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Research paper

EFFECT of *Ananas comosus* crown and *Punica granatum* peel mixture to inhibit post-harvest *Mangifera indica* fungal (*Aspergillus niger*)

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Abstract

Aspergillus niger is a predominant fungus that grow on post-harvest mango (*Mangifera indica*) which lead to crops failure and quality reduction. Therefore, this study was carried out to utilize the fruit waste, *Ananas comosus* crown and *Punica granatum* peel to inhibit *A. niger*. The wastes were extracted by soxhlet extraction method. The antifungal activity of pineapple crown and pomegranate peel crude extracts was tested on *A. niger* using well diffusion method at different ratio; initial ratio (2:1, 1:1 and 1:2 respectively); and further ratio (4:6, 3:7, 2:8 and 1:9 respectively). The positive control (commercial fungicide) and negative control (100% acetone, methanol and sterile distilled water) were tested against *A. niger*. The findings revealed that the best further ratio, 2:8 (34.33±1.04 mm) posses better effectiveness to inhibit *A. niger* compared to the best initial ratio, 1:2 (30.17±0.76 mm) of pineapple crown to pomegranate peel respectively (p=0.01). While for positive controls, Brand X (34.17±1.04 mm) showed the highest effectiveness to inhibit *A. niger* and by comparing with 2:8 of pineapple crown to pomegranate peel ratio showed no significant value (p=0.85). