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In vitro antifungal properties of *Aloe vera* (L.) Burm. f. films incorporated with cinnamon essential oil against *Lasiodoplodia theobromae* in wax apple

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ABSTRACT

Aims: Wax apple is an important fruit crop in Malaysia and other tropical countries. However, the black spot disease caused by *Lasiodoplodia theobromae* can damage the wax apple plants, reducing fruit production and quality. Chemical fungicides are commonly used to overcome this disease. However, their overuse might increase fungal resistance to chemicals. Therefore, this study was aimed to evaluate the *in vitro* antifungal properties of an *Aloe vera* film incorporated with cinnamon oil on *L. theobromae* in wax apples.
Methodology and results: *In vitro* antifungal tests were conducted using the poisoned food. This present study found that the inhibition of *L. theobromae* mycelia when treated with *A. vera* film with different percentages of cinnamon oil was significantly different compared to the control film at ($P<0.05$). Results suggest that *A. vera* film without cinnamon oil showed the highest percentage of inhibition (37.31%) than other films with cinnamon oil due to the antagonistic and less synergistic effect. However, *A. vera* film with 0.07% of cinnamon oil showed the highest percentage of mycelia inhibition (36.15%) compared to the film with 0.06%, 0.05% and 0.04% cinnamon oil, with mycelia inhibition of 30.55%, 24.25% and 18.82%, respectively, while the mancozeb (positive control) showed 100% inhibition.
Conclusion, significance and impact of study: In conclusion, *A. vera* film alone had 37.31% inhibition compared to 36.15% inhibition in *A. vera* film with 0.7% cinnamon oil. As a result, a tiny amount of cinnamon oil added to the mixture might aid in controlling the black spot disease in wax fruits.

Keywords: *Aloe vera* film, antifungal activity, cinnamon oil, *Lasiodoplodia theobromae*, wax apple

INTRODUCTION

The wax apple is known as *jambu air madu* in the vernacular language of Malaysia. It is a non-climacteric humid fruit belonging to the family Myrtaceae and its botanical name is *Syzygium samarangense* (Blume) Merr. & L.M. Perry (Morton, 1987a). According to Khandaker *et al.* (2015), the wax apple species most probably originated from Malaysia and other Southeast Asian countries. It is commercially grown throughout Malaysia and neighbouring countries such as Thailand, Indonesia, Taiwan and other tropical countries (Moneruzzaman *et al.*, 2011). The wax apple is well-known for its crunchy texture and delicate, sugary and aromatic taste (Morton, 1987b). Furthermore, the fruits can be eaten raw with salt or cooked as a sauce with thirst-quenching characteristics (Khandaker and Boyce, 2016).

Moreover, Khandaker *et al.* (2015) reported it as a healthy food since it contains high amounts of vitamins, minerals, organics, flavonoids, phenolics and sesquiterpene compounds, and a smaller quantity of carotenoids, all of which can act as antioxidants. There is an increasing demand for wax apples in tropical countries, fetching a price up to USD3/kilogram, which might be very profitable for domestic farmers and the local economy (Khandaker *et al.*, 2012). However, infection by phytopathogenic fungi such as *Lasiodoplodia theobromae* causes black spot disease in harvested wax apples, as reported by Yang *et al.* (2009) and Che *et al.* (2015). This will affect the quality and reduce the yield of this crop. Some actions recommended compromising treated wax apple with fungicides such as carbendazim, chlorothalonil and thiophanate-methyl (Yang *et al.*, 2009). Even synthetic fungicides are an effective treatment to

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Table 1: Film-forming composition.

Film sample's number	Film composition (v/v %)				
	Aloe vera gel	Glycerol	Gelatine	Cinnamon oil	Tween 80
1	5.0	2.0	2.0	0	0
2	5.0	2.0	2.0	0.04	0.06
3	5.0	2.0	2.0	0.05	0.05
4	5.0	2.0	2.0	0.06	0.04
5	5.0	2.0	2.0	0.07	0.03

control but still important to find non-fungicidal, which are efficient and safe ways to handle pathogens mostly because synthetic fungicide residues are toxic to the health of humans and the environment (Droby, 2006).

