.75 g of AG, where once again it was found that organophilized bentonite does not have the ability to inhibit the growth or kill microorganisms in test, fungal growth when evaluated this material occurred abundantly, receiving the maximum rating on the growth scale, completely filling the surface of the plate. After the insertion of the antimicrobial agent geranyl acetate, there was a marked decrease in cell density. When the composite Bent/ODA/0.75 g of AG was evaluated against *Aspergillus flavus* it was noticed the presence of growth traces and for *Aspergillus niger* no growth was observed. The classification of each of the tested composites can be seen in Table 2.

Table 2. Classification of fungal growth according to ASTM G21-15.

Samples	Rating	
	<i>A. flavus</i>	<i>A. niger</i>
Organo-bentonites	4	4
Bent/ODA/0.75 g de GA	1	0

Source: Authors.

3.2 Adsorption Experiments

Through the analyzes carried out in LCMS it was verified that the initial concentration of Aflatoxin B1 was 107 $\mu\text{g}\cdot\text{L}^{-1}$ and that after 17 hours of contact with organo-bentonites and Bent/ODA/0.75 g of GA this concentration was below the limit of quantification of the equipment, which is 20 $\mu\text{g}\cdot\text{L}^{-1}$, thus ensuring that the final concentration of Aflatoxin B1 is below 50 $\mu\text{g}\cdot\text{Kg}^{-1}$, which is what the Brazilian feed legislation provides (Brasil, 1988).

4. Conclusion

The results obtained indicate that composite based bentonite and geranyl acetate is antibacterial, antifungal and Aflatoxin B1 adsorbent, which can be used concomitantly in the elimination of fungi that produce Aflatoxins, as well as adsorbing them if they are present. This result is very interesting, as it allows the use of this material for applications in animal feed. Future works suggest the study of in vivo toxicity, release of geranyl acetate present in bentonite and incorporation of the material developed in the feed.

Acknowledgments

This work was supported by the Research and Innovation Support Foundation of the State of Santa Catarina (FAPESC, Brazil). We also thank Unochapecó to provide its laboratory structure.

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