

## Potential agrifood application of seriguela (*Spondias purpurea L.*) residues extract and nanoZnO as antimicrobial, antipathogenic and antivirulence agents

Potencial aplicação agroalimentar do extrato de resíduos de seriguela (*Spondias purpurea L.*) e nanoZnO como agentes antimicrobianos, antipatogênicos e antivirulência

Posible aplicación agroalimentaria del extracto de residuos de seriguela (*Spondias purpurea L.*) y nanoZnO como agentes antimicrobianos, antipatógenos y antivirulencia

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### Abstract

The impact of phytopathogenic microorganisms in several crops causes significant losses in agrifood industry, spoilage throughout food chain and storage. Nanoparticles and plant extracts have been highlighted by their antimicrobial properties applied in food packaging, agriculture, drug delivery systems and other medical approaches. Over the past few years, this group have studied the application of ZnO nanoparticles and agroindustrial wastes in edible food coatings/films. This study aimed to evaluate active characteristics from the extract of seriguela processing wastes and nanoZnO regarding to their inhibitory activity against bacterial pathogenicity and virulence systems TTSS (Type Three Secretion System) and QS (Quorum Sensing) for *Pseudomonas savastanoi*. Also, antibacterial action (inhibition area) against species of *Curtobacterium*, *Clavibacter*, *E. coli*, *Xanthomonas* and *Serratia*, and antifungal against *Botrytis cinerea* (reduction in colony size). The 60% extract inhibited the activation of QS and TTSS system in 20.26% and 13.54%, respectively; while nanoZnO at 3% reduced 46.77% QS and increased 302.88% TTSS. Extract without dilution inhibited the growth of *Clavibacter michiganensis* pv *michiganensis* (Gram-positive) and *Xanthomonas phaseoli* (Gram-negative), inhibitory zone of 94.25 mm<sup>2</sup> and 452.39 mm<sup>2</sup> respectively. The latter also being inhibited by nanoZnO 1 and 2% (138.23 mm<sup>2</sup>) and 3% (275.67 mm<sup>2</sup>). Pure extract inhibited 17.38% growth of fungal colony and nanoZnO (1 and 3%) in 33.08%. Finally, the active agents studied showed to be promising in the prevention of phytopathogenic diseases and consequently economic losses, food films/coatings and the extract as a biopesticide, reducing the environmental impact.

**Keywords:** Vegetal source bioactives; Nanoparticles; Antimicrobial activity; Anti-pathogenicity; Antivirulence; Secretion system type three; Quorum sensing.

### Resumo

O impacto de microrganismos fitopatogênicos em diversas safras causa perdas significativas para indústria agroalimentícia, deterioração na cadeia alimentar e de armazenamento. Nanopartículas e extratos vegetais têm se

destacado por suas propriedades antimicrobianas aplicadas em embalagens, agricultura, sistemas de distribuição de medicamentos e outras abordagens médicas. Nos últimos anos, este grupo tem estudado a aplicação de nanopartículas de ZnO e resíduos agroindustriais em revestimentos/filmes comestíveis para alimentos. Este trabalho teve como objetivo avaliar características ativas do extrato de resíduos do processamento da seriguela e do nanoZnO quanto à atividade inibitória contra sistemas de patogenicidade e virulência bacteriana: TTSS (Sistema de Secreção Tipo Três) e QS (Quorum Sensing) para *Pseudomonas savastanoi*. Além da ação antibacteriana (área de inibição) contra espécies de *Curtobacterium*, *Clavibacter*, *E. coli*, *Xanthomonas* e *Serratia*, e da antifúngica contra *Botrytis cinerea* (redução do tamanho da colônia). Extrato a 60% inibiu a ativação do sistema QS e TTSS em 20,26% e 13,54%, respectivamente; enquanto nanoZnO a 3% reduziu 46,77% QS e aumentou 302,88% TTSS. Extrato sem diluição inibiu o crescimento de *Clavibacter michiganensis* pv *michiganensis* (Gram-positiva) e *Xanthomonas phaseoli* (Gram-negativa), zona inibitória 94,25 mm<sup>2</sup> e 452,39 mm<sup>2</sup> respectivamente. Esta última também inibida por nanoZnO 1 e 2% (138,23 mm<sup>2</sup>) e 3% (275,67 mm<sup>2</sup>). Extrato puro inibiu em 17,38% o crescimento da colônia fúngica e o nanoZnO (1 e 3%) em 33,08%. Por fim, os agentes ativos estudados mostraram-se promissores na prevenção de doenças fitopatogênicas consequentemente perdas econômicas, filmes/revestimentos de alimentos e o extrato como biopesticida reduzindo o impacto ambiental.

**Palavras-chave:** Bioativos de fonte vegetal; Nanopartículas; Atividade antimicrobiana; Antipatogenicidade; Antivirulência; Sistema de secreção tipo três; Quorum sensing.

### Resumen

El impacto de microorganismos fitopatógenos en varios cultivos provoca importantes pérdidas en industria agroalimentaria, deterioro a lo largo de cadena alimentaria y almacenamiento. Nanopartículas y extractos de plantas se han destacado por sus propiedades antimicrobianas aplicadas en envasado de alimentos, agricultura, sistemas de administración de fármacos y otros enfoques médicos. En los últimos años, este grupo ha estudiado aplicación de nanopartículas de ZnO y desechos agroindustriales en recubrimientos/películas comestibles de alimentos. Este estudio tuvo como objetivo evaluar características activas del extracto de residuos de procesamiento de seriguela y nanoZnO con respecto a actividad inhibitoria contra sistemas de virulencia y patogenicidad bacteriana TTSS (Sistema de Secreción Tipo Tres) y QS (Quorum Sensing) para *Pseudomonas savastanoi*. También, acción antibacteriana (zona de inhibición) frente a especies de *Curtobacterium*, *Clavibacter*, *E. coli*, *Xanthomonas* y *Serratia*, y antifúngica frente a *Botrytis cinerea* (reducción del tamaño de colonia). Extracto al 60% inhibió activación del sistema QS y TTSS en 20,26% y 13,54%, respectivamente; mientras nanoZnO al 3% redujo 46,77% QS y aumentó 302,88% TTSS. Extracto sin diluir inhibió crecimiento de *Clavibacter michiganensis* pv *michiganensis* (Gram-positivo) y *Xanthomonas phaseoli* (Gram-negativo), zona inhibitora 94,25 mm<sup>2</sup> y 452,39 mm<sup>2</sup> respectivamente. Esta última también inibida por nanoZnO 1 y 2% (138,23 mm<sup>2</sup>) y 3% (275,67 mm<sup>2</sup>). Extracto puro inhibió 17.38% del crecimiento de colonia de hongos, y nanoZnO (1 y 3%) en 33.08%. Finalmente, los agentes activos estudiados demostraron ser prometedores en prevención de enfermedades fitopatogénicas y consecuentemente pérdidas económicas, películas/recubrimientos alimentarios, y el extracto como biopesticida reduciendo impacto ambiental.