Aloe vera (*Aloe barbadensis* Miller) belongs to the Liliaceae family and the plant resembles the cactus (Ahlawat and Khatkar, 2011). *A. vera* is commonly believed to originate from Arabia, Somalia, Sudan and Oman. It is a succulent xerophytic plant and is broadly grown in Africa, India and other dry regions of the globe (Kedarnath *et al.*, 2013). Aloe gel contains about 55% polysaccharides, 17% sugars, 16% minerals, 7% proteins, 4% lipids and 1% phenolic compounds (Ahlawat and Khatkar, 2011). The plant is commonly used in pharmaceuticals, healthcare, remedies, cosmetic care and food products (Abdollahi *et al.*, 2011). In current years, *A. vera* gel is proposed as a possible constituent in edible films or coatings that can lengthen the shelf life of various fruits and vegetables. In addition, previous research by Saks and Barkai-Golan (1995), de Rodríguez *et al.* (2005) and Castillo *et al.* (2010) found that *A. vera* pulp or liquid extracts were effective against a variety of fungal infections in fruits and vegetables. This property is highly desirable since using natural resources, particularly plant extracts against plant pathogens, is safe for humans and the environment (Hernández-Albíter *et al.*, 2007).

Cinnamon (*Cinnamomum verum* J. Presl) belongs to the family Lauraceae and is traditionally harvested in Asian countries (Suppakul, 2012). Cinnamon is known to contain active substances in the form of trans-cinnamaldehyde (60.72%), eugenol (17.62%) and coumarin (13.39%) (Wang *et al.*, 2009). Furthermore, cinnamon oil could be applied as a natural food additive and incorporated into edible coatings to develop safety and increase the shelf life of food products (Zhang *et al.*, 2017). The major bioactive component in cinnamon oil is cinnamaldehyde, which shows broad-spectrum antimicrobial activity against bacteria, yeasts and fungi (Chen *et al.*, 2017). Several studies have demonstrated the antifungal activity of cinnamaldehyde against *Aspergillus* spp., *Penicillium* spp. and *Colletotrichum* spp. (Pina-Pérez *et al.*, 2012; Noshirvani *et al.*, 2017).

Despite the above-mentioned studies, it was realized that there still exists a research gap on the antifungal effects of *A. vera* films incorporated with cinnamon oil against phytopathogenic fungi, especially *L. theobromae*. Therefore, the present study was carried out with the main objective of evaluating the *in vitro* inhibitory effect of *A. vera* film incorporated with different percentages of cinnamon oil against *L. theobromae*.

MATERIALS AND METHODS

Collection of plant materials

Wax apples (red wax apples) were collected from a farm in Labis, Johor. *Aloe vera* plants were obtained from a local nursery in Kuala Terengganu. Cinnamon oil was purchased from the company Sigma-Aldrich, USA.

Extraction of *Aloe vera* gel

Firstly, *A. barbadensis* Miller leaves were washed with water and 2% (v/v) sodium hypochlorite (Sigma-Aldrich, USA) to remove aloin and dirt. Later, the leaves were dissected longitudinally and the colourless parenchymatous tissue (Aloe gel) was extracted using a sterile blade. This colourless hydro-parenchyma gel was homogenised using a blender (Panasonic-MX GS1WSP, Malaysia) for 1 min at high speed. Next, the homogenized gel was filtered with a plastic sieve (6.14 mm) to remove any fibres to obtain the liquid.

Aloe vera film preparation

Aloe vera film was prepared based on the method of Razifah *et al*. (2017). The edible film was prepared based on the film-forming composition as stated in Table 1. Firstly, 100 mL of sterile distilled water was added to 2 mL of glycerol (HmbG, US). To this solution, 2 mL of gelatin (PC Laboratory reagent, Malaysia) was added to block gases (O_2 and CO_2) and the solution was homogenized at 250 rpm, 80 °C for 1 h by constant heating and stirring on a WiseStir MSH-20 D digital hot plate stirrer. After the stirring, the mixture was added with 25 mL of *A. vera* gel and cinnamon oil (Sigma-Aldrich, USA) at percentages of 0.04%, 0.0%, 0.06%, 0.07% and Tween 80 (Scharlau, Spain) separately. Finally, the *A. vera* films incorporated with cinnamon were kept in a chiller at 4 °C to maintain their effectiveness.

