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Potential of the Extract from the Nut of *Areca catechu* to Control Mango Anthracnose

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ABSTRACT

Anthracnose is a common disease that attacks mangoes in many regions, including Malaysia. In this study, extracts from the nuts of *Areca catechu* were tested for their antifungal activities in controlling the disease. Antifungal screening tests were done using six extracts i.e. hexane, chloroform and methanol from ripe and unripe nuts of *A. catechu* to determine their ability to inhibit mycelium growth and spore germination of *Colletotrichum gloeosporioides* isolated from mango. Of the six extracts, the chloroform extract from unripe nuts at a concentration of 10 mg/mL showed the best antifungal activity, inhibiting about 52% of mycelium growth and 100% of spore germination. Thus, this particular extract was selected to treat the fruit against anthracnose in two different ways, namely, by dipping them in the extract solution at 27°C for one hour (normal dip) and also at 52°C for five minutes (hot dip). Meanwhile, control and benomyl solutions (each applied in both dipping methods) were used as comparisons. The test proved that the treatment using the extract reduced 34% of disease infection and 27% of disease rate from the control. However, the treatment using benomyl was slightly effective compared to using the extract, reducing around 47% of disease infection and 38% of disease rate from the control. Hence, results from test also proved that the treatment applied at 52°C reducing 51% of disease infection and 35% of disease rate than those conducted at 27°C. Compound screening tests on the chloroform extract of the unripe nuts revealed that the extract contained alkaloids and phenolics. Many previous studies

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have proven that alkaloids and phenolics from various plants could cause antifungal activities and these substances might be responsible for controlling anthracnose development in the study done.

Keywords: Mango, anthracnose, betel nut extract

INTRODUCTION

Mangoes grow in the tropic and sub-tropic regions (Sangeetha & Rawal, 2008). One of the known varieties grown in Malaysia is the Chok Anan. Anthracnose is one of the common diseases that infect mango during the post-harvest period. This common disease is caused by fungal pathogen, *Colletotrichum gloeosporioides*. Several methods and techniques have been used in controlling mango anthracnose including using resistant varieties of the plant, doing the sanitation process by pruning and removing debris to reduce inoculums and applying fungicide sprays in the field on a regular basis (Nishijima, 1993). After harvesting, suitable treatments have to be applied to reduce potential disease development. The common methods applied to treat the disease are hot water dip and fungicide dip (Ploetz, 2003). Furthermore, fungicides can be added to the hot water to increase the effectiveness of the method. This method, which is also known as fungicide dip, is applied by dipping the mango in hot water of 52^{o}C added with 500 to 1000 ppm benomyl for a duration of 3 to 5 minutes (Lim & Khoo, 1985). However, the usage of chemical fungicides may cause many negative implications, such as bad effects to health and environmental pollution and increasing the resistance mechanisms in pathogens due to frequent application. Many studies have discovered the potential of plant extracts in controlling plant diseases or growth of plant pathogens. One of the potential is the nut of *Areca catechu*, commonly known as the betel nut. The plant can be found in many regions including East Africa, the Arabian Peninsula, the tropical regions of Asia and Indonesia, as well as the central Pacific and New Guinea (Staples & Bevacqua, 2006). The potential of the *A. catechu* nut as an alternative antifungal agent can be related to the presence of its important substituents, alkaloids and phenolics. According to Wang and Lee (1996), there are various kinds of phenolic compound found in the nut including tannin. Meanwhile, the medicine and stimulant brought by consuming the nut can be related to the alkaloid content, which produces euphoria and can treat pain (Petterson *et al*., 1991). Many alkaloids and phenolics extracted from various plants are potential antifungal substances, as has been proved (Deng *et al*., 2011; Veloz-Garcia *et al*., 2010; Hussin *et al*., 2009; Nissanka *et al*., 2001; Baumgatner *et al*., 1990). In Malaysia, however, there has been no research done to determine the potential of the *A. catechu* nut as a biopesticide for post-harvest diseases. The objective of this study was to determine the potential of *A.catechu* nut extract to control mango anthracnose by applying the normal dip method and integrated with the hot water dip technique.