**Palabras clave:** Bioactivos de origen vegetal; Nanopartículas; Actividad antimicrobiana; Antipatogenicidad; Antivirulencia; Sistema de secreción tipo tres; Quorum sensing.

## 1. Introduction

The global agroindustry undergoes great economic losses caused by the action of phytopathogenic bacteria in the production, since the bacterial diseases are responsible for significant losses in several crops, which has consequences on its yield and quality (Biancalani et al., 2016; Gutiérrez-Pacheco et al., 2019; Ombiro et al., 2018), and alongside with other phytopathogens (such as fungi and viruses) and abiotic factors (environmental degradation and climate changes) represent a threat to the agrifood field. These losses are estimated at more than US\$ 1 billion each year and also have consequences for the entire food production chain as well as their supply and demand (Martins et al., 2018), highlighting that at least 10% of global food production is lost due to phytopathogenic diseases (Kannan et al., 2015). Furthermore, the real costs of losses and phytopathogenic diseases in agroindustry are reflected multidirectionally for consumers, public health, the environment, farmers, the economy and society (Savary et al., 2012).

Extremely effective methods (for example, quarantine) have a strong impact on the economy and conventional substances to avoid these diseases, such as chemicals and antibiotics, may lead to problems with safety, ecotoxicity, and resistant bacteria. Thus, the search for sustainable alternatives against phytopathogenic bacteria has been receiving highlight in scientific research (Biancalani et al., 2016). Furthermore, food industry has been seeking more sustainable options against the

action of pathogenic microorganisms aiming to replace the synthetic additives or preservatives commonly used by natural compounds in the preservation of food (Dannenberg et al., 2019; Poveda et al., 2018).

In this perspective, natural extracts from different plant sources have received attention due to their antioxidant action (Poveda et al., 2018), but also to their antimicrobial activity against a broad spectrum such as fungi (*Aspergillus niger*), Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) (Steiner et al., 2017). The use of the active compounds, such as phenolics obtained from agroindustrial residues, comes up with great potential for technological application, especially due to the low cost (Haas et al., 2018; V. Silva et al., 2018).

Some action mechanisms by these bioactive compounds are: destabilization of the cytoplasmic membrane, changes in cell membrane permeability and interference in microbial metabolism. Flavan-3-ols (flavanols, such as catechin), flavonols and tannins demonstrated be able to eliminate virulence factors, neutralize toxins and act in synergy with antibiotics (Pina-Pérez & Ferrús Pérez, 2018). Non-flavonoid phenolic compounds, such as gallic acid and vanillic acid, showed to inhibit *E. coli* (V. Silva et al., 2018; Vaquero et al., 2007). Thus, phenolic compounds available in tropical and exotic fruits, such as seriguela (*Spondias purpurea L.*), have arisen interest due to their rich bioactive composition (Dutra et al., 2017).

In this approach, the search for inhibitors that affect the bacterial systems of virulence and pathogenicity such as those regulated by the Quorum Sensing (QS) and Type Three Secretion System (TTSS), has been reaching strength (Biancalani et al., 2016). The TTSS is the main virulence determinant of several pathogenic bacteria, such as *Shigella*, *Salmonella*, *Chlamydia*, *Yersinia*, *Escherichia* and *Pseudomonas*, and this system mediates a transport process through the cytoplasmic membrane in which bacteria introduce bacterial cytosol proteins into the host cell able to cause pathogenicity and virulence, which leads to bacterial infections (C. Wang et al., 2016). In other words, it works like a syringe that injects these proteins, generating infections (Thakur et al., 2021). Regarding to Quorum Sensing (QS), this is a process of communication between bacterial cells that occurs based on generation and identification of signaling molecules and on group-level response (Zhang et al., 2018). Also, by the QS the bacteria can modulate their gene expression according to the biotic and those from the environment (abiotic) factors (Biancalani et al., 2016; Joshi et al., 2016). In addition, QS also can trigger the biofilm production which may lead to an increase in antibiotic resistance (Zhang et al., 2018). This type of control through these two systems is an advantageous strategy also because it would reduce the risk of generating resistant bacteria as they are not essential for viability (Biancalani et al., 2016).

Some compounds from plant sources have already been shown to interfere with the QS and TTSS of pathogens. In the study by Zhang et al. (2018), coumarin, a plant-derived phenolic compound, was able to inhibit QS and biofilm formation as well as alter the expression of genes related to TTSS of *Pseudomonas aeruginosa*. Gutiérrez-Barranquero, Reen, McCarthy, & O’Gara (2015) also identified coumarin as an inhibitor of QS with a strong anti-virulence action against a broad spectrum of pathogens, mainly Gram-negative bacteria. Biancalani et al. (2016) recognized that the extracts of grape seeds and green tea leaves inhibited the TTSS system of *Pseudomonas savastanoi* as well as the extracts of artichoke leaves, olive leaves and green tea leaves acted as inhibitors of the QS system.

Besides extracts from plant sources, zinc oxide (ZnO) nanoparticles (NPs), classified as safe substances (GRAS) by the Food and Drug Administration – FDA (Kanmani & Rhim, 2014; Shankar et al., 2015), also have been receiving attention due to its antimicrobial activity against a range of microorganisms (Kanmani & Rhim, 2014). These include *Salmonella* and *Campylobacter* (Duffy et al., 2018); *Staphylococcus aureus* (Akbar & Anal, 2014); *L. monocytogenes* and *E. coli* (Shankar et al., 2015); *Bacillus subtilis* and *Enterobacter aerogenes* (Esmailzadeh et al., 2016). The NPs of ZnO have as main mechanisms of action: irretrievable damages to the membranes, DNA and mitochondria caused by oxidative stress; changes in cell morphology; increased permeability of the membrane; changes in the cell wall, among others (Duffy et al., 2018; Hajipour et

al., 2012). Moreover, zinc oxide nanoparticles have shown potential as an eco-friendly application to promote plant growth and disease resistance (Nandhini et al., 2019).