Growth media preparation

According to the manufacturer's instructions (Oxoid, UK), potato dextrose agar (PDA) growth media were weighed and diluted in distilled water in a Schott blue cap container. At room temperature, the PDA solution was allowed to cool to 60 °C after being autoclaved at 121 °C for 30 min at 15 psi. Then, Brandon poured the sterile molten PDA into plastic Petri dishes in a biosafety container. The plates were left unattended for 30 min to

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allow the molten PDA to solidify. The parafilm was used to seal the plates, which were then chilled at 4 °C until their next usage.

Isolation and purification of a fungal pathogen

The wax apple's contaminated skin was peeled off in small parts using a sterilized blade while avoiding the flesh to isolate the fungus that caused it. The infected skins were sterilized for 1 min using a solution of 1% (v/v) sodium hypochlorite (Sigma-Aldrich, USA) before being rinsed in sterile distilled water to eliminate any remaining sodium hypochlorite. Following the placement of the contaminated skins in the centre of the sterile PDA plates, the plates underwent a 7-day incubation period at 25 °C to promote the development of mycelia and spores. To create pure cultures, Peintner *et al.* (2019) applied a technique that involved slicing 1-3 mm mycelial plugs off the edge of the mycelium on the isolation plate and transplanting them onto brand-new PDA plates. The plates were then incubated at 25 °C for 7-12 days. The cultures were routinely inspected to prevent contamination.

Morphological and cultural characterization

Next, three 5-mm plugs were aseptically punched from the edges of actively growing areas of 5-day of pure cultures using a cork borer. Each plug was then transferred onto fresh PDA plates and incubated at 25 °C for 7 days. The colony shape and border were documented after 7 days by inspecting the colony's edge in the plate with naked eyes to see if it was whole, undulate, filiform, curled or lobate. In addition, the colour and texture of the culture (whether fluffy, gritty, rhizomorphic and so forth) were documented. To calculate growth rate, the colony diameter of each culture was measured and recorded every day (in mm) for 7 days. The growth rate was calculated as the 7-day average of mean daily growth (mm per day). Three cultures of each isolate were investigated. For examination of conidial morphology, all isolates were subcultures as mentioned above. Next, the cultures were washed with sterile water and drops of the suspension were placed on microscope slides and mixed with lactophenol cotton blue to stain the conidia. Then, the shape of the conidia was observed and recorded. Lastly, the length and width for 10 conidia per isolate were measured. Only one isolate was used in this research, and three cultures from one isolate were investigated.

Pathogenicity test

The pathogenicity test was done according to the method of Al-Obaidi *et al.* (2017) with some modifications. Firstly, the surface of healthy and detached wax apple fruits was sterilized with 6% (v/v) sodium hypochlorite (Sigma-Aldrich, USA) for 3-5 min and rinsed with sterile distilled water and then dried with sterile tissue paper. After that, the fruit surface was wounded by the pin-pricking method. In this method, a sterile toothpick was used to wound the fruit surface to a 1 mm depth to facilitate the pathogen's entrance, followed by inoculation of the wound with a 5 mm^2 agar plug with mycelia from 7 days old *L. theobromae* culture. Control fruits were inoculated with a sterile PDA plug. Next, the fruits were incubated in a sterile plastic container at room temperature (25 °C) and 95% RH. Sterile wet cotton wool was put into the cinnamon container to provide humidity. The fruits were incubated until anthracnose symptoms appeared on the inoculated fruits. After 5 days of incubation, treated and controlled wax apples were photographed. The pathogen was re-isolated from diseased fruits, pure cultured and morphology identification was done. Finally, the colony and spore morphologies of the re-isolated fungus were compared with the original culture to confirm that the apparent symptoms fulfilled Koch's postulates. The test was done in triplicate and only one fungal isolate was used in this pathogenicity test.

***In vitro* antifungal activity of the film**

Antifungal activity of the *A. vera* films was tested using the poisoned food technique Raza *et al.* (2019) with some modifications. The ratio of molten PDA medium and *A. vera* film mixture used in this study was 1:1. Firstly, 10 mL of the PDA medium was carefully poured into a tube containing 10 mL of the *A. vera* film without cinnamon oil and *A. vera* film with 0.04, 0.05, 0.06 and 0.07% of cinnamon oil. After that, the tube was manually stirred to mix the film in the medium. Then, the mixture was poured slowly into a sterile Petri dish to avoid trapping any air bubbles and left at room temperature (25 °C) for one hour to solidify. After it had solidified, agar discs with mycelia (6 mm in diameter) were cut from the edge regions of 7-day-old cultures using a sterile cork borer and inoculated at the centre of the plate in an inverted position so that the mycelia growth touched the medium's surface. A medium mixed with the mancozeb fungicide (Golden goose), a positive control at 0.08% (100 ppm) was also inoculated with a fungus disk separately. Whereas a medium without the *A. vera* film inoculated with the fungus acted as the negative control. The test was performed in triplicate for each cinnamon oil concentration. The plates were then incubated at 28 °C and the incubation period was stopped when the mycelia had completely grown and covered the negative control plate (3 days). The diameter of mycelia growth was measured in mm. The percentage inhibition of mycelia growth by the films was solution calculated using the formula by Philippe *et al.* (2012):