MATERIALS AND METHODS

Isolation of Colletotrichum gloeosporioides

The mango variety, Chok Anan, with common symptoms of anthracnose (black spots and necrotic lesion on the skin) (Fig.1), were bought from a wet market, Pasar Borong Selangor. The fruits were dipped into 10% of Clorox solution for 15 minutes before being dried inside a running laminar flow. The half infected and half visibly healthy fruit skins were cut with a sterile blade knife and placed inside the prepared potato dextrose agar (PDA) medium in a petri dish. All the cultures were placed in a culture chamber at 27°C. After two to three days, potential cultures of *C. gloeosporioides* were transferred into a new PDA medium. The pure cultures were observed each day and identified as *C. gloeosporioides* (Fig.2). The process of culture identification was conducted based on morphological characteristics. The isolated mature culture was observed and its morphological characteristics were compared with common *C. gloeosporioides*. Spore identification was also conducted by placing the slight portion of mycelium from the culture on a drop of lactophenol cotton blue (LCB) on a glass slide and the spores were observed using a light microscope (Fig.3). The spores were compared with the common shape of the *C. gloeosporioides* spores.

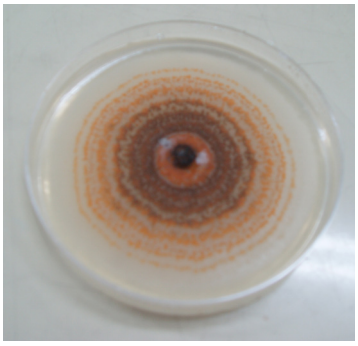

Fig.2: The pure cultures of *C. gloeosporioides*

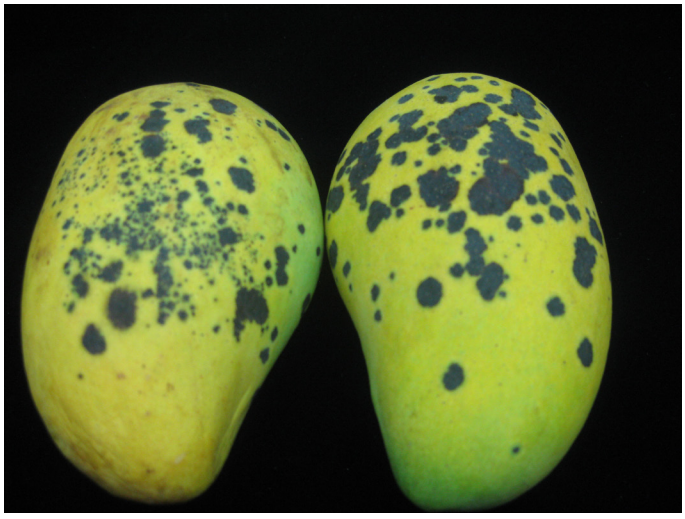

Fig.1: Chok Anan, with common symptoms of anthracnose (black spots and necrotic lesion on the skin)

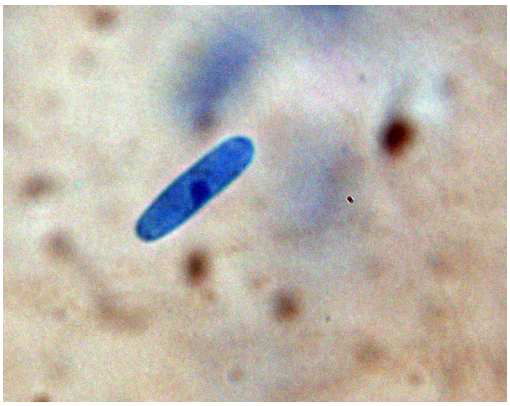

Fig.3: Spore of *C. gloeosporioides*

The Process of Sample Extraction

The ripe and unripe fruits of *A.catechu* were harvested in Kuala Kangsar, Perak, in Peninsular Malaysia. The skin of the ripe fruits was red-orange in colour, while the unripe fruits were dark green. The peel and husk of the fruits were separated from the nuts. The nuts were cut into pieces and dried at normal room temperature for approximately one month to eliminate all the water content. Pieces of the nuts were ground using a blender. Three different solvents were used to extract both the ripe and unripe nuts, which were hexane, chloroform and methanol.

