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Research Article

Identification of Fungal Pathogens of Mango and Soursop Fruits Using Morphological and Molecular Tools and Their Control Using Papaya and Soursop Leaf and Seed Extracts

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Fruit and vegetable products are susceptible to the attack of fungi during postharvest handling. Chemical fungicides are the most commonly used technique to control fungal diseases. However, an alternative product is the use of plant extracts, which have been reported in *in vitro* and *in vivo* conditions. The objective of this investigation was to identify one of the main pathogens of mango and soursop fruits using morphological and molecular tools as well as to evaluate the *in vitro* inhibitory effect of papaya and soursop leaf and seed extracts. Two pathogens were isolated and identified by their morphological and molecular characteristics from mango and soursop fruits. We obtained extracts from leaves and seeds of soursop and papaya using five solvents of increasing polarity (hexane, acetone, ethanol, methanol, and water) through the ultrasound-assisted extraction technique at a frequency of 35 kHz and 160 W for 14 min. *In vitro* evaluations of the extracts were performed using the Kirby–Bauer technique. The extracts with the highest percentage of inhibition were analyzed qualitatively and quantitatively using standardized techniques of colorimetry and spectrophotometry. Furthermore, we determined the content of total phenols, flavonoids, alkaloids, terpenoids, anthraquinones, coumarins, and saponins. As a result, we identified the pathogens as *Colletotrichum fructicola* and *Nectria haematococca*. Aqueous extracts (water as a solvent) showed a higher percentage of inhibition of both pathogens compared with the other extracts. Furthermore, the aqueous extract of papaya leaf was the most effective among all extracts. The aqueous papaya leaf extract exhibited a percentage of inhibition of 49.86% for *C. fructicola* and 47.89% for *N. haematococca*. The aqueous extracts of papaya leaf and seed (AqEPL and AqEPS) presented the greatest amount of metabolites (except anthraquinones and coumarins). The aqueous soursop leaf extract (AqESL) presented the greatest amount of phenols, tannins, and flavonoids (219.14 ± 8.52 mg GAE/L, 159.84 ± 10 mg GAE/g dm and $0.13 \pm 1.12 \times 10^{-4}$, respectively). The aqueous soursop seed extract (AqESS) had the highest saponin content with 1.2 ± 0.1 mg QSES/g dm and the papaya leaf accusative extract (AqEPL) had the highest alkaloid content ($6.413 \pm 1 \times 10^{-3}$ mg AE/g dm) compared with the other extracts. The AqESS had a lower content of secondary metabolites (sterols, alkaloids, and saponins), while AqESL showed no presence of alkaloids and coumarins.

1. Introduction

The postharvest losses of fruits and vegetables can be up to 50%, and diseases caused by fungi represent up to 70% of the total losses [1, 2]. Fruits (especially the tropical ones) are susceptible to the attack of pathogenic fungi during postharvest storage, such as species of *Colletotrichum*, *Fusarium*, *Botrytis*, *Rhizopus*, *Penicillium,* and *Phytophthora* [3, 4]. Several fungi have been identified that infect soursop (*Annona muricata* L.) fruits, such as *Aspergillus flavus*, *Aspergillus niger*, *Botryodiplodia theobromae*, *Colletotrichum* sp., *Fusarium solani*, *Mucor* sp., *Penicillium chrysogenium*, *Penicillium* sp., and *Rhizopus stolonifer,* among others [5]. On the other hand, *Colletotrichum gloeosporioides* (anthracnose disease), *Alternaria alternata* (black spot disease), and *Lasiodiplodia theobromae* (stem rot disease) are the most common pathogens that attack mango fruit during postharvest, leading to low fruit quality and severe economic losses [6].

Fungal diseases are usually controlled by chemical fungicides. Improper handling of these products has resulted in environmental pollution and the development of resistance by the organism [7] which has led to the emergence of alternative products such as the use of plant extracts. These natural products have been obtained using different solvents based on the polarity of the solute of interest [8].

Plant extracts contain a large number of bioactive compounds with biological activity that are classified into three main categories: terpenes and terpenoids (approximately 25,000 types), alkaloids (approximately 12,000 types), and phenolic compounds (approximately 8,000 types) that are synthesized by four routes: shikimic acid route, malonic acid route, mevalonic acid route, and nonmevalonate route (MEP) [1, 9]. Sathya et al. [10] reported that flavonoids, alkaloids, steroids, terpenoids, saponins, phenolic compounds, and other secondary metabolites are present in different parts of the plant: leaves, stems, roots, inflorescences, flowers, fruits, and seeds.

The use of plant extracts has been widely reported *in vitro* and *in vivo* for the control of fungi during the postharvest handling of fruit and vegetable products. Kator et al. [11] tested *in vivo* (tomato fruits) an aqueous extract of Moringa leaf against *A. flavus*, *Penicillium waksmanii*, *B. theobromae*, *Fusarium oxysporum*, and *Colletotrichum asianum*. These researchers reported that the aqueous extract of Moringa leaf has antifungal potential and can increase shelf life, as well as maintaining the quality of tomato fruits during storage. Ochoa et al. [12] performed *in vitro* evaluations of methanolic extracts of lime leaves (*Shinus molle*), chirimoy*a (Annona cherimola*), tabaquillo (*Nicotiana glauca*), and cinnamon bark (*Cinnamomum zeylanicum*) on the mycelial growth and sporulation of *F. oxysporum*, *Fusarium culmorum*, and *F. solani*, reporting that cinnamon and chirimoya extracts affected mycelial inhibition and sporulation of *F. oxysporum*, *F. culmorum*, and *F. solani*. Likewise, Butia et al. [13] evaluated the *in vitro* and *in vivo* antifungal activity of 42 plant extracts from leaves, buds, rhizomes, bulbs, seeds, and fruits with different solvents against the anthracnose of the banana (*Colletotrichum musae*). These authors concluded that the rhizome methanolic extract of *Zingiber officinale* can be used as an effective alternative for the control of postharvest banana anthracnose. Bautista-Baños et al. [14] evaluated the *in vitro* and *in vivo* antifungal activity of aqueous extracts of leaves and stems of *Achras sapota*, *Annona reticulata*, *Bromelia hemisphaerica*, *Carica papaya*, *Citrus limon*, *Chrysophylum cainito*, *Dyospiros ebenaster*, *Mangifera indica*, *Persea americana*, *Pouteria sapota*, *Spondias purpurea*, and *Tamarindus indicus* from the state of Morelos, Mexico against *C. gloeosporioides* in mango and papaya fruits in postharvest handling. The authors reported that the aqueous leaf extract of *C. limon* and *P. americana* completely inhibited the *in vitro* development of *C. gloeosporioides*. The *in vivo* results showed that the leaf and stem extracts of *D. ebenaster* had fungicidal effects on mango fruits, and the leaf extract of *C. papaya* completely inhibited decay in papaya fruits.

Akhila and Vijayalakshmi [15] performed a phytochemical profile of the aqueous extract of papaya leaf using the liquid chromatography-mass spectroscopy (LC-MS) technique, identifying 21 compounds: tocopherol, ascorbic acid, carpaine, deoxykaempferol, kaempferol, deoxyquercetin, quercetin, dicoumarol, coumaroylquinic acid, coumarin, folic acid, cystine, homocysteine, cysteine sulphoxide, L-glutamic acid, *p*-coumaroyl alcohol, dimethoxy phenol, umbel, ferone, phenylalanine, caffeoyl alcohol, and methyl nonyl ketone. Another constituent of greater importance in papaya leaves is latex, which seems to be responsible for the antifungal action due to the chitinase content, which has shown high antifungal activity according to biochemical assays [16].

