Fine Chemical Engineering
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Research Article

Sustainable Starch-*Calotropis procera* Extract Bio-Packing for Protection Against Fungus Attack and Fruit Fast Maturation

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Received: 27 April 2023; **Revised:** 10 August 2023; **Accepted:** 23 August 2023

Abstract: The proliferation of diseases in plantations, control of fruit ripening, and damages in fruits during transportation are significant agricultural concerns. Thus, this research is based on the production of a sustainable packaging film based on starch conjugated to different concentrations of natural extract from *Calotropis procera* seeds for agricultural applications. The films were characterized by Fourier Transform Infrared (FTIR), Nuclear Magnetic Resonance (NMR) and Surface Contact Angle. In addition, antifungal activity against *Colletotrichum musae* were evaluated *in vitro* and *in vivo*, as the control of fruit fast maturing were figured using banana as a model of fruit. The cytotoxicity were also analyzed. The results showed that some compounds may have an important role in this protections, as proteins, calactin and calotoxin. Moreover, it demonstrated cytocompatibility using *in vitro* bioassays (> 80%). The analysis of fruit fast maturing showed that the film with 50 $\mu L \cdot mL^{-1}$ of natural extract presented the best results, controlling the fruit ripening and protecting against diseases. Thus, for the first time, a packaging film based on starch and natural extract from *Calotropis procera* seed demonstrated a versatile action towards agriculture applications.

***Keywords*:** eco-friendly packaging, anti fungicide, starch derivative, sustainable agriculture

1. Introduction

The control of fruit ripening during the transportation is crucial, especially when the fruit is destined for export in ships and taking a few months to reach the destination.[1] The European Commission Regulation (EC, 1221/2008) establishes that in fresh fruits injuries and degenerative processes can not be observed over 10% of fruit.[2] In this sense, diseases caused by fungi are the leading cause of fruit degeneration due to the excessive use of fungicides leading to increased resistance by fungi.[3] Thus, promoting materials which provide good protection against fungi, control of fast maturing, and eco-friendly is vital. For this reason, the development of new approaches to expedite the implementation of new material to sustainable agricultural processes is essential.

Banana is one of the main fruits linked to international agribusiness owing to its worldwide consumption. Its production generates approximately US$ 5 billion/year, and Brazil is the second-largest producer. The cultivation

DOI: https://doi.org/10.37256/fce.4220232930

is especially lucrative for the Brazilian agriculture, diffused throughout the national territory, demosntrating great socioeconomic and cultural impacts.[4] Only the North of Minas Gerais State produces over 346 tons in an area of 16,000 hectares. However, despite the high production, several factors, e.g. many types of fungi hindering fruit storage are still a problem, as the high room temperature that causes fast maturing of fruit, injuring the transportation and exportation.[1,5]

Many fungi species can provoke serious loss to plantations (around 50% in each harvest).[6,7] One of those fungus is called *Colletotrichum musae*, associated with the anthracnose disease. In the North of Minas Gerais, that species is responsible for 30 to 40% of marketable fruit loss.[8] Moreover, the transportation and export are often hampered by factors related to the onset of diseases, like those caused by fungi, in addition to the ripening of the fruit before reaching its destination. Then, control of fast maturing of fruit during the transportation is crucial, mainly because of regulatory bodies impositives.[1,2]

Despite the situation, in the North of Minas Gerais semiarid, a plant with low water demand and abundant occurrence in the region presents a great potential as antifungal supply. *Calotropis procera* (Asclepiadaceae) is a species originated from Asia (Afghanistan, Arabia, India, Iran, Pakistan), which has great adaptability and grows mostly in semiarid and arid climates, producing fruits throughout the year. In addition, it has a successful establishment in arid, degraded, and nutrient-poor soils.[9] In some areas, the dry biomass of *Calotropis procera* is already used as animal feed, and previous studies indicated that this species may accumulate many chemical elements.[10-12] Its antifungal activity has already been studied against some types of fungi species, including *Alternaria alternata* using *Calotropis procera* flower,[12] *Candida albicans*,[13] *Epidermophyton floccosum*, *Tricophyton gypseum*,[14] *Fusarium oxysporum*,[15] and *Colletotrichum dematium* URM 3315.[16] Moreover, as the control of diseases, mainly those from fungus attacks, are very difficult, developing materials that allow more excellent protection against this disease and not harmful to the environment is fundamentally important. In addition, although the literature has already indicated the anti fungicide activity of *Calotropis procera*, as reported by Amini et al. (2021),[11] there is a lack of comprehensive antimicrobial characteristics of *Calotropis procera*.

In this context, this research proposes a production of packaging films based on starch conjugated to different concentrations of natural extract from *Calotropis procera* seeds for agricultural propose as a sustainable alternative. These Starch-*Calotropis procera* seed packaging films are eco-friendly, low-cost, its source is widely available, and quickly produced. The antifungal activity was assessed based on *in vitro* and *in vivo* tests against *Colletotrichum musae*, as the fast maturing was performed *in vivo* using banana as a model of fruit. In addition, an extensive characterization was performed using different spectroscopic analyses, Bradford, and cytotoxicity. The results revealed that these packaging films have excellent potential for protection against fast maturing and fungus attack, which implies in its use in agricultural applications. Furthermore, to the best of the authors' knowledge, the antifungal activity of *Calotropis procera* seeds against *Colletotrichum musae* has not been explored yet (*in vitro* or *in vivo*), as as well as the evaluation of protection against fast maturing of banana fruit.