Each nut sample comprising 1kg in weight was extracted continuously starting with hexane followed by chloroform and, finally, methanol. Six extracts comprising hexane, chloroform and methanol were produced from both nut samples. The extracts were then dried using a rotary evaporator. The concentrated extract suspensions were kept at 24 ± 2°C to let the remaining solvents evaporate, leaving only the crude dry extract.

Preparation of Extract Suspensions

Extract stock solutions with concentration of 80mg/mL were prepared by dissolving the solid crude extracts into hot dimetylsulfoxide (DMSO) heated using a hot plate. A 200-µL (0.2mL) extract solution with final concentrations of 20, 40, 60 and 80 mg/mL was obtained by diluting 50, 100, 150 and 200µL stock solutions with 150, 100, 50 and 0µL of DMSO, respectively.

Antifungal Screening Tests

All 1.6mL extract solutions were prepared by diluting each of the 0.2mL already prepared extract solutions, each with the concentration of 20, 40, 60 and 80mg/ml, prepared as described in preparation of extract suspensions, with 1.4mL of sterile distilled water to make four concentrations of extract solutions, which were 2.5, 5.0, 7.5, 10.0mg/mL (all with 12.5% DMSO concentrations), respectively. This method was done to prepare all the extract solutions. A control solution was prepared by dissolving 0.2mL DMSO into 1.4mL sterile distilled water to make 1.6mL of 12.5% DMSO solution. A mycelium growth test was done based on procedure detailed by Rahman (2008) with some modifications. The fungal plug measuring 0.5cm diameter from a seven-day culture of *C. gloeosporioides* was dipped into the extract and control solutions in a sterile glass tube for four hours at 25 ± 2°C. After that, the plugs were placed at the centre of the PDA medium plate in a 5.5-mm diameter Petri dish. Four replications were done for each treatment. The average diameter of the fungal growth was measured

and recorded from day two until day five. The inhibition percentage (%) of mycelium growth was calculated using the following formula:

$$\frac{\text{Mycelium diameter of control culture-Mycelium diameter of treated culture}}{\text{Mycelium diameter of control culture}} \times 100$$

In the spores germination test, spore suspensions were prepared by a flood of a seven-day-old culture of *C. gloeosporioides* with sterile distilled water before being streaked using a sterile L-bent glass rod. The suspension was filtered using double layers of sterile muslin cloth into a sterile flask. Later, 1.4mL of the suspension with concentration of 2.4 x 10^5 conidia mL^{-1} was transferred into glass tubes containing 0.2mL extract solutions, each with a concentration of 20, 40, 60 and 80mg/ml, prepared as described in the earlier section, to make four concentrations of extract mixed with the spore suspension i.e. 2.5, 5.0, 7.5, 10.0mg/mL respectively, all with 12.5% DMSO concentrations,. A control suspension was prepared by dissolving 0.2mL DMSO into 1.4mL spore suspensions. All the mixed suspensions were incubated for 30 minutes at 25 ± 2°C. After that, 0.1mL was transferred from the suspensions and spread over prepared PDA medium plates. All the plates were incubated for 12 hours at 24 ± 2°C. A drop of LCB was used to inhibit any germination after the completion of the incubation period. Spore was considered as being germinated if the germ tube was half the length of the spore (Sariah, 1994). Light microscope was used to observe the germination. A total of 250 spores were counted randomly on each plate. Meanwhile, four replications of the plate were used for each treatment in the test. The inhibition percentages (%) of spore germinations were calculated using the following formula:

$$\frac{\text{Spore germination of control-Spore germination of extract treatment}}{\text{Spore germination of control}} \times 100$$