Extracts and bioactive compounds are commonly obtained by conventional methods such as maceration, hydrodistillation, pressing, decoction, infusions, percolation, and extraction through soxhlet. Nevertheless, these methods are time-consuming, high energy supply, and require a large number of solvents, leading to a low yield [17]. Therefore, more efficient extraction techniques have been utilized considering the yield and economical and environmental conditions. Taking this into account, safe and nontoxic solvents for plant extractions, such as water, carbon dioxide, and ethanol, have been used [9, 17].

Water is the safest and most environmentally friendly solvent in different separation processes [18]. Among the modern and sustainable techniques, the ultrasound-assisted extraction (UAE) is a green technology that allows the extraction of bioactive compounds efficiently [19, 20].

UAE is carried out in a short time, which facilitates the recovery of thermosensitive compounds [19]. The UAE process is capable of breaking cell membranes and walls, allowing greater solvent penetration into the matrix, reducing solvent consumption in combination with agitation and/or heat [20, 21].

The efficiency of the UAE is generated by the effects of cavitation, which results from the creation, growth, and implosion of gas bubbles, which collapse when high pressures and temperatures occur, causing microfractures in the materials being cavitated. Moreover, frequency, time, and

acidity also usually affect the recovery of the compounds [22]. Based on the previously mentioned, the objective of this investigation was to identify one of the main pathogens of mango and soursop fruits using morphological and molecular tools as well as to evaluate the *in vitro* inhibitory effect of papaya and soursop leaf and seed extracts.

2. Materials and Methods

2.1. Plant Material. Fruits at physiological maturity and juvenile leaves of papaya cv. Maradol were collected in Ejido La Libertad, San Blas, Nayarit (21°32′23″N, 105°17′8″ W; 220 mamsl). On the other hand, soursop fruits at physiological maturity and juvenile leaves were collected in Ejido El Tonino, Compostela, Nayarit (21°14′N, 104°54′W; 301 mamsl). Plant material was transported to the laboratories of the Food Technology Unit of the Autonomous University of Nayarit. Fruits and leaves were washed with distilled water to remove traces of any foreign material that could be found.

2.2. Pathogens

2.2.1. Isolation of the Pathogens. Pathogens were isolated from soursop and mango fruits at physiological maturity, without visible mechanical damage or signs of disease. Fruits were washed, disinfected, and then incubated at 28°C and HR ≥90%. Once the fruits showed signs of disease, segments of necrotic cuticular tissue and unaffected tissue were taken and treated with a 1% sodium hypochlorite solution for 3 min, washed with sterile distilled water, and then placed in the center of Petri dishes with potato dextrose agar (PDA). Petri dishes were incubated for seven days with daily observations of color, texture, and colony formation [23]. Frequent reisolations were performed to preserve the purity of the strains. The pure isolates were grown in PDA, incubated at 28 ± 2°C for six days, and then stored at 4°C until further use. Eight days before the start of the bioassay, these isolates were grown in fresh PDA.

2.2.2. Morphological Identification. The morphological identification was carried out through the use of dichotomous keys [24]. Five to ten microcultures were performed with PDA medium on a slide, incubated at 28 ± 2°C (RH >95%) for six days, and then observed on a Motic model BA310 microscope (Motic, British Columbia, Canada) at 40x to record the structure of the pathogens.

2.2.3. Molecular Identification

(1) Genomic DNA Extraction, PCR Amplification, and Sequencing. Segments of purified mycelium were placed in 20 mL of Broth-Potato-Dextrose medium. The pathogens were incubated on a mechanical shaker (Orbital Shaker OS-200, China) for four days at 150 rpm at room temperature. Genomic DNA was extracted from the mycelium using the technique reported by Allers and Lichten [25].

Magnetic beads and 700 μL of the CTAB extraction buffer were added to the mycelium, incubated for 1 h at 65°C in an Accublock (Labnet®, USA), and then stirred in a vortex for 10 min. Subsequently, 700 μL of chloroform-octanol (24 : 1 v/v) was added, stirred for 30 s, and centrifuged at 16,000 × g using a Mini Spin centrifuge (Eppendorf®, Germany) for 10 min. CTAB buffer was added in 1 : 10 ratio to the supernatant, mixed for 30 s and then an equal volume of chloroform-octanol solution was added. Next, an equal volume of cold isopropyl alcohol (<−20°C) was added and centrifuged at 16,000 × g for 15 min at 4°C. The supernatant was removed and the pellet was washed with 750 μL of 75% ethanol, mixed by inversion, and then centrifuged at 16000 × g for 3 min. Finally, DNA was resuspended with 50 μL of sterile Mili-Q water. The concentration of the DNA was determined in a spectrophotometer (Biotek®, USA) with the absorbance ratios A260/A280 nm and A260/A230 nm.

The molecular test was performed to identify the pathogens. The polymerase chain reaction (PCR) was used to amplify the ITS-5.8S region of the rDNA using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-CCTCCGCTTATTGATATGC-3′) [26, 27]. PCR was performed in a T-100 thermocycler (Bio-Rad, California, USA) under the following conditions: initial denaturation at 94°C of 5 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 10 min. The amplification products were separated by 1% agarose gel electrophoresis at 80 V for one h. The gel was visualized in the transilluminator Benchtop UV and a PhotoDoc-it system was used for the capture of the images (Laboratory Equipment, California, USA). PCR products were sequenced by Macrogen Humanizing Genomics (Seoul, Korea). The nucleotide sequences were compared with the NCBI (National Center for Biotechnology Information) database using the BLAST tool. Then, we constructed a phylogenetic tree with the MEGA 7.0 software using the Neighbor-Joining method with a Bootstrap analysis of 1000 repetitions.

2.3. Plant Extract from Soursop and Mango. The plant material (seeds and leaves) was stored at −80°C in a Thermo Scientific freezer, model ULT 1.3-86-3-A41, LLC (USA) and then lyophilized in LABCONCO Free zone 2.5 (Kansas City, USA) at −45°C/0.020 mBar for 24 and 40 h for leaves and seeds, respectively. After, the plant material was crushed in a mill with Krups steel blades model GX4100 (Germany). The resulting powder was stored at −20°C until further use. 100 mL of each of the solvents of increasing polarity (hexane, acetone, ethanol, methanol, and water) were mixed with 20 g of plant material (soursop and papaya leaf and seed) to obtain the extracts. Later, the mixture was sonicated in a Luzerner® model CD-4820 ultrasonic bath at 35 kHz and 160 W for 14 min. Once the sample was sonicated, the supernatant was filtered under vacuum (20 Torr) using Whatman No. 1 paper. Next, the solvent was removed in a rotary evaporator (IKA RV 10) at a temperature of ≤35°C, 110 rpm and a vacuum of 20 Torr [26].