In view of the above considerations, it was verified the need for a more detailed study regarding to the active property of the extracts from fruits agroindustrial residues and zinc oxide nanoparticles, since these have been widely explored by the Food Science and Technology Research Group of the Federal Rural University of Pernambuco (UFRPE), which in recent years has been carrying out research applying these compounds in films and coatings for food packaging or as microencapsulated materials. Furthermore, the use of these substances of simple obtaining with low cost brings the possibility of application in agrifood industry. After characterization of the seriguela residue extract phenolic profile by HPLC (High Performance Liquid Chromatography), this study evaluated the extract and ZnO nanoparticles (nanoZnO) with respect to their inhibitory activity on TTSS (pT3) and QS systems of the Gram-negative phytopathogenic bacteria *Pseudomonas savastanoi*, and to their antibacterial action (against species of *Curtobacterium*, *Clavibacter*, *Escherichia*, *Xanthomonas*, *Serratia*) - by the inhibition zone method - and antifungal - by the decrease in colony size (against *Botrytis cinerea*), in collaboration with the University of Florence in Italy.

## 2. Methodology

This study was developed in the Laboratory of Food Physicochemical Analysis located in the Department of Home Economics of Federal Rural University of Pernambuco – UFRPE (Brazil) in collaboration with the Molecular Plant Pathology Laboratory, belonging to the Department of AgriFood Production and Environmental Sciences (DISPAA) of the University of Florence (Italy). This work was an experimental lab research, quantitative in nature, with methodological support provided by Pereira et al. (2018).

### 2.1 Obtaining and Preparing Raw Materials

The seriguela residue (skin, pulp residues and seed pieces) was provided by the Fruta Pluss® (a frozen fruit pulp industry, located in the city of Recife - PE/Brazil). The seriguela residue was collected at the processing area and immediately transported in a styrofoam box to the Laboratory of Food Physicochemical Analysis, where they were stored under freezing conditions (-18°C) until drying. The residue was then dried at 50°C in an air circulating oven (MA035/5 - MARCONI) until it reached 10% moisture content or less, and then milled in a multipurpose mill - TECNAL TE 631/2. The obtained flour was sieved (100 mesh) to uniformize particles size, and stored under freezing conditions at -18°C in high density polyethylene bags covered with aluminum foil to protect the active compounds from light. This flour was used for the preparation of the ethanolic extract of seriguela residue.

Concerning the zinc oxide nanoparticles (nanoZnO), these were synthesized in the Laboratory of Devices and Nanostructures (LDN/CTG/UFPE) according to the methodology described by Santos and Santos (2012).

### 2.2 Obtaining the Seriguela Residue Extract

The method proposed by Andrade et al. (2015) and Portugal Zegarra et al. (2018), with modifications, was used for the extraction of the phenolic compounds of the seriguela residue flour, in which 10g of the flour (item 2.1) were mixed with 50mL of 60% ethanol solution under constant stirring (400 rpm) for 30 minutes on a digital mechanical stirrer (TE - 039/1 - Tecnal) at  $23 \pm 2^\circ\text{C}$ . Then, the mixture was centrifuged (centrifuge CT-6000 R-CIENTEC) at 4000 rpm for 15 minutes and the precipitate was resuspended in 50 mL of 60% ethanol solution and centrifuged again. The supernatants obtained from the consecutive centrifugations were collected and their final volume measured to 100mL. Subsequently the extract was stored under freezing condition at (-18°C) in amber glasses, for further delineation of the phenolic profile.

### 2.2.1 Phenolic profile design

The phenolic profile design of the hydroethanolic extract was carried out by high performance liquid chromatography (*Ultimate 3000 Dionex®* HPLC). An aliquot of the extract (20  $\mu$ L) was injected into the HPLC, equipped with degasifier, quaternary pump, automatic sampler, UV/Visible molecular absorption detector oven, *Chromeleon software*, with column temperature at 35°C. Chromatographic analysis was performed using *Acclaim®120 Dionex* C18, 250 X 4,6mm X 5 $\mu$ m column (SHIMPACK CLC-ODS); as the mobile phase the mixture of formic acid: acetonitrile: ultra pure water 0.5: 12.5: 87 v/v (solvent A) and 10:50:40 v/v (solvent B), in concentration gradient obtained by changing the ratio of solvent B in solvent A as follows: 0-10 min, 100 to 90% A; 10 to 30min, 90 to 60% A; 30 to 40 min, 60 to 40% A; 55min, 40% A, with flow rate of 0.6mL.min<sup>-1</sup> at 220nm, 280nm, 306nm and 368nm. The phenolic compounds were identified based on their retention time and spectral property, and quantified by standard curve of each compound (Tolun et al., 2016).

### 2.3 Inhibitory Activity of Seriguela Residue Extract and Nanozno on the TTSS (T3) and Qs Systems of Gram-Negative Phytopathogenic Bacteria *Pseudomonas savastanoi*

The inhibitory effect of extract and nanoZnO on the activity related to the TTSS (or T3) and QS systems of the bacterium *Pseudomonas savastanoi* was determined based on gene coding for the green fluorescent protein (GFP). This assay was based on the method suggested by Biancalani et al. (2016), with some modifications. Bacterial cells carrying the T3 and QS promoters were cultured overnight on KB (King's B) medium at 26°C, with their final pellet being washed twice in sterile physiological solution and inoculated into *Pseudomonas* culture medium (minimal medium - MM) with final bacterial concentration at OD600 (optical density at 600 nm) = 0.3. Then, the extract and nanoZnO were added in different concentrations. For the extract, contents of 10, 20, 40 and 60% were tested, and for nanoZnO 1, 2 and 3% (aqueous solution using sterile water). The measurements during this assay were carried out in zero-time and after 24 hours. The bacteria carrying the empty vector (without T3 and QS promoters) were used as negative control. The assay was performed in multiple well plates (BIOFIL®, Guangzhou, China) for 24 hours with the promoter activity (T3 e QS) assessed quantitatively by simultaneous measuring (GFP fluorescence linked to anti virulence/pathogenicity effect and absorbance at 600nm related to bacterial growth or antibiotic activity) on reader Infinite® M200PRO Quad4 Monochromators™ (TECAN).