2. Materials and methods

2.1 *Materials*

Sodium hydroxide (Sigma, USA, ≥ 99%, NaOH), hydrochloric acid (Sigma-Aldrich, USA, 36.5-38.0%, HCl), urea (Sigma-Aldrich, USA, anhydrous 99.5% CH_4N_2O), Acetic acid (Synth, Brazil, 99.8%, M = 60.05 g·mol^{-1}, CH_3CO_2H), sodium phosphate dibasic (Sigma-Aldrich, USA, ≥ 99.0%, M = 141.96 g·mol^{-1}, Na_2HPO_4), potassium phosphate monobasic (Sigma-Aldrich, USA, ≥ 99.0%, M = 136.09 g·mol^{-1}, KH_2PO_4), potassium chloride (Sigma-Aldrich, USA, ≥ 99.0%, M = 74.55 g·mol^{-1}, KCl), sodium chloride (Sigma-Aldrich, USA, ≥ 99.0%, M = 58.44 g·mol^{-1}, NaCl), Bovine Serum Albumin (Sigma-Aldrich, USA, lyophilized powder, ≥ 96% (agarose gel electrophoresis) were used as-received without any further purification. Deionized water (DI-water) (Millipore Simplicity™) with a resistivity of 18 MΩ·cm was used to prepare all solutions. All preparations and syntheses were performed at Room Temperature (RT, 25 ± 2 °C) unless otherwise stated.

2.2 *Natural extract from Calotropis procera*

The natural extract was obtained from *Calotropis procera* seeds with procedure described in our previous research.[17] The seeds were collected at the North of Minas Gerais, Brazil, in the municipality of *Janaúba* and was dried at 45 ± 2 °C in an oven for 7 days. In sequence, the seeds were crushed using a knife mill (MA350, Marconi, Brazil). Briefly, the seeds were macerated with 1 L of deionized water using an industrial blender and stored at room temperature with humidity control. After 7 days, the extract was dialyzed with deionized water at RT using a membrane (12-14 kDa, Sigma, USA) to remove any water-soluble contaminants. Finally, the colloid was placed into a rotary evaporator at 45 ± 2 °C to remove most of remaining water. The final material was poured into *Eppendorfs* tubes (1.5 mL) and stored at 4 ± 2 °C until further use.

2.3 *Packaging films production*

The packaging films was fabricated using the natural extract from *Calotropis procera* seed prepared as described before mixed with starch from manioc extracted to similar procedure into literature.[18] Briefly, 45 g of Starch from manioc was dissolved at 13.5 g/L of glycerol under moderate stirring for 1 hour for complete solubilization. Then, 25, 50, 75 and 100 µL/mL of natural extract was added to the solution maintained under moderate stirring at 35 ± 2 °C for 24 h. Finally, 1.5 mL of final solution (starch + glycerol + colloidal extract) was poured into petri dish and frozen at -4 ± 2 °C for 72 h and freeze-dried (ModulyoD, Thermo Electron Corporation, Wathan, Massachusetts, USA) at -50 °C and 200 ± 50 µbar to complete solvent extraction (glycerol) and the films had been formed. Then, these film could be applied to fruit packaging protection against fast maturing and fungus attack against *Colletotrichum musae*.

2.4 *Characterization of the packaging films from Starch-Calotropis procera*

2.4.1 *Spectroscopic analyses based on infrared spectroscopy and nuclear magnetic resonance spectroscopy*

Chemical and structural characterization of packaging film was performed using spectroscopy analyses to determine the chemical groups present at Starch-*Calotropis procera* seed film. Then, Fourier Transform Infrared (FTIR) spectra were obtained using an Attenuated Total Reflectance method (ATR, ZnSe crystal prism, 4,000-650 cm^{-1} using 32 scans and a 4 cm^{-1} resolution - Nicolet 6700, Thermo-Fischer). All characterization were conducted in triplicate (n = 3).

In addition, characterization by NMR spectroscopy was conducted according to the hydrogen nucleus (^1H NMR). For this process, 2% of the film were weighed, and D_2O and HCl (100/1, v/v) solutions were prepared, with stirring for 24 hours for full solubilization. After 24 hours, the samples were placed into tubes for NMR analysis and then subjected to hydrogen experiments. All experiments were conducted in triplicate (n = 3).

2.4.2 *Surface Contact Angle (SCA)*

The effect of the packaging films hydrophilic/hydrophobic characteristics were evaluated via contact angle measurements. The tests were performed by pouring DI water droplets using a microsyringe (50 µL) onto the films and capturing the images for contact angle calculations (Lumix FZ-47 digital camera, Panasonic, Tokyo, Japan, and open-source image processing program, ImageJ Fijji, NIH). All analyses were performed six times for statistical purposes.

2.5 *Antifungal activity of packaging films*

Firstly, the films with same average mass and size were sterilized using Ultraviolet (UV) radiation at 10 cm of distance from the light source (4.78 $mW \cdot cm^{-2}$, 6 W, at λ = 254 nm, Boitton Instruments). Then, *Colletotrichum musae* was isolated from bananas, which exhibited dark spots on the bark and the presence of an orange conidia mass, symptoms that characterize anthracnose, disease caused by *Colletotrichum musae*. For the fungus isolation, little pieces of tissue were removed from the lesions of the banana fruit. Then, the fragments were disinfected superficially in an alcohol solution, and 1% sodium hypochlorite solution for 2 minutes, and it was washed in two consecutive fractions of distilled water and sterilized. Soon after in agar-agar medium, the fragments were plated and incubated at 25 °C with 12 hours photoperiod. After this period, the hyphae were transferred to the medium of Potato-Dextrose-Agar (PDA) culture

and the fungus was incubated at 25 °C, with a photoperiod of 12 hours. In sequence, the inoculums fungus were plated and incubated at 25 °C for 12 hours. Subsequentially, the hyphae were transferred to the agar diffusion. The antifungal activity of films were checked out using agar-well diffusion method according to Clinical Laboratory Standards Institute M2-A8 (2003) method. Finally, the antifungal activity was evaluated at 1, 2, 3, 4, 5 days. All analyses were performed four times for each concentration. Polyvinyl Chloride (PVC) film with commercial fungicide (imibenconazole, 150 g/L) were used as comparative.