Screening for Potential Compounds in the Extracts

Two screening tests were carried out to detect the presence of alkaloids and phenolic compounds in the extracts. The screening for alkaloids was based on Touchstone and Dobbins (1983). A stock solution of Dragendorf reagent was prepared by mixing a solution of 0.85g bismuth sub-nitrate in 10mL acetic acid and 40mL water with a solution of 8g potassium iodide in 20mL water. The spray solution was prepared by mixing 1mL of the stock solution with 2mL acetic acid and 10mL water before use. The crude extracts were spotted on a thin layer chromatography (TLC) plate. The solvent systems used for hexane, chloroform and methanol extracts for both ripe and unripe nuts were hexane:chloroform (5:5), chloroform:methanol (7:3) and 100% methanol, respectively. The plate was developed and sprayed with the Dragendorf reagent. Orange coloured spots indicated the presence of alkaloids. For phenolics screening, an iron (III) chloride solution was prepared by dissolving 1.0g iron (III) chloride in 100mL methanol. The

crude extracts were similarly spotted on a developed TLC plate end. The solvent systems used were the same as that applied in the screening of the alkaloids. The developed plate was stained with an iron (III) chloride solution. Blue greyish coloured spots indicated the presence of phenols.

In vivo Test on Mango Using the Selected Extract

The extract causing the best antifungal reaction against *C. gloeosporioides* in both the mycelium growth and spore germination tests was applied in controlling the anthracnose infection on the Chok Anan mangoes. The test was done on the artificially inoculated fruit. Two different treatments using the extract were done on the mangoes, and this was carried out by dipping the fruit in the extract solutions at 27°C for one hour and dipping them in the extract solutions at 52°C for 5 minutes. In this study, unripe green mangoes were used. The three treatments selected were: i) Extract solution; ii) Benomyl solution; and iii) Control solution. The extract solution was prepared by dissolving 250mL extract stock solution with concentration of 80mg/mL (prepared by dissolving 20g extract in 250mL DMSO) in 1750mL sterile distilled water. A benomyl solution was prepared according to suggestion (according to manufacturer recommendations) by dissolving 100mg benomyl powder into 2L sterile distilled water to make a 0.05g/L benomyl solution. A control solution was prepared by dissolving 250mL DMSO in 1750mL sterile distilled water.

The mangoes were cleaned and surface sterilised by dipping them in 10% Clorox solution for 15 minutes; this was followed by drying in a running laminar flow. Spore suspensions, in the concentration of 6.7 x 10^7 conidia mL^{-1}, which had been prepared following the procedure described in the earlier section, were sprayed on the whole fruit. After three hours, the mangoes were treated using two different dipping methods. The first method (normal dipping) was done by dipping the fruits into the prepared extract solution at 27°C for one hour, while the other method (hot dip) was carried out by dipping the fruits in the extract solution at 52°C for 5 minutes using a hot-water bath. For each method, a 12.5% DMSO solution and a 0.05mg/mL benomyl solution were used as the negative control and the positive control, respectively. The mangoes were then dried under a running laminar flow after being treated before being incubated in a chamber. The fruits were sprayed with sterile distilled water. Conditions in the chamber were maintained with relative humidity at 88 ± 2% RH in a temperature of 24 ± 2°C. Five replicates of the fruit were used for each treatment. The experiment was done twice. The spots that appeared on the fruit skin were counted every day for seven days. Disease severity was scored on a 1–5 scale based on Koomen and Jeffries (1993) and Pordesimo (1979) with some modifications, where 1=1-5 spots, 2=6-10 spots, 3=11-20 spots, 4=21-30 spots, and 5=>30 spots. Disease severity percentages (%) were calculated based on the following formula:

$$\frac{\Sigma\ (\text{Disease scale} \times \text{Total fruits in the scale})}{\text{Total fruits in the experiment} \times \text{Highest scale}} \times 100$$

The graphs of the disease severity on the mangoes during the seven days of treatments were plotted. The area under the disease progress curve (AUDPC) was determined from the graphs based on the formula as described by Madden *et al*. (2007).

$$\text{AUDPC} = \sum_{i=1}^{N_i-1} \left(\frac{y_i + y_{i+1}}{2}\right)(t_i + t_{i+1})$$

y = data of disease severity or disease incidence collected

t = time of data collected

The disease rate was determined by using a regression of transformed diseased severity values using logistic model ln (y/(1-y)) (Berger, 1981). Percentage data were first transformed using an arc sine transformation before analysis.

Statistical Analysis

All the experiments were carried out in a Completely Randomized Design (CRD). The analysis of variance (ANOVA) using SAS statistical software was utilised to analyse the data. The results showing significant differences were subjected to the mean separation of the Duncan multiple range test (DMRT) at $P \leq 0.05$.