Inhibition of the mycelia growth (%): [(dc - dt)/dc] × 100

Where, dc is the average diameter of mycelia growth in the control sample and dt is the average diameter of mycelia growth in the treated sample.

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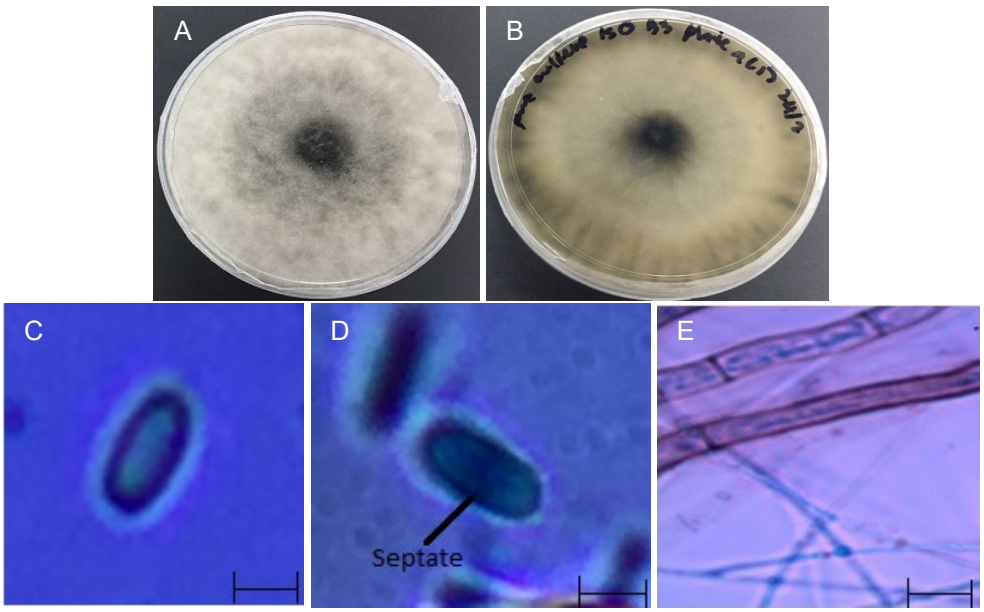

Figure 1: Morphological characteristics of isolated *Lasiodiplodia theobromae* from infected wax apple. (A) Upper surface; (B) Lower surface; (C) Immatured conidia; (D) matured conidia; (E) Hyphae. (Scale bar=10 µm).

Table 2: Morphological characterization of *Lasiodiplodia theobromae* species.

Species	Colony shape	Margin	Colony colour	Texture	Growth rate (mm/day)
Lasiodiplodia theobromae	Circular	Entire	White greyish mycelium	Fluffy and compact	4.34-4.61

Table 3: Conidial morphology of *Lasiodiplodia theobromae* species.

Species	Conidial (mean)		
Lasiodiplodia theobromae	Shape	Length (µm)	Width (µm)
	Oval shaped	11.03-13.38	5.68-6.82

Statistical analysis

The raw data were analysed using the SPSS 20.0 statistical software. Data were expressed as mean ± standard deviation. Data for the inhibition of *L. theobromae* mycelia growth on *A. vera* films with different percentages of cinnamon oil were analyzed using one-way analysis of variance (ANOVA). The significance was accepted at the 0.05 level of probability ($P<0.05$).

RESULTS AND DISCUSSION

Morphological and cultural characterization of *Lasiodiplodia* spp. isolate

The colony form, border, colour, texture and growth rate of *Lasiodiplodia theobromae* species on PDA media were reported as morphological parameters (Table 2). A colony with a circular form and a complete edge was seen in cultures from one isolate. Furthermore, the isolate's cultures exhibited white and grey mycelium on the top surface, with white and light-greenish mycelium on the bottom surface (Figure 1). Furthermore, the isolate's cultures were found to have a fluffy and compact structure. Furthermore, the isolate's cultures grew at a pace ranging from 4.34 to 4.61 mm/day.