2.6 *Analysis of fruit packaging protection against fast maturing and fungus attack*

Unripe bananas were chosen as a fruit model because it is the most consumed type of banana in the world and were obtained in a local market in *Janaúba*, North of *Minas Gerais*, Brazil. The choice of the individuals was standardized based on essential characteristics, e.g. size, no mechanical damage, no pests or diseases, and two bananas types (apple banana or silver banana). Then, the bananas were washed with distilled water and sanitized using 1% of sodium hypochlorite solution for 15 minutes. Next, the bananas were rewashed using distilled water and dried at room temperature. In sequence, the packaging film were used to cover all banana. The protection against fast maturing and fungus attack were evaluated at 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 days based on visual appearance of the color of banana and the presence of dark spots on the bark and the presence of an orange comidia mass, symptoms that characterize anthracnose caused by *Colletotrichum musae*. Bananas not covered with the Starch-*Calotropis procera* seed film, covered by polyvinyl chloride with commercial fungicide (imibenconazole, 150 g/L) and no covered were used as comparative. These experiments were performed with 20 bananas for each type (silver and apple) and films concentrations (n = 100 of each banana type, films of 4 different natural extract concentrations).

After 20 days, parts that visualized injuries were removed to antifungal analysis against *Colletotrichum musae*. Briefly, little pieces of tissue were removed from the lesions of the banana fruit. Then, the fragments were disinfected superficially in an alcohol solution, and 1% sodium hypochlorite solution for 2 minutes, and it was washed in two consecutive fractions of distilled water and sterilized. Soon after in agar-agar medium, the fragments were plated and incubated at 25 °C with 12 hours photoperiod. After this period, the hyphae was transferred to the medium of Potato-Dextrose-Agar (PDA) culture and the fungus was incubated at 25 °C, with a photoperiod of 12 hours. In sequence, the inoculums fungus were plated and incubated at 25 °C for 12 hours. Subsequentially, the hyphae were transferred to the agar diffusion. The antifungal activity of films was checked out using agar-well diffusion method according to Clinical Laboratory Standards Institute M2-A8 (2003) method.

2.7 *Toxicity evaluation of Starch-Calotropis procera seeds films*

The human embryonic kidney cell line (HEK 293 T) was kindly provided by the Department of Immunology and Biochemistry, UFMG. HEK 293 T was used as models of toxicity evaluation, since it share similarities with cells that are involved in the production of ECM components essential for human body.[19-21] The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), penicillin G sodium (10 units·mL^{-1}), streptomycin sulfate (10 mg·mL^{-1}), and 25 µg·mL^{-1} amphotericin-b (all from Gibco BRL, NY, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were used for the experiments at passage twelve. All of the biological tests were conducted according to ISO standards 10993-5:1999 (Biological evaluation of medical devices; Part 5: tests for *in vitro* cytotoxicity). HEK 293 T cells were plated (3×10^4 cells) on each packaging film produced. UV radiation was used to sterilized for 60 minutes. Controls were created using cells and DMEM medium (10%); Triton x-100 (1%; Sigma-Aldrich, St. Louis, MO, USA) was the positive control, and chips of sterile polypropylene (1 mg·mL^{-1}; Eppendorf, Hamburg, Germany) were the negative control. After 48 h, all medium was aspirated and replaced with 210 µL of culture medium with serum. Then, 3-(4,5-dimethylthiazol-2yl) 2,5-diphenyl tetrazolium bromide (MTT) (170 µL, 5 mg·mL^{-1}; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 4 h followed by incubation for 16 h with SDS/4% HCl. Subsequently, the absorbance was quantified at 595 nm in a Varioskan Reader (Thermo Scientific). The absorbance values were expressed as the percentage of viable HEK 293 T cells (Equation 1). The values of the controls (wells with cells, and no films) were set to 100% cell viability. All of the biological experiments were performed with 6 samples for each system (n = 6).

$$\text{Cell viability (\%)} = (\text{Abs of sample and cells/Abs of control}) \times 100 \quad (1)$$

2.8 *Statistical analysis*

The results of all experiments were averaged and statistical analyses were performed using ANOVA (one way included Tukey's test, $p < 0.05$, software Origin v.8.1, OriginLab Corporation, USA) unless specifically noted.

3. Results and discussion

3.1 *Spectroscopy analysis of Starch-Calotropis procera seeds packaging films*

The infrared spectroscopy analysis elucidated the presence of different chemical groups (Figure 1). For the Starch, centered bands were identified at 3,269, 2,896, 1,590 and 1,027 cm^{-1}, related to -OH stretching groups, CH_2 vibrations, -OH bending groups, and arising from C-O, C-C and C-O-H stretching and C-O-H bending, respectively.[22,23] In the case of natural extract, presence of groups associated to various chemical compounds was identified; the band at 1,717 cm^{-1} corresponding to carbonyl (C = O) groups which may be related to xylan components of hemicellulose,[24] or amino acids in protein backbone chains, *e.g.* glutamine,[25] or cardenolides derivatives, such as calactin and calotoxin.[26] In addition, bands related to CH_2 vibrations between 2,950-2,830 cm^{-1} was observed, as at 1,109 cm^{-1}, and in the range of 1,374-1396 cm^{-1} correlated to in-plane scissoring of CH_3 bands may be related to groups present in Threonine (Thr) associated with serine/threonine-protein kinase, responsible for phosphorylation in the plant.[25] The native cellulose presents two different forms depending on the origin, one-chain triclinic structure, Iα, and two-chain monoclinic structure, Iβ;[24] at 932 cm^{-1}, a band was detected and associated to Iβ crystalline cellulose in natural extract, which may also be related to Cellulose Nanocrystals (CNCs).[27] The broad band at 3,339 cm^{-1} is associated with O-H stretching vibrations of cellulose and CNC.[27,28] Additionally, at 1,459 cm^{-1} and 1,429 cm^{-1}, bands that are linked to C-C stretches and may be related to fatty acids present in natural extract, respectively.[29,30] Then, at 1,551 and 1,573 cm^{-1}, bands related to C = C were perceived may be correlated with groups present in Threonine (Thr), from serine/threonine-protein kinase.[31] Moreover, in packaging film of Starch-*Calotropis procera* seed bands associated to both compounds, natural extract and starch, were perceived. At 3,313 cm^{-1} appeared a band related to -OH groups overlapping with band related to C-H stretching displaced compared to starch and natural extract, which indicated that was a linked to among both materials (starch and natural extract) by these chemical groups away hydrogen bonding. In addition, bands at 1,944 and 2,833 cm^{-1} were visualized in the Starch-*Calotropis procera* seed film related to CH_2 vibrations displaced comparing with starch and natural extract, indicating the linked between the natural extract and starch in the packaging film. Moreover, at 1,698 cm^{-1} band associated to amide I and carbonyl group in the starch and natural extract, respectively, was presented with shift comparing with both materials, which may also indicate the conjugation by these two materials. Furthermore, at 1,412 cm^{-1} bands linked to C-C stretches associated to fatty acids were visualized.[29,30] No significative difference were founded into the FTIR spectra of Starch-*Calotropis procera* seed film with different concentrations of natural extract, probably, because the low concentration ($\mu L \cdot mL^{-1}$) of natural extract used to produced all packaging films. Additionally, bands linked to cellulose and cellulose nanocrystal, similar to natural extract, were envisioned to packaging film.