### 2.4 Antibacterial Assay for The Extract and Nanozno

The antibacterial activity was evaluated by the agar well diffusion method, and the extract of the seriguela residues and the nanoZnO were tested according to the methodology proposed by Dahech et al. (2013) and Arulmozhi et al. (2019) with changes. The culture medium LB (Luria Bertani) with agar addition was previously prepared and distributed in sterile and disposable Petri dishes. After its solidification, 100  $\mu$ L of the bacterial culture (with concentration of 0.5 OD - McFarland Scale) was inoculated by spreading this volume over the entire agar surface using disposable Drigalski handle. Thereafter, wells/holes of 7 mm diameter were punched aseptically with a sterile cork borer, and 50  $\mu$ L of the extract (for each concentration: 100% or without dilution, 10 and 5%) or nanoZnO dispersed in sterile water (at 1, 2 and 3%) were injected into the wells using an automatic pipette, and the plates were incubated at 26  $\pm$  1°C for 24 hours. The antibacterial action was identified by the presence of the inhibition growth zone around the wells. The area of the entire zone (inhibition + well) subtracted from the well area provided the zone of inhibition in mm<sup>2</sup>. The bacterial strains tested were those obtained from the International Collection of Microorganisms from Plants (ICMP) - Auckland, New Zealand (WDCM 589): *Curtobacterium flaccumfaciens* pv *flaccumfaciens* (ICMP 2584); *Curtobacterium flaccumfaciens* pv *betae* (ICMP 2594); *Curtobacterium flaccumfaciens* pv *oortii* (ICMP 2632) and *Clavibacter michiganensis* pv *michiganensis* (ICMP 2550); in addition those

obtained from the collection of the Molecular Plant Pathology Laboratory at the University of Florence: *Escherichia coli* DH5 $\alpha$ ; *Xanthomonas phaseoli*; *Serratia marcescens*.

## 2.5 Antifungal Assay for the Extract and Nanozno

The evaluation of the antifungal activity was performed according to the method suggested by Khaldi, Daami-Remadi, Hamada, Somai, & Cherif (2015) with modifications, to verify the effect of seriguela residue extract and nanoZnO on the growth of phytopathogenic fungus *Botrytis cinerea* (from the Molecular Plant Pathology Laboratory's collection). A 7 mm diameter mycelial plug of this actively growing fungus was cut aseptically with a sterile cork borer and placed 2 cm away from a well containing 50  $\mu$ l of extract (without dilution) or nanoZnO (1 and 3% dispersed in sterile water) in the PDA medium (Potato Dextrose Agar) previously prepared and solidified in a sterile and disposable Petri dish of 90 mm. The control plate consisted of the same scheme, but without the presence of extract or nanoZnO into the well. The plates were then incubated at  $26 \pm 1^\circ\text{C}$  for 7 days to evaluate the inhibition of fungal growth, measured by the percentage reduction in colony size (circular area) in the presence of nanoZnO and extract with respect to the control.

## 3. Results and Discussion

Through the assays performed it was possible to obtain important results regarding the inhibitory and antimicrobial activity of the seriguela residue extract and nanoZnO. These results may guide the development of new technologies for plant disease prevention avoiding large economic losses to agriculture as well as the use of pesticides for example, and as active agents for packaging in many fields especially food industry.

### 3.1 Phenolic Profile of the Seriguela Residue Extract

The compounds identified from the phenolic profile analysis (HPLC assay) of the extract obtained from the seriguela residue are shown in Table 1. The content found was expressed with respect to the flour of this residue and to the its extract. Gallic acid was the predominant compound quantitatively, followed by lower amounts of p-coumaric acid, resveratrol (-*trans*), quercetin, syringic and ferulic acid.

**Table 1** - Phenolic profile obtained from the chromatographic analysis (HPLC assay) of the seriguela residue extract (matrix).

Compounds	Content in mg.100g <sup>-1</sup> of seriguela residue flour or $\mu\text{g.mL}^{-1}$ of extract *
Gallic acid	479,93
Vanillic acid	ND
Syringic acid	5,57
p-Coumaric acid	50,26
Caffeic acid	ND
Ferulic acid	4,04
Ellagic acid	ND
Rutin	ND
Resveratrol ( <i>trans</i> -)	29,50
Myricetin	ND
Quercetin	19,35
Kaempferol	ND

\* Values reported for one sample injection; ND: Not Detected. Source: Authors (2021).

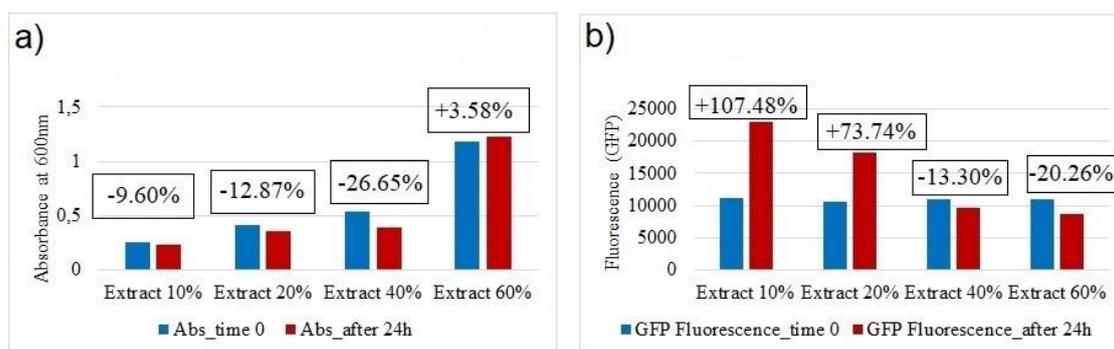
First, analysing the phenolic profile of extract, it is important to mention that the parameters of the extraction process can affect its efficiency and thus the availability of the bioactive substances – phenolic compounds (Haas et al., 2018) which may lead to qualitative and quantitative variations, being the fruit portion and its place of origin also relevant. Despite the differences in the amounts of each phenolic compound identified by HPLC and the standards used for chromatographic assay, a phenolic profile relatively similar to that found in the seriguela (fresh fruit) and in its frozen pulp by Dutra et al. (2017) was noticed, as well as in the umbu-cajá (fruit and pulp) also from genus *Spondias*. Moreover, Engels et al. (2012) previously reported the presence of phenolic compounds such as gallic acid, quercetin, rutin, and kaemperol in seriguela skin, being the first two compounds also identified in this study (Table 1).