Conidial morphology, conidia *L. theobromae* shape and mean size (length and width) were recorded (Table 3). Immatured conidia are hyaline (pale colour) and aseptate, while matured conidia have thick walls and septate. Both *L. theobromae* conidia types were oval-shaped (Figure 1). The length and width of the conidia were 11.25-13.38 µm and 5.68-6.87 µm, respectively (Table 3).

Pathogenicity test

After five days of incubation, infected wax apples developed round, black patches produced by *L. theobromae*, but control wax apples showed no symptoms (Figure 2). The fungus re-isolated from damaged apples had the same colony morphology, spore

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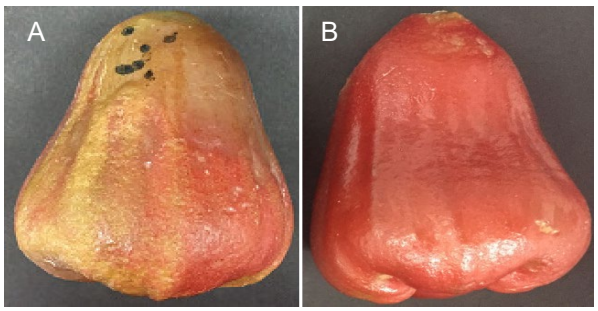

Figure 2: Symptoms of black spots disease on wax apples after wound inoculation for five days. (A) Round, black spots typical of infection; (B) Control fruits did not show any symptoms of *Lasiodiplodia theobromae* infection.

Table 4: Antifungal activity of *Aloe vera* film incorporated with different percentages of cinnamon oil.

Percentage of cinnamon oil (%)	Mycelia growth diameter (mm)	Percentage of inhibition mycelia growth (%)
0 (Only *Aloe vera* gel)	24.13^{b}	37.31 ± 3.48^{b}
0.04	31.21^{e}	18.82 ± 2.79^{e}
0.05	29.13^{de}	24.25 ± 2.06^{de}
0.06	26.71^{cd}	30.55 ± 1.02^{cd}
0.07	24.54^{bc}	36.15 ± 3.52^{bc}
Positive control (mancozeb 0.08%)	0^{f}	100.00 ± 0.00^{a}
Negative control	38.46^{a}	0.00 ± 0.00^{f}

*Different alphabets indicated significantly different between control and treatment.

shape and size as the original isolate. After seven days of incubation, mycelia colour emerged white grey on the upper surface of the plate and yellowish black on the lower surface of the plate. Therefore, Koch's postulates were met and the pathogenicity test verified that the isolated fungus was *L. theobromae*.

In vitro antifungal activity of film

In vitro antifungal activity of *A. vera* films was investigated using the poisoned food method. The study showed that the percentage of inhibition *L. theobromae* mycelial growth by *A. vera* films without cinnamon oil (control) was significantly different from *A. vera* films with different percentages of cinnamon oil ($P<0.05$) after three days of incubation (Table 4). Compared to *A. vera* films with different amounts of cinnamon oil, *A. vera* film without cinnamon oil had the smallest mycelia growth diameter (24.13 mm) and the most significant percentage of inhibition *L. theobromae* (37.31%).