RESULTS

Antifungal Screening Tests

The chloroform extract from the unripe nut had the best antifungal activities against *C. gloeosporioides*. In the spore germination test (Table 1), both chloroform extracts from the ripe and unripe nuts inhibited 100% of spore germination when applied at the concentrations of 7.5 and 10.0mg/mL. Both hexane extracts from the unripe nut inhibited 65.2% and 44.5% of germinations respectively when applied at the highest concentration of 10mg/mL. Methanol extracts from both nuts had the least inhibition among others. In the mycelium growth test (Table 2), the chloroform extract from the unripe nut showed the best result, which inhibited the mycelium growth at 52.2% when applied at the highest concentration of 10 mg/mL. This was followed by the chloroform extract from the ripe nut (44.5%), the hexane extract from the ripe nut (36.7%), the hexane extract from the unripe nut (36.0%), the methanol extract from the ripe nut (12.4%) and the methanol extract from the unripe nut (12.4%).

Screening for Potential Compounds in the Extracts

From both screening tests, positive results indicated the presence of phenolics and alkaloids in the crude extracts (Table 3). Both compounds were present in the two chloroform and the two methanol extracts. However, both compounds were not detected in the two hexane extracts.

TABLE 1
Percentage Inhibition (%) of Spore Germination of *C. gloeosporioides* Treated with Extracts from the *A. catechu* Nuts

Extracts	Concentrations (mg/mL)			
	2.5	5.0	7.5	10.0
Hexane ripe	19.2 Ca[z]	26.8 Cb	45.0 Cc	65.2 Cd
Chloroform ripe	73.7 Da	94.0 Db	100.0 Dc	100.0 Dc
Methanol ripe	5.8 Aa	10.3 Ab	19.2 Ac	26.1 Ac
Hexane unripe	11.0 Ba	19.2 Bb	33.9 Bc	44.5 Bd
Chloroform unripe	82.2 Ea	94.7 Db	100.0 Dc	100.0 Dc
Methanol unripe	7.6 Aa	8.2 Aa	18.5 Ab	25.2 Ac

[z]Means followed by the same uppercase or lowercase letter, for each fungus, within each column or row did not differ significantly at DMRT $P \leq 0.05$.

TABLE 2
Percentage Inhibition (%) of Mycelium Growth of *C. gloeosporioides* Treated with Extracts from the *A. catechu* Nuts

Extracts	Concentrations (mg/mL)			
	2.5	5.0	7.5	10.0
Hexane ripe	2.9 Ba[z]	18.7 Bb	20.5 Cc	26.5 Bd
Chloroform ripe	15.7 Ca	28.9 Cb	36.9 Dc	44.5 Cd
Methanol ripe	2.2 Ba	4.8 Aa	8.9 Bb	12.4 Ac
Hexane unripe	1.0 Ba	5.4 Ab	9.2 Bc	13.0 Ad
Chloroform unripe	18.3 Da	28.6 Cb	43.4 Ec	52.2 Dd
Methanol unripe	0.0 Aa	5.1 Ab	6.7 Ab	12.4 Ac

[z]Means followed by the same uppercase or lowercase letter, for each fungus, within each column or row did not differ significantly at DMRT $P \leq 0.05$.

TABLE 3
Results on Alkaloids and Phenolics Presence in Crude Extracts of Ripe and Unripe Nuts of *A. catechu*

Crude extracts	Alkaloids present	Phenolics present
Hexane ripe nut	-	-
Chloroform ripe nut	+	+
Methanol ripe nut	+	+
Hexane unripe nut	-	-
Chloroform unripe nut	+	+
Methanol unripe nut	+	+

The positive sign (+) indicates the presence of the compounds in the extracts and the negative sign (-) indicates absence of the compounds in the extracts.

In vivo Test on Mango Using the Selected Extract

Interaction between treatments solutions and dipping methods is significant (Table 5 and Table 6). In both hot and normal dip methods, the fruits that had been treated with the control solution were found to be the most infected by anthracnose; this was followed by the fruits treated with the extract and benomyl solutions (see Fig.4). The control fruits also had the fastest disease development, followed by the extract, and benomyl, with the least disease rate (Table 4). The fruits treated with the extract had 34% less disease infection and 27% less disease rate, as compared with the control. However, benomyl-treated fruits had a slightly better result with 47% disease infection reduction and a 38% disease rate from the controlled fruits.

The fruits treated with the normal dip were found to be severely infected by anthracnose during the experiment whereas those treated with the hot water dip had the least disease development. Fruits treated with normal dips also had a higher disease rate compared to fruits treated with the hot dip method (Fig.5). Thus, the hot dip method was proven to have effectively reduced 51% of the anthracnose infections and to have slowed down more than 35% of the disease rates as from the normal dip method (Table 4).

TABLE 4
Effects of the Treatments Applied on Anthracnose Development on Artificially Inoculated Mangoes for Seven Days after Inoculation

	AUDPC	Disease rate
Solution		
Control	226.0 a[z]	0.85 a
Extract	148.5 b	0.62 b
Benomyl	119.0 c	0.53 c
Temperature		
Normal temperature dip	221.0 a	0.81 a
Hot dip	108.0 b	0.52 b

[z]For each treatment, the means within a column followed by the same letter are not significantly different by DMRT at P≤0.05.

TABLE 5
ANOVA Table on Effects of the Treatments Applied on AUDPC

Source of Variation	df	SS	MS	F
Treatment	5	64639		
Solution	2	24434	12217	268**
Temperature	1	38307	38307	839**
Solution x Temperature	2	1898	949	21**
Error	6	274	46	
Total	11	64913		

** are significant at P≤0.01.

TABLE 6
ANOVA Table on Effects of the Treatments Applied on Disease Rate

Source of Variation	df	SS	MS	F
Treatment	5	0.5442		
Solution	2	0.2135	0.1067	143.90**
Temperature	1	0.2611	0.2611	352.01**
Solution x Temperature	2	0.0695	0.0348	46.96**
Error	6	0.0045	0.0007	
Total	11	0.5486		

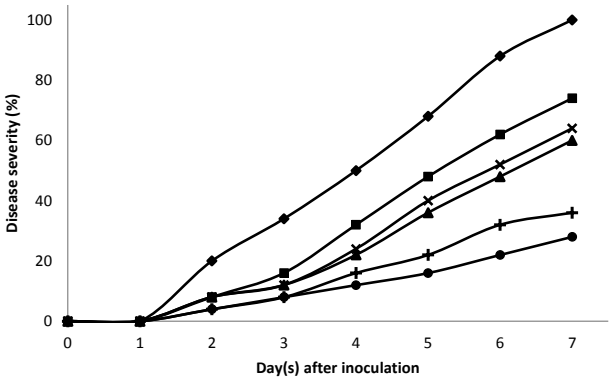

Fig.4: Disease progress curve of anthracnose by *C. gloeosporioides* on mango treated by dipping into control (♦), extract (■) and benomyl solutions (×) at 27°C temperature for 1 hour and dipping into control (▲), extract (+) and benomyl solutions (•) at 52°C temperature for 5 minutes

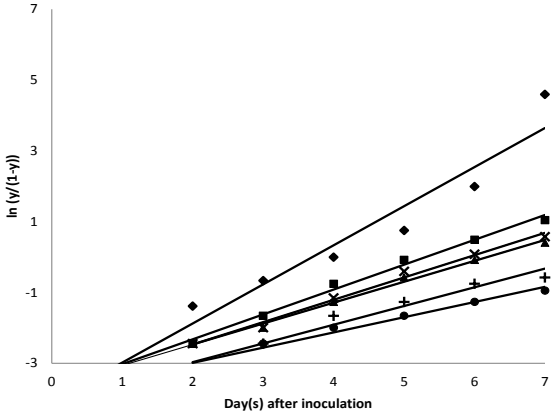

Fig.5: Regression of transformed diseased severity values of disease progress curve of anthracnose by *C. gloeosporioides* on mango treated by dipping into control (♦), extract (■) and benomyl solutions (×) at 27°C temperature for 1 hour and dipping into control (▲), extract (+) and benomyl solutions (•) at 52°C temperature for 5 minutes using logistic model ln (y/(1-y), the equation for the line being Y=1.10x-4.08, R^2=0.91, Y=0.70x-3.72, R^2=0.99, Y=0.63x-3.73, R2=0.99, Y=0.57x-3.61, R^2=0.99, Y=0.53x-4.02, R^2=0.96 and Y=0.43x-3.85, R^2=0.98, respectively

DISCUSSION

Many studies including by Oxenham *et al*. (2002), Holdsworth *et al*. (1998), Wang *et al*. (1997), Wang and Lee (1996) and Huang and McLeish (1989) proved both alkaloids and phenolics are the two substances in *A.catechu* nut most well studied and discovered. As these two groups of compounds are important in the nut, it is possible to relate the antimicrobial effects brought by both alkaloids and phenolics in the extracts. During both mycelium growth and the spore germination tests, both hexane extracts from ripe and unripe nuts were seen to be less effective; this might be due to the absence of alkaloids and phenolics in both extracts. The chloroform extracts of both the ripe and unripe nuts of *A.catechu* gave the best antifungal activities against *C. gloeosporioides*. Between the two, the extracts from the unripe nut proved to be better in inhibiting the growth of the pathogen. A study by Wang *et al*. (1997) found that the unripe nuts contained higher concentrations of alkaloids as compared to the ripe nuts. Since the chloroform extract of the unripe nut had better antifungal activity compared to the chloroform extract of the ripe nuts, the higher concentration of alkaloids it contained might be one of the reasons. Many kinds of alkaloids are able to cause fungitoxic and bacteriostatic actions (Petterson *et al*., 1991).

During the screening test, phenolics were detected in both the ripe and unripe nuts of the chloroform extracts. A study by Wang *et al*. (1997) revealed that the contents of total phenolics and condensed tannins in the nuts increased upon maturity. Due to a better inhibition by the chloroform extract from the unripe nuts compared to the chloroform extract of the ripe nuts, phenolic compound concentrations might have little effect on the antifungal activity of the nuts. Based on the spots on the TLC plates, both methanol extracts from the ripe and unripe nuts contained both phenolics and alkaloids. However, both were found to be ineffective in controlling the growth of *C. gloeosporioides*. Among all the solvents used in the extraction, methanol, being a polar solvent extract, revealed the highest quantity of compounds as compared to the chloroform and the hexane extracts, which can be said to be ineffective. The abundance of compounds in both the methanol extracts might cause each compound in the extracts to act against each other or interfere with each other's mechanisms (Dellavalle *et al*., 2011).

The results from the *in vivo* tests had proven that dipping the fruits in the extract solution did reduce the disease severity of anthracnose. First, the extract solution treatment might induce the resistance level of the fruits. Other than affecting the defence mechanism of the host plant, the plant extracts might cause action mechanisms on fungal pathogens. The mechanisms of crude plant extracts might be due to several different actions on pathogens (Niño *et al*., 2012). The results from the *in vivo* test showed that the treatment using fungicides of the benomyl solution had proven to be effective in slowing down the anthracnose development on the fruits. However, it did

not stop the infection from deteriorating the fruits.

The results from the above study also showed that hot water dipping significantly reduced anthracnose infection on mangoes compared to normal dipping. Antifungal mechanisms by hot water treatment might directly damage the pathogen cells and indirectly increase the resistance level of the host (Karabulut *et al*., 2010). Many plant materials have been successfully controlled using a hot water dip but not many studies have been carried out on integrating the extract application and hot water dipping. Using integration methods in controlling plant diseases causes a difficulty in the pathogen defence mechanism due to the different kinds of antifungal mechanisms caused by each of the treatment (Sharma & Tripathi, 2008).

CONCLUSION

The chloroform extract of the unripe nut reduced disease severity of anthracnose on the mangoes. Both alkaloids and phenolics were found to be present in the extract. These compounds might be involved in the antifungal properties of the extract. However, there have been no reports done on the antifungal activities of these compounds in the study. In the future, a study has to be done on the use of specific compounds from the nut in controlling plant diseases. Dipping fruits in the extracts at 52^{o}C increases the effectiveness of controlling the infections on fruits. More studies have to be carried out in the future to maximise the potential of the extracts by integrating the treatment using the extracts with other available methods.

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