The extracts were deposited in amber bottles and taken to a drying chamber (MMM VC 55 STD) for 72 h at a temperature below 35°C to evaporate the solvent residues and then stored at −20°C until further use. The aqueous

extracts of papaya and soursop leaf and seed were centrifuged at 9 000 rpm for 15 min, at −4°C in a HERMLE® centrifuge model Z326K (Wehingen, Germany) and then filtered through Whatman filter paper Nos. 1 and 5. The aqueous extracts were stored at −80°C and then lyophilized in the LABCONCO Free zone 2.5 at −45°C/0.420°mBar. The nomenclature used for the identification of plant extracts was as follows.

The first letter refers to the extraction solvent (H = hexane, A = acetone, E = ethanol, M = methanol and Aq = aqueous), the second letter means that is an extract (E), the third letter denotes the source from where the extract was obtained (P = papaya and S = soursop), and the last letter signifies the part of the plant from which the extract was obtained (L = leaf and S = seed). According to this, the nomenclature to identify the extract using hexane as a solvent from papaya leaf is HEPL.

2.4. In Vitro Test. We performed an *in vitro* test to evaluate the antifungal effect of the 20 extracts against the pathogens. The test was performed according to the well technique in the modified Kirby–Bauer agar [28]. 0.3 g of the extracts was resuspended in 1 mL of DMSO (dimethyl sulphoxide) ACS Fermont® [29] and stirred in a vortex for 1 min. We performed four wells of 6 mm diameter with a punch (a well in the center of the Petri dish and the remaining three wells were located equidistantly around the plate) in a 90 mm polystyrene Petri dish with PDA. In the central well, a 6 mm segment of mycelium of the pathogens was placed and in the rest of the wells, 100 μL of each of the crude and dissolved extracts in DMSO was placed. The positive control was used with the concentration recommended by the manufacturer (625 g·100·l^{-1} for pear) of a commercial fungicide with the following active ingredients: streptomycin sulfate, oxytetracycline hydrochloride and copper oxychloride, a DMSO control (reagent grade) and negative control (no extract and no fungicide).

The mycelial growth of the pathogens was measured for eight days after 48 h of sowing. The measurements were carried out every 24 h and the treatments were incubated at 28 ± 2°C. The clear areas that formed around the plant extract were considered as indicative of the antifungal activity of the extract. The percentage of mycelial inhibition of the extracts was obtained by the formula proposed by Ozgonen and Gulcu [30]:

$$I = \frac{(\mathrm{GR1} - \mathrm{GR2})}{\mathrm{GR1}} * 100, \quad (1)$$

where I = percent inhibition, GR1 = control radial mycelial growth, GR2 = treatment radial mycelial growth.

Likewise, for the report of the mycelial growth of pathogens in the initial, intermediate, and final stages, basic mathematical formula (equation (2)) for an irregular figure was used and expressed in mm^2:

$$\pi * \frac{0.5 * D}{2}, \quad (2)$$

where D = diameter of mycelial growth of the pathogen.

Qualitative and quantitative phytochemical analyses were carried out on the extracts that presented a greater inhibition against the pathogens.

2.5. Qualitative Chemical Analysis of Secondary Metabolites. 0.3 g of lyophilized plant material (aqueous extracts of papaya and soursop leaf and seed) was diluted in 1 mL of deionized water (stock solution). An aliquot of 300 μL of the extracts (papaya and soursop leaf and seed) was used. Standardized procedures were used for the detection of phenols, tannins and flavonoids (ferric trichloride), alkaloids (Mayer's reagent), steroids (Liebermann–Burchard), saponins (foam production), anthraquinones, and coumarins, using the Sofowara [31], Harborne [32], and Evans [33] methodologies. For the description of the trials, the crossing system was used to specify the presence or absence of secondary metabolites present in leaf and seed. The analyses were performed in triplicate.

2.6. Quantitative Chemical Analysis of Secondary Metabolites. 0.1 g of lyophilized plant material (aqueous extracts of papaya and soursop leaf and seed) was dissolved in 2 mL of distilled water (stock solution). The quantification of secondary metabolites (total phenols, nontannin phenols, flavonoids, total saponins, and alkaloids) was performed by standardized spectrophotometric techniques in a ThermoFisher Scientific™ Multiskan™ GO model 1510 microplate reader (Ov, Vantaa Finland). The analyses were performed in triplicate.

2.6.1. Total Phenols. The quantification of total phenols was carried out using the methodology proposed by Maksimović et al. [34] using the Folin–Ciocalteu reagent. The absorbance of the final solution at 725 nm was measured. Gallic acid was used for the calibration curve (Sigma-Aldrich, China). The results were expressed in mg GAE·g^{-1} dm.

2.6.2. Total Tannins. The content was determined according to the Folin–Ciocalteu method described by Maksimović et al. [34]. In order to perform this, we measured the absorbance of the final solution at 725 nm. A calibration curve was performed with gallic acid (Sigma-Aldrich, China). The results were expressed in mg GAE·g^{-1} dm. The total tannin content was calculated as follows:

$$\text{total tannins} = \text{total phenols} - \text{nontannin phenols}. \quad (3)$$

2.6.3. Flavonoids. The methodology proposed by Maksimović et al. [34] based on the reaction of aluminum trichloride ($AlCl_3$) with the flavonoids present in an alkaline medium was used. The absorbance was recorded at 430 nm. The flavonoids were expressed as routine equivalent in a calibration curve of a standard routine solution expressed as mg RE·g^{-1} dm.

2.6.4. Total Saponins. The analysis was carried out with the methodology proposed by Hernández et al. [35] using the DNS test (3.5 dinitro-salicylic acid). The sample was

hydrolyzed and placed in a water bath until reaching 60–70°C. Then, 3 mL of HCl was added and kept under these conditions for 15 min. We stopped the reaction with an ice bath and then the pH was adjusted to 6.5–7.2. Next, it was adjusted to a volume of 10 mL with distilled water (sample A). The same procedure was repeated without adding HCl (sample B). From samples A and B, an aliquot of 0.5 mL was taken and 0.5 mL of DNS was added to each one and then subjected to a water bath at 100°C for 5 min. The reaction was stopped with a cold-water bath and simultaneously 5 mL of distilled water was added. The samples were allowed to stand until they reached room temperature. An aliquot of 300 μL aliquot was taken from each sample, and its absorbance and sugars were measured by the DNS method proposed by Miller (1959) [35]. The absorbance was read at 540 nm, and the results were expressed as mg QSES·g $^{-1}$ dm. The calibration curve was performed using *Quillaja saponaria*.

2.6.5. Alkaloids. Total alkaloids assay was performed following the methodology proposed by Shamsa et al. [36], which is based on the reaction of the alkaloid with bromocresol green (BCG), reading at maximum absorption of 470 nm. The extract was dissolved in 2N HCl (1 : 1 v/v) and then we performed three washes with chloroform and the sample was adjusted to neutrality. Next, 5 mL of BCG and 5 mL of phosphate buffer with a pH of 4.7 were added. The mixture was stirred and the alkaloid complex was extracted with 1, 2, 3, and 4 mL of chloroform. The yellow complex was recovered, and chloroform was added until a final volume of 10 mL. An aliquot of 200 μL aliquot was taken and then the absorbance was measured. The results were expressed as mg AE·g^{-1} MS. The calibration curve was performed using a standard atropine solution.