Complementing the infrared spectroscopy analysis, ^1H NMR was conducted to verify the chemical groups in the Starch-*Calotropis procera* seed films. Figure 2A shows the ^1H NMR of starch. Signals at 4.5-5.0 ppm for α-(1,6)-linkages, 5.1-5.6 ppm for α-(1,4)-linkages reltade to hydroxyl groups, as at 3.07-3.66 ppm is possible visualized H-chemical shifts.[32-35] In the case of natural extract (Figure 2B), similar to FTIR, the ^1H NMR analysis revealed the presence of groups associated to Thr (serine/threonine protein kinase) at 1.258 ppm[36] and aliphatic chains related to the signals corresponding to acyl chain protons at 1.3-1.0 ppm (greater δ related to α -CH_2- and β -CH_2- and smaller δ for -$(CH_2)_{d\text{-}i}$-).[37-39] The bands related to the -CH group present at xylan hemicellulose components were observed at 3.513 ppm, close to 2.6 ppm, and 2.050 ppm.[37] In addition, bands related to the two-chain monoclinic structure, Iβ, were observed close to 2.6 ppm.[37] In the Starch-*Calotropis procera* seed films (Figure 2C) are possible vizualide signal associated to both materials, natural extract and starch. Signals in the range of 1.3-1.0 ppm, 3.5 ppm, in the range of 2.0-2.7 ppm and 4.3 ppm were visualized related to acyl chain, CH groups present at xylan components of hemicellulose, the two-chain monoclinic structure, Iβ, and an anomeric carbon is observed in relation to double-linked

cardiac glycoside present at cardenolides derivatives, such as calactin and calotoxin, respectively.[26,36-40] Similar to FTIR, no significative difference were founded into the ^{1}H NMR spectra of Starch-*Calotropis procera* seed films with different concentrations of natural extract. These results indicated that the linked among natural extract to starch to form a packaging film was successful.

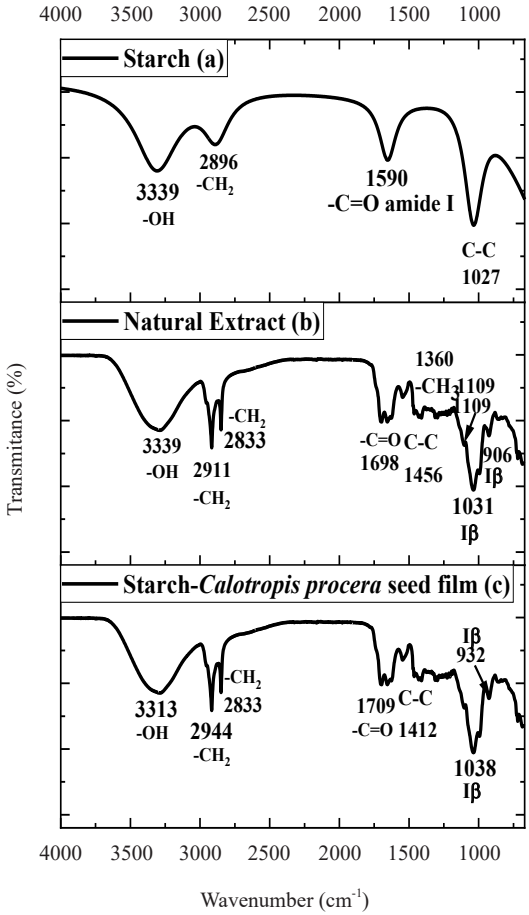


Figure 1. FTIR of (a) Starch, (b) Natural extract and (c) Starch-*Calotropis procera* seed film with natural concentration of 25 μL·mL^{-1} used as a model; indicating the priority chemical groups

3.2 *Surface contact angle analysis*

The correct and adequate storage of fruits is essential to transportation, mainly to exportation. Temperature and humidity influence the fruit ripening at the right time. In addition, these effects can provide an environment conducive to the emergence of diseases, such as those caused by fungi and other microorganisms.[1,3] In this context, using hydrophobic protection in fruit storage is an interesting solution. The surface contact angle was evaluated in this research to understand the Starch-*Calotropis procera* seed packaging films qualitative hydrophobic/hydrophilic behavior to improve the fruit storage. The films are composed of many chemical groups which increase the hydrophobic behavior, like proteins, cellulose, or even aliphatic chains, as described in spectroscopy analyses, and may guarantee an adequate environment for the storage and transportation of fruits. These chemical groups improve the hydrophobic behavior, important factor in the case of fruit protection to transportation, exportation, and diseases like fungi.[1,41] The results of the contact angles are reported in Figure 3 comparing with starch film indicating an increase of hydrophobic character to Starch-*Calotropis procera* seed packaging films without no significative statistical difference among the

films with different natural extract concentration, probably, because the low concentration (μL·mL^{-1}) of natural extract used to produced all packaging films, demonstrating that the conjugation between natural extract to starch to form a packaging film was successful.