Silva et al. (2016) identified in seriguela skin the flavonols quercetin and rutin, previously detected by Engels et al. (2012), and different compounds such as caffeic acid (not identified in the seriguela residues analyzed here). Finally, it is worth mentioning that some constituents found in the seriguela residues such as gallic acid, p-coumaric acid and quercetin have also been identified in cajá or taperebá (*Spondias mombin L.*) pulp as shown in the study by Bataglioni, Da Silva, Eberlin, & Koolen (2015), with higher prevalence for gallic acid, which shows a certain likeness with the profile of Table 1.

### 3.2 Inhibitory Activity of Seriguela Residue Extract and nanoZnO on The TTSS and QS Systems of Gram-Negative Phytopathogenic Bacteria *Pseudomonas savastanoi*

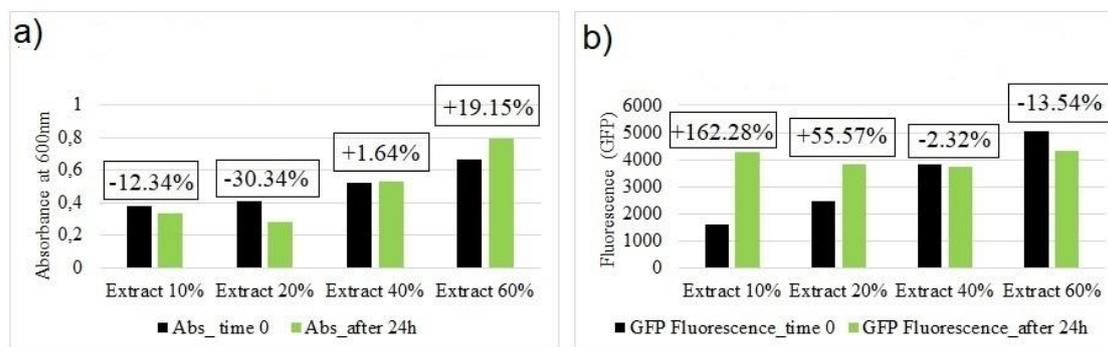
The action of the seriguela residue extract and nanoZnO on TTSS and QS systems based on the fluorescence of the GFP protein was verified as well as the antibiotic effect in each of them based on the measurement of the absorbance (600 nm) that is related to the bacterial growth. The obtained results for the inhibitory activity of the extract can be seen in Figure 1 and 2, for the QS and TTSS systems, respectively.

**Figure 1** Antibiotic (absorbance at 600nm) (a) and anti virulent (fluorescence - GFP) (b) activities of the seriguela residue extract related to the QS system of the bacterium *Pseudomonas savastanoi*.



Source: Authors (2021).

**Figure 2** Antibiotic (absorbance at 600nm) (a) and anti virulent (fluorescence - GFP) (b) activities of the seriguela residue extract related to the TTSS (T3) system of the bacterium *Pseudomonas savastanoi*.



Source: Authors (2021).

Regarding to Figure 1a, it was found that despite the reduction in bacterial growth (antibiotic effect) after 24h for the QS system, the 10 and 20% extracts were not able to reduce virulence related to Quorum Sensing as there was a relevant increase in the fluorescence of GFP protein (Figure 1b). As for the 40% extract, there was a greater reduction of bacterial growth (more than double) when compared to 10 and 20% concentrations (Figure 1a), as well as a decrease in the QS virulence system of 13.30% (Figure 1b). Comparing to the 60% extract, the reduction related to Quorum Sensing was even higher (20.26%) – Figure 1b, despite the small increase in bacterial growth – Figure 1a.

In Figure 2, when it comes to the TTSS (T3) system, it is possible to identify again that the extracts at 10 and 20%, despite being able to reduce bacterial growth – Figure 2a - (most expressively for the 20%), are not efficient to avoid pathogenicity and virulence related to the transport of proteins that can cause infections and diseases, since there was an expressive increase in GFP protein fluorescence (Figure 2b). At the 40% concentration, there was a increase of 1.64% in bacterial growth, as well as a expressionless reduction (2.32%) in the T3 system (Figure 2a and 2b, respectively). The 60% extract provided a more significant increase in the number of bacteria of 19.15% (Figure 2a), but nevertheless again reduced the virulent and pathogenic effect by a higher percentage (13.54%) than the 40% extract (2.32%), (Figure 2b).

The inhibitory activity of different compounds on systems, as for example TTSS and QS, became a new strategy to reduce bacterial pathogenicity or virulence (Biancalani et al., 2016). By take a look at TTSS/QS and phenolic profile results, it is possible to infer that these phenolic compounds (bioactives) were responsible for the inhibitory activity showed by the extract against these antibacterial pathogenic systems studied here. Yin et al. (2015), for instance, demonstrated the high potential for inhibition of green tea polyphenols in the QS system regulating the pathogenicity of *Pseudomonas aeruginosa*, including an inhibition greater than 80% in biofilm formation. Khokhani et al. (2013) identified many phenolic compounds able to act as an inhibitor in TTSS system of the plant pathogen *Erwinia amylovora*.

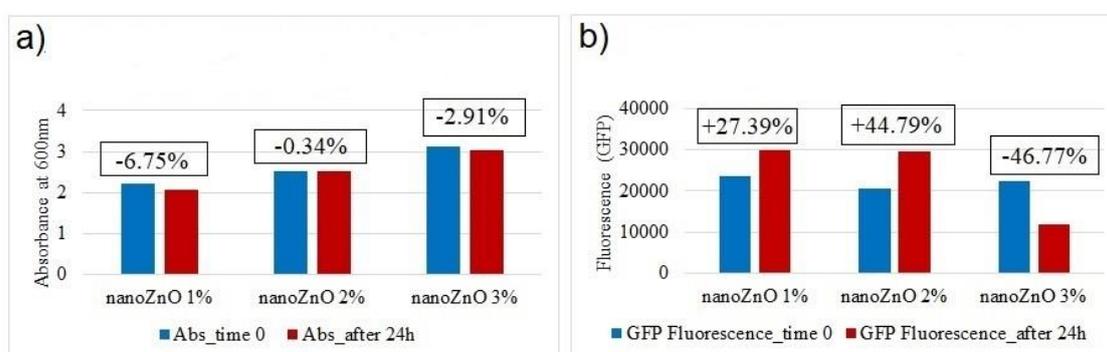
For the lower concentrations of the extract (10 and 20%) it was possible to notice that they showed antibiotic effect but were not able to reduce virulence and pathogenicity related to the QS system, instead of that there was a relevant increase. So, this indicated that the presence of the extract at these concentrations leads to an increase in communication between bacterial cells and to a better adaptation of their genetic expression to biotic and abiotic changes that may occur in the environment, considering the definition of Quorum Sensing process presented by Zhang et al. (2018) and Biancalani et al. (2016). Thus, extracts at the two lowest concentrations limited the growth, but they acted as inducers of QS system and the remaining bacteria were able to adapt and increase their virulence and pathogenicity in this system. Yang et al. (2008) identified the induction of TTSS gene expression by plant phenolic compounds for opportunistic plant pathogen *Dickeya dadantii*.