The poisoned food approach was used to test the antifungal activity of *A. vera* films *in vitro*. After three days of incubation, analysis of variance (ANOVA) revealed that the percentage of suppression of *L. theobromae* mycelia development, when treated with *A. vera* films containing various percentages of cinnamon essential oil, was substantially different from both group controls ($P<0.05$) (Table 4). It is thought that combining cinnamon oil with *A. vera* film produces a synergistic effect, increasing antifungal activity. However, compared to *A. vera* films with various percentages of cinnamon oil and negative control, Figure 3 and Table 4 show that the *A. vera* film without cinnamon oil produced the smallest mycelia growth diameter (24.13 mm) and the highest percentage inhibition of *L. theobromae* (37.31%). According to Al-Bayati and Mohammed (2009), this might be because cinnamon oil contains a significant component known as cinnamaldehyde. Even though cinnamaldehyde has an outstanding antifungal outcome, it has features such as being easily volatile and oxidised that limit its function and impact its antifungal effect (Neves *et al*., 2019). As a result, when cinnamon oil is mixed with *A. vera* film, its antifungal effects are reduced. Another reason why *A. vera* films without cinnamon oil had the highest percentage suppression of mycelia compared to films with various concentrations of cinnamon oil because of the antimicrobial component in *A. vera* gel and cinnamon oil had a less synergistic effect. Anthraquinones, for example, an antibacterial ingredient in *A. vera* gel, but cinnamaldehyde is an antimicrobial compound in cinnamon oil. When two antimicrobial components are combined, the overall antimicrobial activity produced is greater than the total antimicrobial activity produced when the compounds are utilized separately (Hyldgaard *et al*., 2012). Furthermore, the antagonistic action induced by the combination of antibacterial chemicals in *A. vera* gel and cinnamon oil is most likely why *A. vera* films without cinnamon oil had the largest percentage inhibition of mycelia compared to films with various concentrations of cinnamon oil. Because of an antagonistic effect, the antibacterial activity of two substances is lower than their antimicrobial activity when administered separately (Bush *et al.*, 2011).

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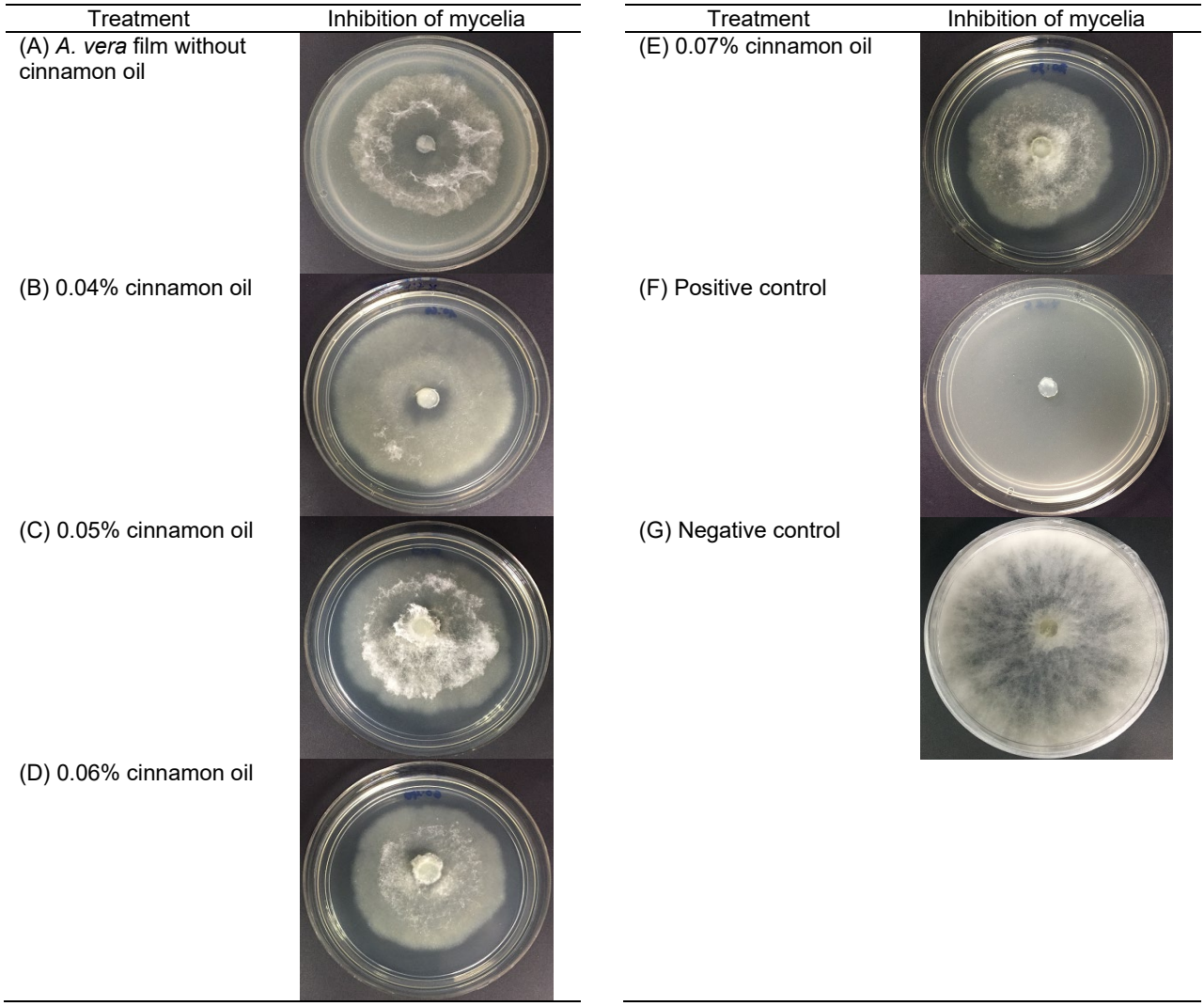