2.7. Statistical Analysis. The *in vitro* test was carried out under a completely randomized design with a $4 \times 5 \times 2$ factorial arrangement including the controls (with fungicide, without fungicide, and without extract and with DMSO reagent). The data obtained were analyzed by analysis of variance (ANOVA) using the Tukey tests with an $\alpha = 0.05$. The analysis was performed using the SAS statistical package version 9.2.

3. Results and Discussion

3.1. Identification of Pathogenic Fungi

3.1.1. Morphological Identification

(1) Colletotrichum fructicola. The pathogen presented a circular shape with concentric rings, abundant cottony texture, and concentric reliefs at the macroscopic level. On the aerial view of the culture, a white-grayish tone was observed with relief in the center and a white tone on the periphery. On the back of the Petri dish, it was seen that the colony had a creamy white color in the center, with a concentric gray and white ring on the periphery. This isolate showed rapid growth, covering the total of the Petri dish in eight days (Figures 1(a) and 1(b)). *C. fructicola* presented elongated conidia with rounded ends of an average size of 11.29 μm × 3.41 μm ($n = 50$) at the microscopic level, as shown in Figure 1(c) (A). We also found appressories with ovoid shape in small groups (Figure 1(c) (B)), setae (Figure 1(e)) acervuli, and formation of conidiophore (Figures 1(f) and 1(g)). The morphological characteristics coincide with those observed by Fuentes-Aragón et al. [37] in avocado fruits from the central part of Mexico. On the other hand, Lima et al. [38] studied five species of *Colletotrichum* infecting mango in Brazil. These authors indicate that *C. fructicola* showed no conidia, while in the present study these structures were observed.

Prihastuti et al. [39] reported *C. fructicola* for the first time in Thailand isolated from coffee cherries (*Coffea* sp.) and peanut leaf spots (*Arachis*). In Brazil, the pathogen *C. fructicola* was also reported in mango by Viera et al. [40] and also it has been reported in Asia, Africa, and America in various hosts as the cause of anthracnose [41]. Likewise, Fuentes-Aragón et al. [37] confirmed that *C. fructicola* was previously reported as *C. gloeosporioides* and then it was reclassified. More than one species of *Colletotrichum* can affect a single plant based only on the morphology of the pathogen becomes a problem due to the high morphological similarity among species such as *C. siamense* and *C. fructicola*, species that have been closely related and morphologically similar. Therefore, the importance of molecular characterization of *Colletotrichum* species [42].

(2) Nectria haematococca. This pathogen presented a circular shape with concentric rings of cottony and abundant texture at the macroscopic level. In the front of the Petri dish, a reddish-purple center was observed after 48 h of growth, which changed to a white-brown tone and a cotton-white tone on the periphery after the third day. On the back of the Petri dish, we observed a dark brown tone in the center of the Petri dish with a yellow-orange tone on the periphery; the culture medium turned into a yellow-orange tone. This pathogen presented a low growth rate compared to *C. fructicola*, covering 60% of the 90 mm Petri dish in eight days (Figures 2(a) and 2(b)).

Furthermore, *N. haematococca* presented extensive mycelium septate (Figure 2(c)), crescent-shaped macroconidia with septa of an approximate average size of 3.69 μm × 0.98 μm ($n = 25$) (Figure 2(d)), microconidia with rounded to oblong ends (Figure 2(e)), and conidiophores (Figure 2(f)) at the microscopic level. The morphological characteristics described by Hanlin [43], as well as those described by Nalim et al. [44], coincide with those of the present study regarding the description of conidiophores, macroconidia, microconidia, and mycelium.

N. haematococca (also called *Haematonectria haematococca*) is commonly known by its asexual name of *F. solani*. It is the most studied species among the species that are in the group known as "Complex species of *Fusarium solani*," which includes about 50 species [45, 46]. The species of this genus can colonize a great variety of hosts of economic importance, such as cereals, ornamentals, and vegetables,

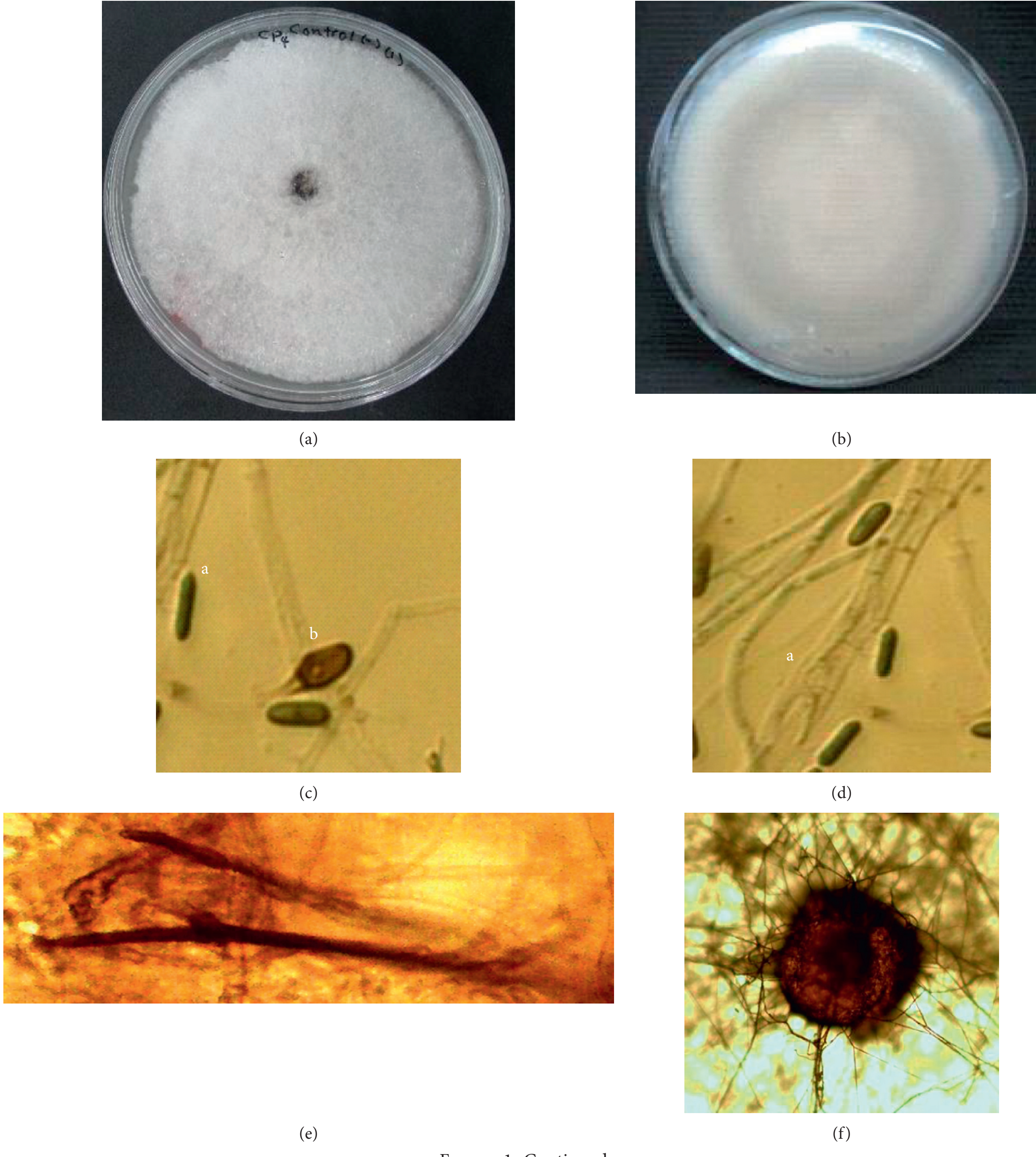
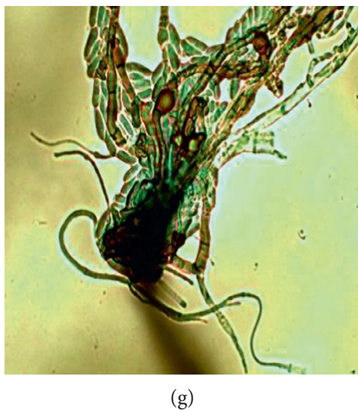


Figure 1: Continued.