Concerning to the higher concentrations, they presented better results for the inhibitory activity. In the case of 40% extract, this concentration had significant results due to the fact that it presented both antibiotic and antivirulent effects. Although the number of bacteria has increased (even slightly), the 60% extract was the most effective in avoiding the activity of the virulence and pathogenicity system related to QS. This means that the amount of bacteria was higher in the presence of 60% extract but this increase was not able to stimulate communication between cells, on the contrary, it was reduced. Other authors also found percentages of inhibition for this system around 20% for compounds from plant sources. Zhang et al. (2018), studying the action of coumarin on the QS system of *Pseudomonas aeruginosa*, found an inhibitory effect ranging from 20 to 40% depending on the concentration of coumarin applied. Biancalani et al. (2016) identified 21% of inhibition for QS (*Pseudomonas savastanoi*) using green tea leaves extract (catechins as predominant compounds).

Similar interference behaviors in the TTSS system were identified for the seriguela residue extract, and all concentrations were able to alter T3 gene expression. Despite the antibiotic effect, the lowest extract concentrations acted as inducers of pathogenicity and virulence caused by TTSS. The highest concentrations of extract did not show antibiotic effect but they behaved as inhibitors of these systems, once again with greater effectiveness for 60% extract. Biancalani et al. (2016) identified percentages of inhibition in the T3 system of the bacterium *Pseudomonas savastanoi* higher than those mentioned in this work, 48 and 54% inhibition by analyzing grape seed and green tea leaves extract respectively.

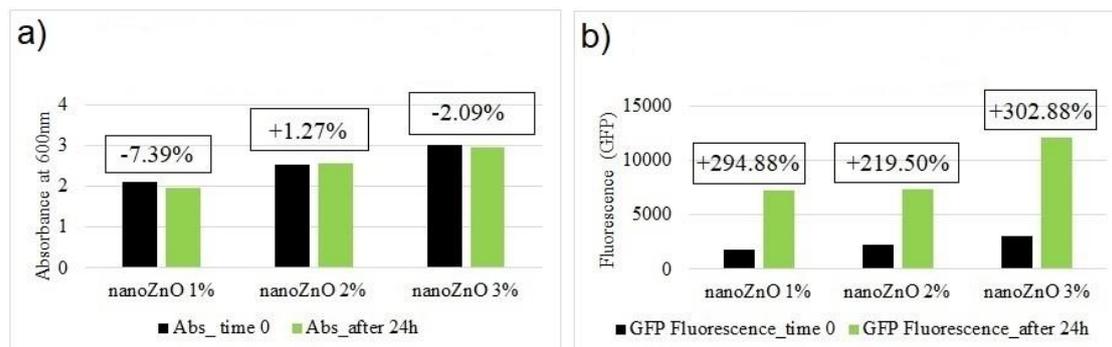
Results related to the inhibitory activity of nanoZnO can be seen in Figure 3 and 4 for both systems. Regarding to the bacterial communication system QS, all concentrations were able to reduce bacterial growth related to this system, but the concentration of 1% obtained the highest antibiotic effect (Figure 3a). However, 1 and 2% nanoZnO were not able to reduce pathogenicity or virulence through this system (Figure 3b), on the contrary, there was an increase in the action of bacteria by the Quorum Sensing system. With respect to 3% nanoZnO, this one obtained prominence to reduce in almost 50% the activation of this protein promoter of the QS system (Figure 3b). Analyzing these results, all concentrations showed slight antibiotic activity against *Pseudomonas savastanoi* which is in accordance with García-Lara et al. (2015) who mentioned that ZnO nanoparticles have a slight or no effect on bacterial growth when studied as a quorum quencher. However, the effect on QS promoter had different behaviors. NanoZnO at 1 and 2% acted as inducers of this pathogenicity/virulence bacterial system unlike nanoZnO 3%, which one was able to inhibit in half the expression of QS. Thus, nanoZnO 3% emerges as an alternative compound to control phytopathogenic diseases related to QS in future agricultural or agrifood applications.

**Figure 3** Antibiotic (absorbance at 600nm) (a) and anti virulent (fluorescence - GFP) (b) activities of nanoZnO related to the QS system of the bacterium *Pseudomonas savastanoi*.



Source: Authors (2021).

**Figure 4** Antibiotic (absorbance at 600nm) (a) and anti virulent (fluorescence - GFP) (b) activities of nanoZnO related to the TTSS (T3) system of the bacterium *Pseudomonas savastanoi*



Source: Authors (2021).

By analyzing the results regarding the Type III Secretion system for nanoZnO (Figure 4), it was observed that there was an antibiotic effect of 7.39% and 2.09% respectively, related to concentrations 1 and 3% (1% had a higher intensity), whereas for 2% there was a small increment of 1.27% (Figure 4a). In view of the inhibitory action to the TTSS (T3) system shown in Figure 4b, it was identified that the presence of nanoZnO at all tested concentrations led to a significant increase (above 200%) in GFP protein fluorescence. Therefore, a slight effect on bacterial growth was also observed but the most significant observation was that nanoZnO (at all concentrations) acted as an inducer of the TTSS system with relevant increasing in the values of GFP fluorescence. This indicated that the activity related to the process of transport of material that causes pathogenicity and virulence to the host cells (TTSS) was stimulated by the presence of these nanoparticles.