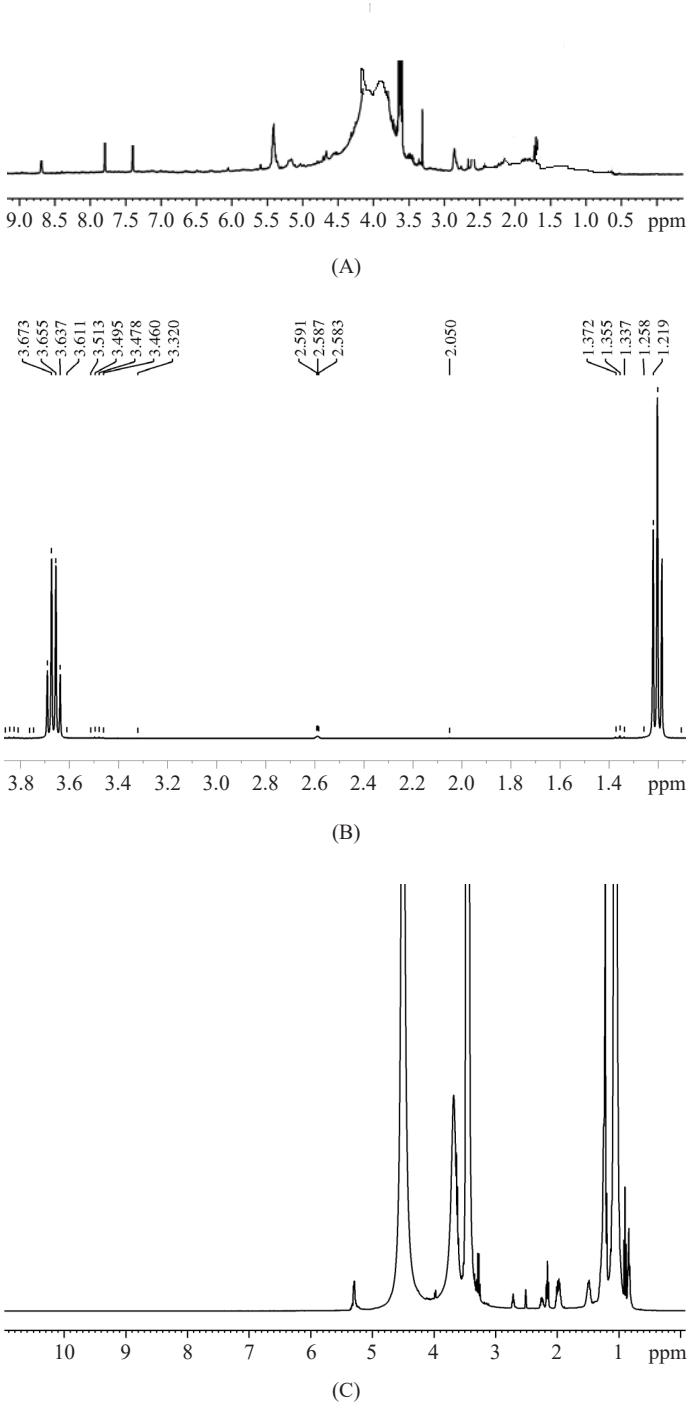


Figure 2. [1]H-NMR spectra of (A) Starch, (B) Natural extract and (C) Starch-*Calotropis procera* seed film with natural concentration of 25 μL·mL^{-1} used as a model

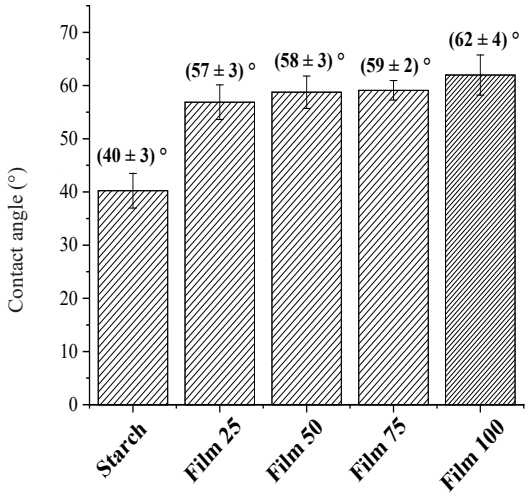


Figure 3. Surface contact angle of (A) Starch, (B) Natural extract and (C) Starch-*Calotropis procera* seed film

3.3 *Antimicrobial activity against Colletotrichum musae of Starch-Calotropis procera seed packaging films*