(g)

Figure 1: *Colletotrichum fructicola* isolated from "Ataulfo" mango fruit. (a) Front plate. (b) Reverse plate. (c) Conidia (a) and appressories (b). (d) Mycelium (a). (e) Setae. (f) Acervuli. (g) Formation of a conidiophore.

(a) (b) (c)

(d) (e) (f)

Figure 2: *Nectria haematococca*: (a) Front plate. (b) Reverse plate. (c) Mycelium. (d) Macroconidia. (e) Microconidia. (f) Conidiophore.

being responsible for diseases such as stem and root rot, sudden death syndrome and wilting, as well as various diseases of approximately 100 different plant genera [47, 48].

3.1.2. Molecular Identification. We amplified DNA fragments of 562 bp and 530 bp as shown in Figure 3. BLAST analysis of the PCR products showed a 92.75% identity for *C. fructicola* and 95.38% identity for *N. haematococca,* respectively. We carried out a phylogenetic tree using the sequences of *C. fructicola* and *N. haematococca* to observe the degree of similarity with other species found by BLAST (Figures 4 and 5). In this sense, we found that the closest distance was observed with the microorganisms *Colletotrichum* and *Nectria* genera. Furthermore, this analysis confirmed the species identified previously. The disease

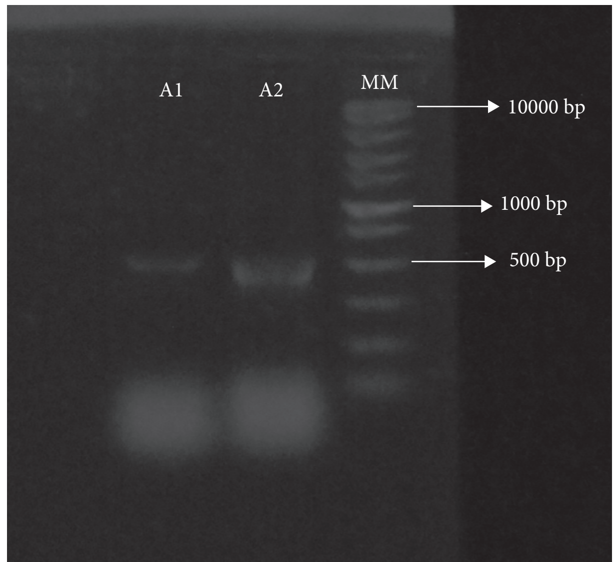


Figure 3: Amplification by PCR. MM = molecular (Fast DNA Ladder), A1 and A2 represent the amplified fragments of 562 bp and 530 bp, respectively.

caused by the different species of the genus *Colletotrichum* and its potential to infect a wide range of hosts is due to the complexity of the pathogen life cycles. These cycles are highly regulated by the information of specific genes and biochemical interactions that occur through specific enzymes and secondary metabolites produced in the host-pathogen interface [49]. On the other hand, *F. solani* can adapt to different environments, reflecting the genetic plasticity and metabolic diversity of the species. This species represents one of the most important groups of pathogens that are associated with opportunistic fungal infections [47, 48].

Various phytopathogenic genera such as *Botryosphaeria, Diaporthe, Mycosphaerella, Fusarium*, and *Colletotrichum* are difficult to identify based solely on a classification of morphotaxonomic characters [42, 50], so it was necessary to perform the molecular identification.

3.1.3. In Vitro Test. Figures 6 and 7 show the effect of the extracts on the inhibition percentage of *C. fructicola* and *N. haematococca* after eight days of incubation, respectively.

The aqueous extracts showed the highest percentage of inhibition for both pathogens.

(1) C. fructicola. The inhibitory effect of the plant extracts indicates that four statistical groups were formed. In group 1, the controls (with fungicide, without fungicide, and with DMSO reagent) and hexane extracts (HESL, HEPL, and HESS) presented a 0% of inhibition.

In the second group, the extracts HEPS, AESL, AEPL, EESL, EEPL, EESS, MESL, MEPL, MESS, and MEPS presented no significant statistical differences compared with the controls ($P < 0.05$) but showed a higher inhibitory percentage that ranged from 11.41 (EESL and MESL) to 16.78% (EEPL).

Extracts that presented a medium percentage of inhibition were located in group 3 (AESS, AEPS EEPS, and MESS). AEPS (27.57%) showed the highest inhibitory percentage in this group. Within the fourth group, there were the aqueous extracts AqESL, AqEPL, AqESS, and AqEPS, which had an important inhibitory effect, highlighting AqEPL with 49.86% of inhibition ($P < 0.0001$).

Regarding the controls, it was observed that the pathogen showed no sensitivity to the commercial fungicide, which may be because the strain evaluated has already developed a tolerance to the active ingredients of the fungicide, while DMSO induced no inhibition of the fungus. Therefore, the DMSO solvent did not interfere with the mycelial growth of the pathogens (Figure 6).

(2) N. haematococca. The results showed that three statistically different groups were formed. Among the 20 plant extracts tested against *N. haematococca*, only some of them showed inhibition (HEPS, AEPL, AEPS, EESL, EESS, EEPS, MESL, and MEPS), while the rest of them stimulated the mycelial growth of the pathogen (Figure 7).

The extracts HESL, HEPL, HESS, AESL, AESS, EEPL, MEPL, and MESL stimulated the mycelial growth of the pathogen presenting a similar behavior to the controls with commercial fungicide and the DMSO reagent. Within this group, HEPL exhibited the highest growth stimulation of the pathogen. Regarding the group of the extracts HEPS, AEPL, AEPS, EESL, EESS, EEPS, MESL, and MEPS, they displayed a similar behavior to the negative control, with an inhibition percentage from 1.5 to 6.4% for the extracts AEPL, AEPS, EESL, EESS, EEPS, and MESL while the extract with the highest inhibition in this group was HEPS with 14.5% ($P < 0.0001$). Figure 4 shows that the aqueous extracts AqESL, AqEPL, AqESS, and AqEPS had a more prominent inhibitory effect than the rest of the extracts. AqEPL presented the highest antifungal activity (47.89%) for *N. haematococca* (Figure 7).

The bioassay carried out in the present study showed that *C. fructicola* was the pathogen with the highest susceptibility to AqEPL. According to Vásquez et al. [50], this behavior can be explained by the chemical composition and the differences in the concentration of the bioactive compounds present in the extracts, which cause the difference in the response of the pathogens.