Figure 3: Antifungal activity of *Aloe vera* film extract with different concentrations of cinnamon oil and positive and negative control.

When the percentage of cinnamon oil in *A. vera* film is increased from 0.04% to 0.07%, the diameter mycelia development reduces and the percentage inhibition *L. theobromae* mycelia rise (Figure 3 and Table 4). The findings of this study correspond with those of Palhano *et al.* (2004), who discovered that as the concentration of essential oils increases, citrus and lemongrass crude oils prevent the germination of *L. theobromae* spore on mango fruits. Furthermore, the film with 0.07% cinnamon oil had the smallest diameter of mycelia development (24.54 mm) and the highest percentage of inhibition (36.15%) compared to the films with 0.04%, 0.05% and 0.06% cinnamon oil (Figure 3 and Table 3). This is most likely due to the antifungal properties of the essential oils derived from cinnamon (Wang *et al.*, 2009). According to Chanthaphon *et al.* (2008), cinnamaldehyde may be responsible for cinnamon's antibacterial properties. In addition, Singh *et al.* (2007) claimed that cinnamaldehyde had the most antifungal action of all the components in cinnamon oil.

In fungus, cinnamaldehyde induces cytoplasm loss, mitochondrial and plasma membrane disintegration, and cell fold and cell wall stability loss, according to Xing *et al.* (2014). Furthermore, Khan and Ahmad (2011) think that cinnamaldehyde inhibits the enzymes (b-(1, 3)-glucan synthase and chitin synthase, which might directly or indirectly decrease cell wall formation. Furthermore, cinnamaldehyde can obstruct the biological process of electron transfer by reacting with nitrogen-containing substances such as proteins and nucleic acids, reducing microbial development (Gupta *et al.*, 2008).

Many *in vitro* investigations have shown that cinnamon oil can suppress fungal development; for example, cinnamon oil at 250 (g/mL) inhibited *C. gloeosporioides* (Penz.), anthracnose in papaya, by 100% (Barrera-Necha *et al.*, 2008). In addition, Wu *et al.* (2017)

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found that gelatine films containing 0.5% cinnamon oil inhibited *Aspergillus niger*, *Rhizopus oryzae* and *Paecilomyces varioti* to a considerable extent. Compared to the percentages of inhibition by *A. vera* films with or without cinnamon oil, the synthetic fungicide mancozeb as a positive control demonstrated complete (100%) suppression of mycelia growth.

CONCLUSION

The *in vitro* antifungal activity of *A. vera* films incorporated with different percentages of cinnamon oil against *L. theobromae* in the wax apple was investigated in this study. We found that the *A. vera* film without cinnamon oil showed the highest percentage inhibition of *L. theobromae* compared to *A. vera* films with different percentages of cinnamon oil. However, no significant difference in inhibition was recorded by *A. vera* film without cinnamon oil and film with 0.07% of cinnamon. The present study also showed that the percentage inhibition of *L. theobromae* increases when the percentage of cinnamon oil in *A. vera* film rises from 0.04% to 0.07%. Furthermore, the *A. vera* film with 0.07% cinnamon oil showed the highest percentage of inhibition than films with 0.04%, 0.05% and 0.06% cinnamon oil. Therefore, it can be concluded that *A. vera* films with different percentages of cinnamon oil could directly inhibit *L. theobromae* mycelia growth *in vitro*. Thus, the present study demonstrated the antifungal properties of *A. vera* films incorporated with cinnamon oil as a possible alternative to chemical fungicides in managing anthracnose disease. However, further *in vivo* studies should be conducted to evaluate the effectiveness of *A. vera* films incorporated with cinnamon oil against phytopathogenic fungi.

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