Zinc oxide nanoparticles have been studied as a potential antimicrobial agent, but their application against phytopathogens is a recent field (Nayantara & Kaur, 2018). Nevertheless, researchers already point to ZnO nanoparticles as a compound able to inhibit biofilm formation and production of Quorum-Sensing-dependent virulence factors in *P. aeruginosa* (Baptista et al., 2018; García-Lara et al., 2015), which is related mainly to the quorum quenching effect of the nanoparticles and not to bacteriostatic or bactericide activity (García-Lara et al., 2015). Therefore, the results obtained in the present work for the nanoZnO can bring new possibilities for nanotechnology in agrifood industry to prevent plant diseases and avoid bacterial resistance. It is important to mention that several ZnO nanoparticles parameters such as size, physicochemical properties and particle dissolution to ionic zinc interfere with inhibitory activity against pathogenicity and virulence factors (García-Lara et al., 2015).

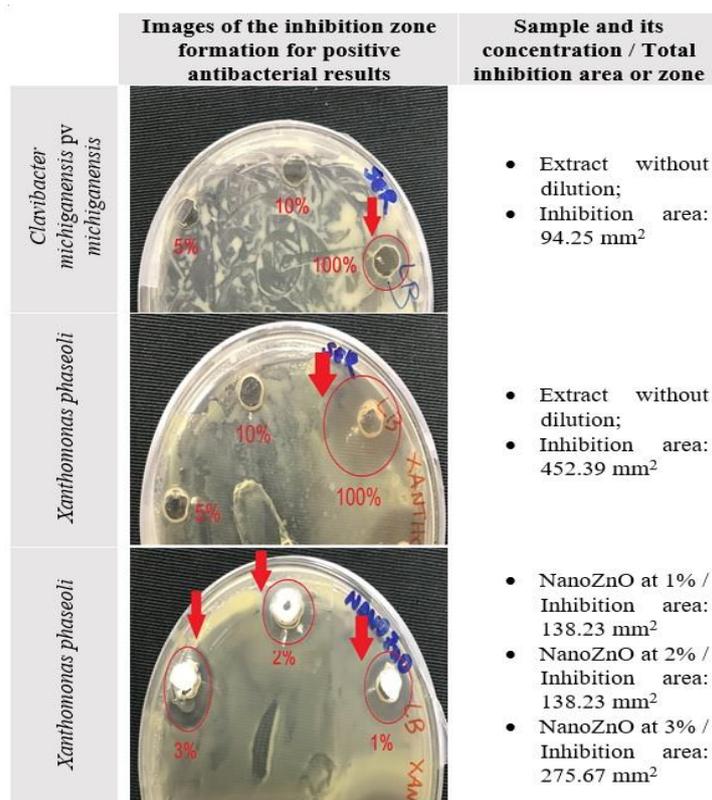
### 3.3 Antibacterial Assay for the Extract and Nanozno

The antibacterial activity of the seriguela residue extract and the ZnO nanoparticles was evaluated against strains of *Curtobacterium*, *Clavibacter*, *Escherichia coli*, *Xanthomonas* and *Serratia*.

The results showed that both seriguela residue extract and nanoZnO had no antimicrobial action (absence of inhibition zone) against strains of: *Curtobacterium flaccumfaciens* pv *flaccumfaciens* - Gram-positive bacterium, phytopathogen responsible for bacterial wilt and large production losses in bean crop worldwide (Huang et al., 2007; Osdaghi et al., 2015); *Curtobacterium flaccumfaciens* pv *beta*; *Curtobacterium flaccumfaciens* pv *oortii* - Gram-positive pathogenic bacteria for plants, specifically for beet (Chen et al., 2007); *Escherichia coli* DH5 $\alpha$  - Gram-negative bacteria that did not exhibit known pathogenic mechanisms according to Chart, Smith, La Ragione, & Woodward (2000) and are widely used for laboratory cloning (Kostylev et al., 2015); *Serratia marcescens* - Gram-negative bacteria belonging to the family *Enterobacteriaceae* and present in soils and plants (Queiroz et al., 2018).

However, the extract exhibited antimicrobial activity against the strains of *Clavibacter michiganensis* pv *michiganensis* and *Xanthomonas phaseoli*, and also the nanoZnO showed action effectiveness against this latter. Figure 5 exhibited the positive results found for the antibacterial activity of the extract and nanoZnO, characterized by the presence of inhibition zone (highlighted with red circles e pointed by arrows of the same color).

**Figure 5** Antibacterial action of extract and nanoZnO against the strains of *Clavibacter michiganensis* pv *michiganensis* and *Xanthomonas phaseoli* characterized by inhibition zone formation.



Source: Authors (2021).

Researches about the antibacterial activity of natural extracts from plant sources has been intensifying over the past few years (Steiner et al., 2017) and many polyphenols showed activity against a broad spectrum of microorganisms (Pina-Pérez & Ferrús Pérez, 2018). Furthermore, ZnO nanoparticles also showed potential use as an antimicrobial agent (Duffy et al., 2018; Kanmani & Rhim, 2014). Concerning these approaches, the research group at UFPE/UFRPE (Brazil) has explored the active properties of components from agroindustrial waste such as acerola described by (Portugal Zegarra et al., 2018), and nanoZnO (Arroyo et al., 2020; Andreina Maria Pinheiro Santos et al., 2019) in edible food coatings and films improving food preservation. Moreover, other researchers have studied applications of nanoZnO and plant extracts in active or drug delivery systems and wound treatments for medical field (Nafchi et al., 2013; Rahman et al., 2020)

By analyzing the results obtained in the antibacterial action assays, it was possible to notice that the pure extract (without dilution) showed antimicrobial action against the strain of *Clavibacter michiganensis* pv *michiganensis*. However the activity of this extract against *Xanthomonas phaseoli* was more effective since the inhibition area of this latter bacterium was four times larger than the area of the first mentioned. The bacterium *Clavibacter michiganensis* pv *michiganensis*, Gram-positive, is responsible for the bacterial disease called tomato bacterial canker that causes significant economic losses in many countries (de León et al., 2008; Hiery et al., 2013). *Xanthomonas phaseoli*, Gram-negative, is also a phytopathogen that can attack especially lima bean pods (Sharma et al., 2014). In view of the above considerations, it was possible to verify that the

extract has potential activity in a spectrum against both Gram-positive and negative phytopathogenic bacteria, with greater efficiency against Gram-negative. This was an important result because Gram-negative bacterial cells have a complex cell membrane structure (outer layer of lipopolysaccharide) that may hinder the action of the bioactive compounds present in the extract (Du et al., 2011). Nonetheless, it was noticed here that the compounds in the seriguela residue extract were able to overcome this barrier and prevent the growth of these bacteria.

Other plant extracts have also shown effective antimicrobial activity against Gram-positive and Gram-negative spectrum, such as the extract of the plant *Hypericum perforatum* in the study of Del Monte et al. (2015), in which the extract demonstrated action against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. In addition, Alkan and Yemencioğlu (2016) identified that active compounds extracted from natural plant sources (oregano, clove, artichoke stem, walnut shells) were able to act against a diversified spectrum of phytopathogenic bacteria, such as *Erwinia amylovora*, *Erwinia carotovora*, *Xanthomonas vesicatoria* and *Pseudomonas syringae*.

Furthermore, nanoZnO at all concentrations (1, 2 and 3%) exhibited antimicrobial activity against *Xanthomonas phaseoli* (Figure 5), indicating that despite the complex membrane structure of these microorganisms, these nanoparticles were able to cause the necessary damage to avoid the growth of this bacterium. The inhibition area for 1 and 2% of nanoZnO was the same, indicating same action effectiveness for both, while the increase of concentration to 3% doubled the size of inhibitory zone showing more intense antimicrobial action of nanoZnO when applied in that condition.

Thus, nanoZnO demonstrated action effectiveness against the development of Gram-negative phytopathogenic bacterium *Xanthomonas phaseoli*, in which the increasing of concentration to 3% amplified the action intensity of these nanoparticles twice. The use of nanoparticles in the control of plant diseases comes as a new and recent strategy for agricultural applications, but some researches have already demonstrated the potential use of silver and zinc oxide nanoparticles against phytopathogens, specifically fungi (Nayantara & Kaur, 2018). However, for other types of pathogens, the antimicrobial activity of nanoZnO has already been proven against a large group of microorganisms: *Salmonella typhimurium*, *Staphylococcus aureus*, *L. monocytogenes*, *E. coli*, *Bacillus subtilis*, *Enterobacter aerogenes* and *Campylobacter* strains (Akbar & Anal, 2014; Duffy et al., 2018; Esmailzadeh et al., 2016; Shankar et al., 2015).

Although there was no antibacterial activity against *Serratia marcescens*, neither through nanoZnO nor extract, this can be seen as a positive result from the agricultural point of view. According to X. Wang et al. (2018), this bacterium is already widely present in soils and has been used in bioremediation of soils contaminated with fungicides and pesticides, and in the removal of heavy metals from the environment, especially the strains that produce a red pigment (like those used in this study) which also have antibacterial, antifungal and antiprotozoal properties against organisms that cause pathogenicity to soil, plant and crops (Queiroz et al., 2018). These endophytic bacteria can contribute to the development of the plant, availability of nutrients, production of phytohormones and nitrogen fixation, besides act as a biological control (Devi et al., 2016) and bioherbicides (J. Yang et al., 2015).

### 3.4 Antifungal Assay for the Extract and Nanozno

The antifungal activity of the extract and nanoZnO in this research was evaluated with respect to the phytopathogenic fungus *Botrytis cinerea*. Regarding the extract obtained from the seriguela residue and the nanoparticles of ZnO, it was verified that both showed antifungal action against the mentioned microorganism.

The ZnO nanoparticles at concentrations of 1 and 3% obtained the same intensity of action, exhibiting the same reduction percentage of 33.08% concerning to the control colony. This value was almost double the percentage of reduction presented by the seriguela residues extract (pure, without dilution) that was 17.38%, being nanoZnO more effective than the

extract. *Botrytis cinerea* is the fungus that causes gray mold in more than 200 plant species around the world, which generates many losses in the production of fruits and vegetables (Ma et al., 2019).

Nayantara and Kaur (2018) reported that zinc oxide nanoparticles have recently been studied as effective fungicides for phytopathogens, especially pathogenic post-harvest fungi *Botrytis cinerea* and *Penicillium expansum* as documented by He, Liu, Mustapha, ND Lin (2011), which found percentages of inhibition ranging from 63 to 80% for *Botrytis cinerea*, a value higher than that found in the present study (33.08%). This difference may possibly be related to the assay methodology applied in the two studies. Since, although the present study has applied higher nanoZnO concentrations than ones used by He et al. (2011), the water-dispersed nanoZnO was possibly not able to diffuse well through the medium because over the incubation period the mixture dried and the nanoZnO remained solid and stuck inside the well. However, in the work of He, Liu, Mustapha and Lin (2011) the nanoparticles were dispersed directly into the liquid culture medium, thus obtaining a larger distribution along the whole plate after the medium solidification, and then a fungus disk or plug was placed on the center of the plate.

Dimkpa, McLean, Britt & Anderson (2013) demonstrated a significant antifungal action of nanoZnO against another phytopathogenic fungus: *Fusarium graminearum*. Lastly et al. (2018) reported that the mechanism used by the nanoparticles is possibly linked to cell wall damage and to the collapse of fungal hyphae.

#### 4. Conclusion

The results highlighted that both extract and nanoZnO demonstrated, although to different extent, inhibition against the promoters of the bacterial pathogenicity and virulence systems studied. The seriguela residue extract was able to partially inhibit the activity of both systems (TTSS and QS) of Gram negative phytopathogenic bacterium *Pseudomonas savastanoi*, whereas nanoZnO exhibited greater effectiveness in reducing promoter activation for the QS system. As for antibacterial activity, our findings indicated a spectrum of action against both Gram-negative and Gram-positive phytopathogenic bacteria for the extract, while nanoZnO proved to be more effective against the development of Gram-negative bacteria. Regarding antifungal action, both compounds were able to inhibit the growth of phytopathogenic fungus, being nanoparticles the most potent. Possibly the inhibitory, antibacterial and antifungal actions of the seriguela residue phenolic extract were promoted due to the presence of gallic acid, p-coumaric acid, resveratrol and quercetin.

Given the above, both seriguela residue extract and nanoZnO have shown great potential for application in the agro-industry in order to avoid economic losses resulting from diseases caused by bacteria and fungi. Also, as active agents in films and coatings for food packaging, both due to the known antioxidant activity of the extract and the antimicrobial action of these two compounds (extract and nanoZnO), that together (additive and synergistic effects) may improve the active property of these materials. Thus, great attention should be given to these compounds which can have a variety of applications. For future researches, the application of these compounds is recommended in order to verify their practical action (as new technologies for plant disease prevention and as active substances for food films/coatings). In addition to carrying out new tests with different concentrations and combinations of the active agents studied here.

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