Chavez-Quintal et al. [16] evaluated the *in vitro* antifungal activity of byproducts ethanolic extracts from *C. papaya* L. cv. Maradol (papaya leaves and seeds of ripe and immature fruits) against *R. stolonifer*, *Fusarium* spp., and *C. gloeosporioides*, obtaining as a result that the papaya leaf extract was the most efficient inhibiting *Fusarium* spp. and *C. gloeosporioides* compared with papaya seed extracts. Likewise, they reported a percentage of inhibition of 24.2% for *Fusarium* and 21.8% for *C. gloeosporioides.* Additionally, these authors reported 0% of inhibition for *Rhizopus.* These results differ from those obtained in this investigation since the inhibition percentage in this study was 49.86 and 47.89% for *C. fructicola* and *N. haematococca,* respectively. Vásquez et al. [50] reported that the differential response of the pathogen to plant extracts is due to resistance mechanisms

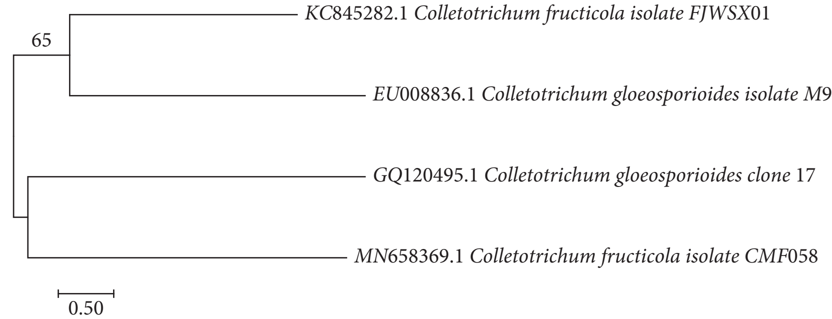


Figure 4: Phylogenetic tree from the sequence of the pathogen isolated from mango.

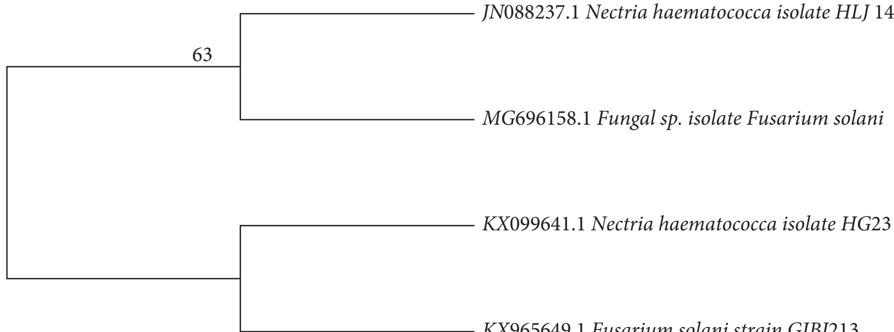


Figure 5: Phylogenetic tree from the sequence of the pathogen isolated from soursop.

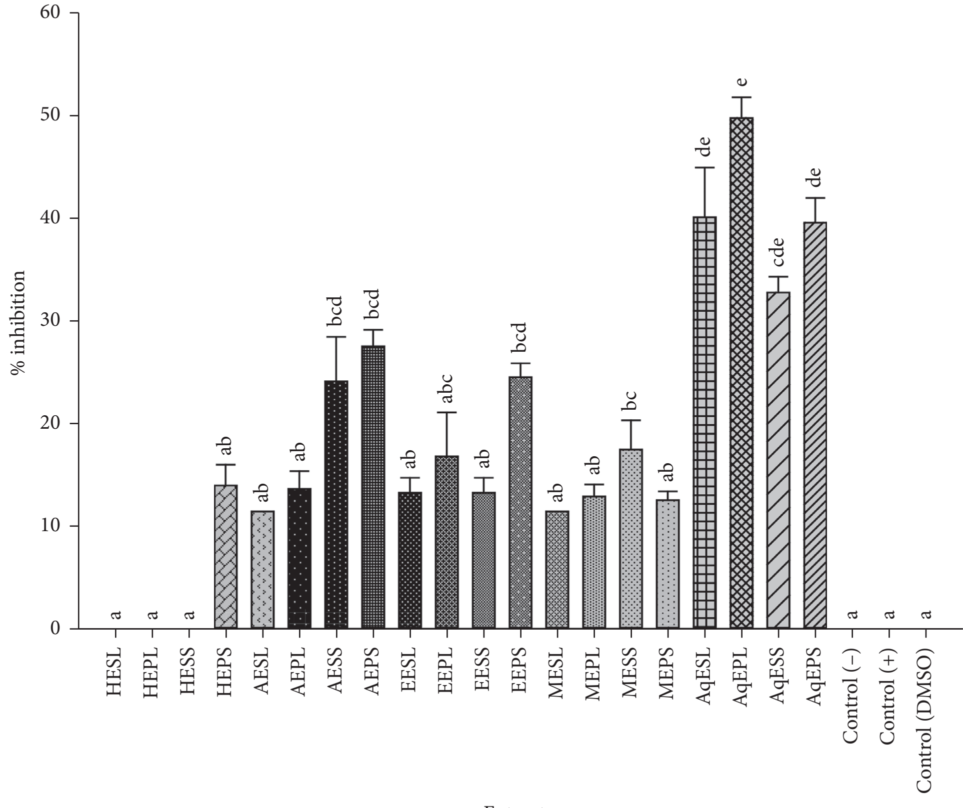


Figure 6: Inhibition percentage at day eight of incubation of the mycelial growth of *C. fructicola* in the presence of papaya and soursop leaf and seed extracts. Means with the same letter are not significantly different according to Tukey's test ($P < 0.05$).