One of the major problems in agriculture is the control of diseases, mainly those from fungi attacks.[8] Many types of fungi can cause substantial damages to plantations, leading great economic losses.[6,7] The *Colletotrichum musae*, responsible for anthracnose is one of this fungis that is of great concern in many rural farmers, this agent can damage various types of fruits, including the banana, the most consumed fruit in the world.[5,11,42] For this reason, developing materials that allow more excellent protection against this disease and not harmful to the environment is fundamentally important. Then, this research studied the *in vitro* antifungal behavior of Starch-*Calotropis procera* seed films. Figure 4 shows the results of the antifungal activity of Starch-*Calotropis procera* seed films with the different natural extract concentrations comparing with PVC film with commercial fungicide and without any material over 5 days. The inhibition of fungus micellar growth was proportional to the increase in concentration of Starch-*Calotropis procera* seed film. The film with concentration of 100 $\mu L \cdot mL^{-1}$ demonstrated the best effect, even comparing with PVC film with commercial fungicide. In addition, except of film with 25 $\mu L \cdot mL^{-1}$ of natural extract, all Starch-*Calotropis procera* seed films presented results better than PVC film with commercial fungicide. These results may be associated with the flavonoid presented into natural extract increasing with the concentration, as the flavonoids' antimicrobial activity is well known.[12,43] However, the natural extract showed the presence of proteins, e.g. serine/threonine-protein kinase (determined in the spectroscopy analyses), and it is known that proteins can act as a chemical barrier in the plant defense against external pathogenic bacteria, fungi, or virus.[44] Then, Starch-*Calotropis procera* seed films showed signals and band related the most of chemical groups into starch and natural extract (as described in the spectroscopy analysis), the presence of these proteins into film may relate to antifungal property. In addition, the protein exercises a vital role through Pattern Recognition Receptors (PRR), participating in the plant physiology process, including antimicrobial protection.[44] The serine/threonine-protein kinase is important in cell differentiation, transcription, and transduction in a diversity of animals and plants. It is also reported in the literature that this protein catalyzes the gene MoPPG1, promoting several defects in fungi hyphal growth and do not allow the gene penetration on plants, which can cause diseases.[45] Then, the serine/threonine-protein kinase in the films may play an essential role in antifungal activity. As an exploratory analysis to determine the presence of proteins in the film, using the film with natural extract concentration of 25 $\mu L \cdot mL^{-1}$ (as the increase of conectration, increase the protein concetration), Bradford assay was performed. The test indicated a high amount of proteins (not specific determined), as visualized in Figure 5, which may contribute to antifungal activity.

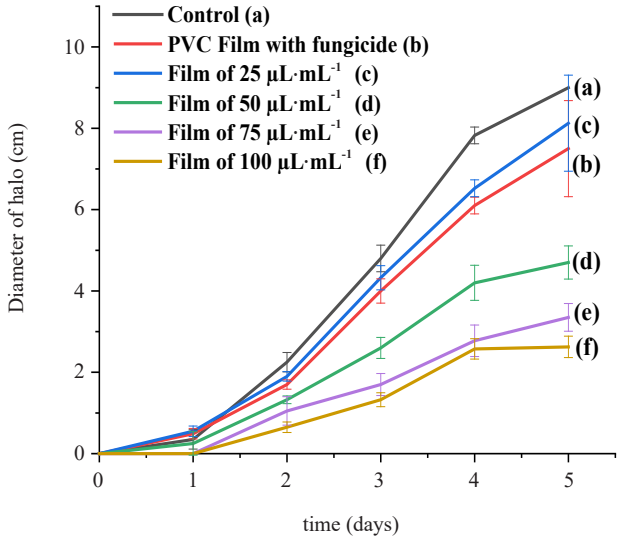
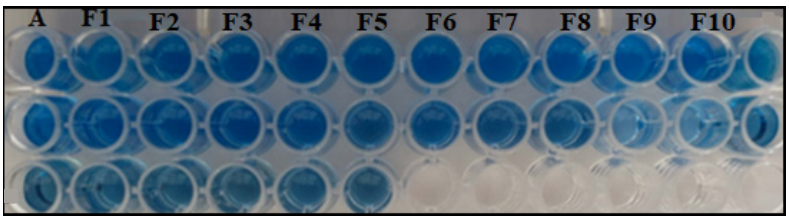
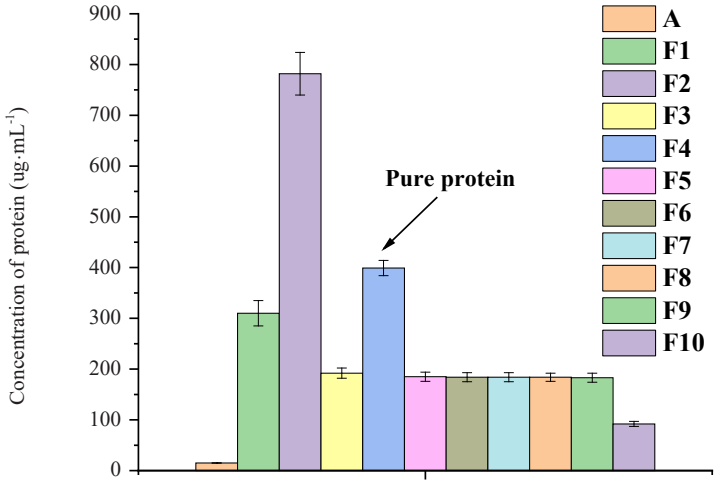


Figure 4. Antifungal activity for (a) Control, (b) PVC film with commercial fungicide, (c) Film of 25 µL·mL^{-1}, (d) Film of 50 µL·mL^{-1}, (e) Film of 75 µL·mL^{-1}, (f) Film of 100 µL·mL^{-1}



(A)



(B)

Figure 5. (A) Bradford analysis of the dissolved Starch-*Calotropis procera* seed film with natural concentration of 25 µL·mL^{-1} used as a model, (B) Qualitative protein quantification using Bradford

3.4 *Analysis of Starch-Calotropis procera seed packaging protection against fast maturing*

The protection of fast maturin during the transportation is crucial, especially when the fruit is destined for

exportation, mainly to Europe, as the EC settled that the fruits can not have over 10% of injuries and degenerative processes.[11] Thus, promoting materials which provide good protection against fungi, control of fast maturing, and eco-friendly is vital. Then, this research studied the Starch-*Calotropis procera* seed packaging films to determine its potential to promote antifungal protection and fast maturing of fruit, using banana as a model, for transportation and exportation. Figure 6 shows the results of experiment over a period of 20 days. The results demonstrated that the Starch-*Calotropis procera* seed films protected fast maturing. In addition, the films also provided control of fast maturing of fruit compared with samples without film (control) and PVC film with commercial fungicide. Moreover, the best result of control of fast maturing were perceived in the film of 50 $\mu L \cdot mL^{-1}$ of natural extract. The results were similar to both types of bananas studied (apple banana or silver banana). Although, the shape and weight of control samples changed differently from samples with Starch-*Calotropis procera* seed films, no significant differences were detected. As Ashitiani et al. (2014) reported, the consumers preferred fruits with similar shape and weight.[46] Thus, guaranteeing protection against fast maturing, and maintaining the shape and weight of fruits are essential in agricultural transportation, especially exports.