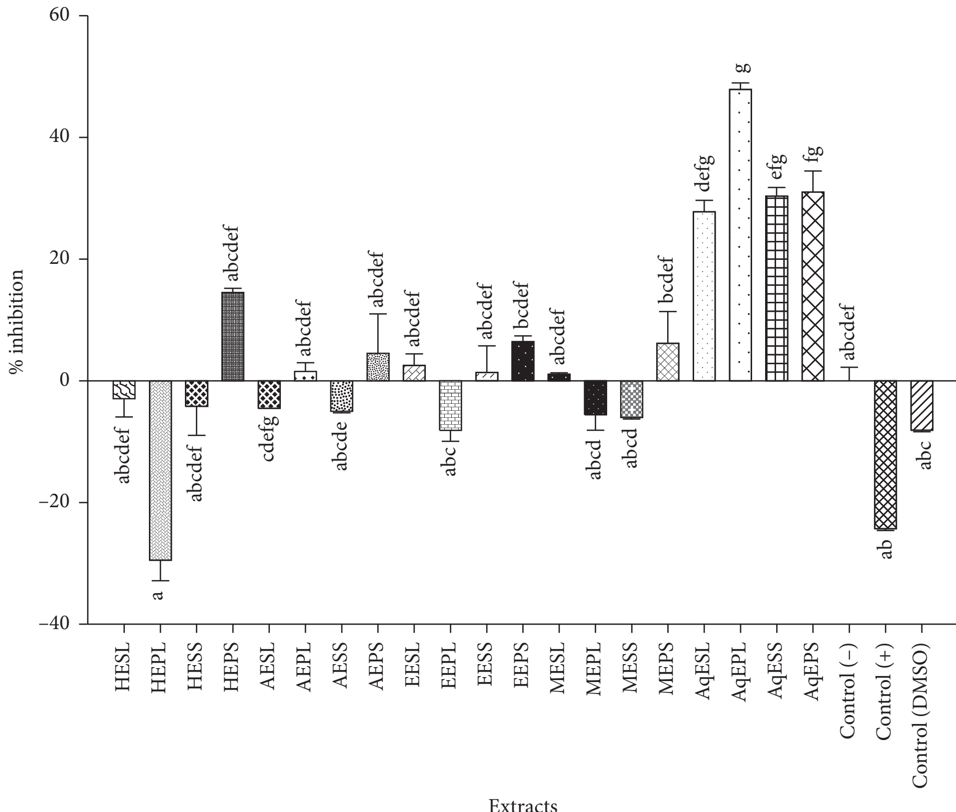


Figure 7: Inhibition percentage at day eight of incubation of the mycelial growth of *N. haematococca* in the presence of papaya and soursop leaf and seed extracts. Means with the same letter are not significantly different according to Tukey's test ($P < 0.05$).

(enzymatic, structural, change in membrane permeability, among others) which allows the pathogen to take advantage or detoxify some of the compounds present in plant extracts. They also mentioned that the susceptibility of the pathogen to plant extracts will largely depend on the species and pathogenicity of the fungus as well as the concentration of the treatment to which the organism is subjected. Alberto and Otanes [51] determined the sensitivity of three phytopathogenic fungi (*C. gloeosporioides*, *C. acutatum*, and *F. chlamydosporum*) from soursop fruits to 12 fungicides, finding that the fungicide containing cupric hydroxide as an active ingredient presented 0% of inhibition for the three phytopathogenic fungi.

The results mentioned above are in agreement with the results of the present study since the pathogens evaluated (*C. fructicola* and *N. haematococca*) were contrasted with a fungicide containing copper oxychloride as an active ingredient, obtaining 0% inhibition in both pathogens. The mentioned researchers suggest that the effectiveness of the fungicide will depend on the mode of action against the pathogen and the spectrum of the fungicide. Gharieb et al. [52] conducted a study where they investigated the activity of fungicide-tolerant fungi with copper oxychloride and the possible mechanisms involved in tolerance, observing that fungicides with a cupric formulation are poorly soluble in water and their antifungal activity will depend on their solubilization and ability to form Cu^{+2} ions. In addition, these researchers mention that the main copper tolerance mechanism developed by fungi is due to the ability to prevent the entry of copper into the cell or to reduce the accumulation of copper in the cell. Also, they mentioned that a pH reduction in the culture medium leads to a reduction in the toxicity of copper to the fungus due to the decrease in the amount of copper that is absorbed by the cell.

Regarding the growth stimulation of *N. haematococca* with the extracts HESL, HEPL, HESS, AESL, AESS, EEPL, MEPL, and MESL and with the fungicide, Oliva et al. [53] evaluated the antifungal activity of *Ruta graveolens* L. extract fractions against seven fungi, reporting that some extracts stimulated the pathogen growth. The authors attributed this behavior to the low levels of potentially toxic agents, a phenomenon known as "hormesis."

Table 1 shows the inhibitory effect of aqueous extracts on *C. fructicola* and *N. haematococca* after different days of incubation.

3.2. Qualitative and Quantitative Phytochemical Analyses. Qualitative and quantitative phytochemical analyses of the aqueous extracts of papaya and soursop leaf and seed were performed since the aqueous extracts presented a greater inhibition against the two pathogens evaluated. The soursop seed extract showed a lower amount of secondary metabolites (sterols, alkaloids, and saponins), the soursop leaf extract was negative (–) to the presence of alkaloids and

Table 1: Effect of aqueous extracts of papaya and soursop leaf and seed on the mycelial growth of *C. fructicola* and *N. haematococca* after different days of incubation.

Extracts	Mycelial growth (mm^2)					
	C. fructicola			*N. haematococca*		
	Day 3	Day 6	Day 8	Day 3	Day 6	Day 8
AqESL	9.81	22.62	30.52	6.16	12.57	23.76
AqEPL	8.55	24.34	29.87	6.61	16.38	18.86
AqESS	10.37	30.19	39.22	5.59	16.14	23.19
AqEPS	9.26	30.19	36.67	5.73	15.21	26.42
Control (+)	9.62	30.52	50.27	7.07	18.60	38.48
Control (−)	11.34	33.52	50.27	6.45	23.19	38.85
Control DMSO	8.04	30.19	50.27	6.31	19.37	28.91

coumarins, while the leaf extract (the one with the highest antifungal activity) and papaya seed presented a positive presence (+) of the majority of secondary metabolites analyzed except for anthraquinones and coumarins in leaf and seed, respectively. It should be noted that coumarins were only present in the papaya leaf extract (Table 2). Sankarganesh et al. [54] reported that papaya leaf contains potent secondary metabolites such as alkaloids, phenolic compounds, flavonoids, saponins, tannins, and glycosides, among other compounds, while Chavez-Quintal et al. [16] found that the bioactive compounds of papaya leaf extract are little known, but are a potential source of secondary metabolites with antifungal properties, because of the presence of alkaloids, flavonoids, triterpenes, and saponins in an ethanolic extract of papaya leaves.

On the other hand, coumarins (secondary metabolites produced in the phenylpropanoid route) are a type of phytoalexins of great importance in the defense of plants that inhibit the growth of phytopathogenic fungi during fruit maturity, having a close relationship of these compounds with the resistance of the plant to fungal diseases [7, 55]. Madinah et al. [56] presented results of the phytochemical analysis of the aqueous extract of papaya seed showing a greater abundance of the phenolic compounds, flavonoids, tannins, and alkaloids. Gavamukulya et al. [57] reported the content of alkaloids, terpenoids, phytosterols, phenols, flavonoids, tannins, saponins, and anthraquinones in aqueous extracts of soursop leaf, which differs from the results of this research due to the absence of alkaloids and coumarins. Coria-Téllez et al. [58] reported 212 bioactive compounds in soursop; among these compounds, phenols, alkaloids, and acetogenins were the most abundant.

Rodríguez et al. [59] indicated that secondary metabolites such as flavonoids have a high range of biological activity, including antimicrobial activity. Also, tannins are responsible for the inhibition of protein synthesis in the cell, while phenols produce the enzymatic inhibition by oxidation of the compounds. The alkaloids inhibitory effects in microorganisms are due to the ability to intercalate with DNA, stop protein synthesis, induce apoptosis, and inhibit carbohydrate metabolism enzymes. Alkaloids such as drinkine, palmatine, and sanginarin are toxic isoquinoline alkaloids that inhibit the growth of bacteria, fungi, and viruses. These alkaloids react with anionic groups and nucleophilic groups of amino acids as receptors and enzymes, inhibiting their function [60], while the mechanism of action of terpenes has not been fully elucidated. Nevertheless, it is suggested that they can cause membrane breakdown through lipophilic compounds. Saponins are nonvolatile compounds, and their primary action concerning fungi is similar to the effect of antibiotics, resulting in the formation of pores and loss of membrane integrity [61, 62].

3.2.1. Phenols. Higher content of phenols in soursop leaf extracts (219.14 ± 8.52 mg GAE/L) and papaya leaf extracts (122.39 ± 6.56 mg GAE/L) was observed compared to soursop and papaya seeds while the papaya seed extract had the lowest phenol content (92.96 ± 5.63 mg GAE/L) (Table 3). As reported by Gavamukulya et al. [57], the total soluble phenols content in aqueous and ethanolic extract of soursop leaf was 683.69 μg/mL GAE and 372.92 μg/mL GAE, respectively. These values are lower than those reported in the present investigation. Adefegha et al. [63] analyzed the phenolic content and antioxidant properties of extracts of the pericarp, pulp, and soursop seed. In that study, they obtained a difference in the content of phenols between the analyzed parts of the soursop fruit, finding that the content of phenols in the pericarp was greater than in the seed and pulp. This was attributed to the fact that the pericarp is exposed to environmental stressors such as the ultraviolet rays of sunlight, which causes an intense synthesis of phenolic compounds in the plant, while the seed is protected by the edible part of the fruit and, therefore, less exposed to stress factors. The results presented by Adefegha et al. [63] have a similarity with the results of the present investigation because the phenols content in papaya and soursop leaves was greater than the content of phenols in papaya and soursop seed.

3.2.2. Tannins. The tannins present in the soursop leaf (159.84 ± 10 mg GAE/g dm) were higher than those obtained in the rest of aqueous extracts. The aqueous extract of papaya leaf (75.64 ± 4.79 GAE/g dm) had the lowest tannin content. Tannins are phenolic compounds found in the plant vacuole surface and are nonspecifically bound to proteins through hydrogen or covalent bond to groups of protein amino acids [64].

3.2.3. Flavonoids. Flavonoids are phenolic compounds with antioxidant properties of multiple biological functions, including antimicrobial and cytotoxic [64]. Flavonoids belong to a large group of polyphenolic compounds and are present in any part of the plant [65]. In the results shown in Table 3, soursop leaf was the biological part that presented the highest flavonoid content with $0.13 \pm 1.12 \times 10^{-4}$ RE/g dm, followed by papaya leaf with a content of $0.14 \pm 9.41 \times 10^{-5}$ RE/g dm, while aqueous extracts of papaya seed and soursop leaf showed a lower content of flavonoids.

3.2.4. Saponins. Soursop seed had the highest content of saponins (1.2 ± 0.1 mg QSES/g dm) followed by papaya leaf ($1 \pm 1.64 \times 10^{-14}$ mg QSES/g dm). Both extracts presented in

Table 2: Qualitative identification of secondary metabolites presents in aqueous extracts of papaya and soursop seed and leaf.

Secondary metabolites	Aqueous extracts			
	Papaya seeds	Soursop seeds	Papaya leaf	Soursop leaf
Phenols and tannins	+	−	+	+
Flavonoids	+	−	+	+
Alkaloids	+	+	+	−
Terpenoids	+	−	+	+
Anthraquinones	+	−	−	−
Saponins	+	+	+	+
Coumarins	−	−	+	−

+, presence; − absence.

Table 3: Quantification of secondary metabolites present in aqueous extracts of papaya and soursop seed and leaf.

Secondary metabolites	Aqueous extracts			
	Papaya seed	Soursop seed	Papaya leaf	Soursop leaf
Phenols (mg GAE/L)	93 ± 5.6	103 ± 10.1	122 ± 6.6	219 ± 8.5
Tannins (mg GAE/g dm)	78 ± 6	83 ± 9.6	76 ± 4.8	160 ± 10
Flavonoids (mg RE/g dm)	$0.13 \pm 3.8 \times 10^{-5}$	$0.16 \pm 9.0 \times 10^{-6}$	$0.14 \pm 9.4 \times 10^{-5}$	$0.13 \pm 1.1 \times 10^{-4}$
Saponins (mg QSES/g dm)	$0.10 \pm 1.6 \times 10^{-14}$	1.2 ± 0.1	$1 \pm 1.6 \times 10^{-14}$	0.33 ± 0.057
Alkaloids (mg AE/g dm)	$6.25 \pm 5.7 \times 10^{-4}$	$6.25 \pm 1.15 \times 10^{-3}$	$6.41 \pm 1 \times 10^{-3}$	$6.25 \pm 5.7 \times 10^{-4}$

the qualitative analysis a stable and consistent foam content, while in the papaya seed and soursop leaf extracts, the presence of foam was minimal and inconsistent. Saponins are secondary metabolites of the steroidal or triterpenoid class responsible for the defense of plants against pathogens and insects [66]. The toxicity of saponins to different organisms is related to the interaction with biological membranes and could be related to the soapy properties of saponins [66].

3.2.5. Alkaloids. Regarding the alkaloid content, the papaya leaf had the highest value among the four aqueous extracts ($6.413 \pm 1 \times 10^{-3}$ mg EA/g dm) while in papaya seed, soursop seed, and leaf extracts, the values obtained were $6.25 \pm 5.7 \times 10^{-4}$, $6.25 \pm 1.15 \times 10^{-3}$, $6.25 \pm 5.7 \times 10^{-4}$ respectively.

Alexander et al. [67] conducted an investigation to evaluate the phytochemicals and antimicrobial activity of leaves of *C. papaya* L. and *Psidium guajava* (two medicinal plants used in Nigeria) performing the extraction of bioactive components with a soxhlet extractor. The results of that investigation showed an alkaloid content in papaya leaf extract of 0.16 ± 0.01 mg/100 g dm, which differs from that obtained in this investigation, which may be due to the extraction method and solvent used. The alkaloids in papaya leaves are found in the form of carpaine, pseudocarpains, piperidine macrocyclic, hydrocarpain I, hydrocarpathin II, and nicotine [54]. Matsuura and Fett-Neto [68] mention that the presence of alkaloids and other secondary metabolites in plants increases the reproductive rate by improving defenses against biotic factors and abiotic stress in plants. These researchers indicate that the greater importance of the alkaloids present in plants is due to the protection against herbivores since some alkaloids have a bitter taste and cause the protein functions to break once the alkaloid has been ingested and metabolized and also alters the nervous system.

Plants are capable of producing a large number of various bioactive compounds; however, the quality of a plant extract will depend on the part of the plant used and the technology used to extract these compounds. The effect of the extract will depend on the nature of the plant material, origin, degree of processing, humidity, particle size, and variation in the extraction method which includes the type of extraction, the time, and temperature [69].

Likewise, another parameter that influences the composition and quantity of secondary metabolites found in an extract is the nature of the solvent used for extraction as well as its polarity [8]. Ncube and Afolayan [69] stated that the amount of chemical substances present in an extract will depend on the method of extraction, age of the plant, crop, and sex of the plant.

4. Conclusion

We identified the pathogens as *C. fructicola* and *N. haematococca* (mango and soursop, respectively) according to their morphological and molecular characteristics. The aqueous extracts (water as a solvent) of papaya and soursop leaf and seed presented the highest percentage of inhibition of both pathogens. Furthermore, the aqueous extract of papaya leaf was the most effective among all extracts.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors contributed equally to the writing of the manuscript and approved the final version.

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