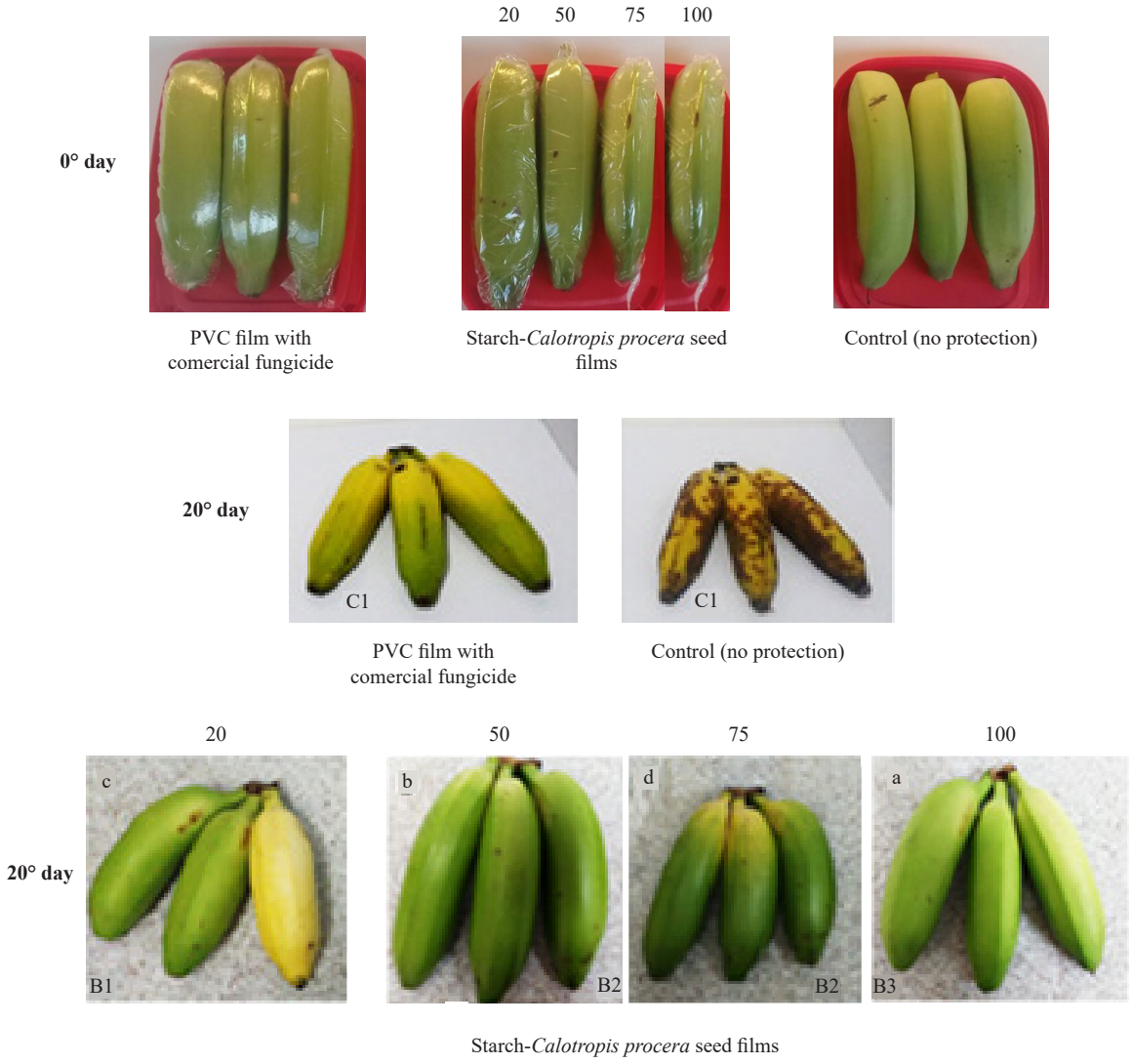


Figure 6. Images of analysis of Starch-*Calotropis procera* seed packaging protection against fast maturing and fungus attack at PVC film with commercial fungicide, Starch-*Calotropis procera* seed films with different natural extract concentration, and control (no protection fungicide or film) in the biggening and after 20 days

3.5 *Analysis of Starch-Calotropis procera seed packaging protection against Colletotrichum musae fungus attack*

In the case of protection against fungus attack, Figure 6 shows the results of experiment over a period of 20 days with *Colletotrichum musae*. The results demonstrated that the Starch-*Calotropis procera* seed films protected fungus attack. It was not perceived any characteristics signs of the presence of fungi visualized, differently from samples without protection (control) that signs of *Colletotrichum musae* were detected. In addition, the best result of anti-fungicide activity was when the concentration of extract increased in the film, differently of fast maturing, which the best result was in the film of 50 $\mu L \cdot mL^{-1}$ of natural extract. Although, similar to analysis of fast maturing, the results were similar to both types of bananas studied (apple banana or silver banana).

Then, it is important highlighted that fungus produces an alginate film protection around the cell. In addition, the cell wall in the fungus is most part compound by chitin, which is a polysaccharide that has glucosamine group that interact with this groups -O, C-C and C-O-H stretching and C-O-H bending, then, probably, these chemical groups presented in Starch-*Calotropis procera* bio-packing linked with the alkyl chain and the fungus cell wall. Moreover, the shape and weight of control samples changed differently from samples with Starch-*Calotropis procera* seed films, no significant differences were detected. Thus, these bio-packings guaranteeing protection against fungus attack, maintaining the shape and weight of fruits, which is very important to agricultural transportation, especially exports.

Moreover, the antifungal activity of Starch-*Calotropis procera* seed films with the different natural extract concentrations were evaluated after 20 days removing the injuries of bananas (Figure 7). The inhibition of fungus micellar growth was proportional to the increase in concentration of Starch-*Calotropis procera* seed film. The film with concentration of 100 $\mu L \cdot mL^{-1}$ demonstrated the best effect. These results may be associated with the flavonoid presented into natural extract increasing with the concentration, as the flavonoids' antimicrobial activity is well known,[12,43,47,48] as described at section above. Furthermore, as reported by literature, there is a lack of comprehensive antimicrobial characteristics of *Calotropis procera*.[49-52]

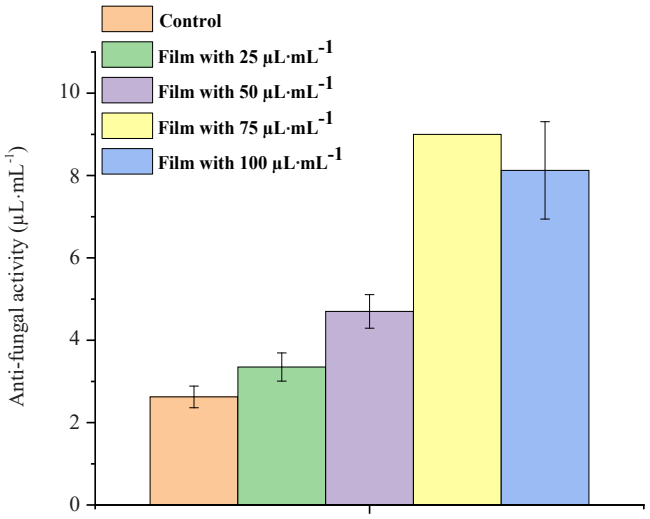


Figure 7. Antifungal activity for (a) Control, injuries removed from bananas after 20 days with films with extract concentration of (b) 25 $\mu L \cdot mL^{-1}$, (d) 50 $\mu L \cdot mL^{-1}$, (e) 75 $\mu L \cdot mL^{-1}$, (f) 100 $\mu L \cdot mL^{-1}$

3.6 *Cytotoxicity evaluation of Starch-Calotropis procera seed packaging film*

The toxicity of the biofilm was characterized using a MTT *in vitro* assay according to the international standard described in section 2.7 as environmentally friendly materials in agriculture applications. HEK 293 T cells were used as a model cell line, since it shares similarities with cells that are involved in the production of ECM components

essential for human body.[19-21] This cell line is extensively adopted in research, as it has facile access, high proliferative performance, and the results are reproducible. Figure 8 shows that the Starch-*Calotropis procera* seed films did not demonstrate any cytotoxic effects since the cell viability responses were ≈ 80%. Thus, the Starch-*Calotropis procera* seed films can be applied to agriculture applications as an eco-friendly protection.

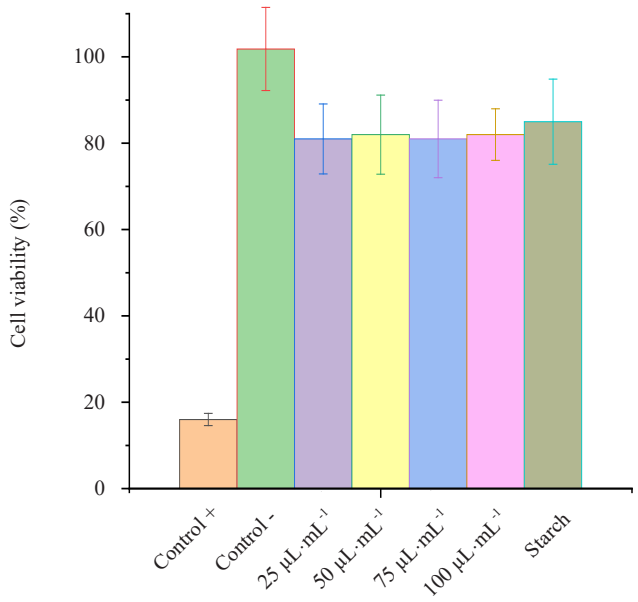


Figure 8. Cell viability response by MTT assay after 48 h of incubation with Starch-*Calotropis procera* seed films using HEK 293T (scale bar = 100 µm)

4. Conclusions

This research reported the production of packaging film based on Starch conjugated to natural extract by *Calotropis procera* seed as a sustainable alternative applications in agriculture. The results indicated the presence of important chemical groups which may play an important role in antifungal activity against *Colletotrichum musae*. Also, The results indicated the protection of fast maturing to fruit, which is an important characteristic to transportation and exportation. The presence of some compounds were detected by spectroscopys analysis, and may have an important role in these protections, as proteins, calactin and calotoxin. In addition, the conjugation of starch of manioc and natural extract by *Calotropis procera* seed were very successful. Furthermore, the Starch-*Calotropis procera* seed films controlled the fast maturing of banana, which is crucial in the transportation and exportation of fruits. The antifungal analysis against *Colletotrichum musae* indicated that the inhibition of fungus micellar growth was proportional to the concentration of the natural extract in the film. Differently, the *in vivo* analysis, showed that the best result was at film with 50 $\mu L \cdot mL^{-1}$ concentration of natural extract. The evaluation of cytotoxicity demonstrated cell viability over 80%, which determines this packaging film as eco-friendly material. Thusly, this Starch-*Calotropis procera* seed films are contemplated as a sustainable alternative to agriculture applications.

Acknowledgments

The authors acknowledge the BIOSEM from the Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM) for all chemical experiments, and they express their gratitude to AMBER Research Centre/School of Chemistry for the spectroscopic analyses. In addition, the authors acknowledge the FAPEMIG (APQ-02561/2021),

CNPq and CAPES for financial support.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Author contributions

The manuscript was written through the contributions of all authors. All authors have given their approval to the final version of the manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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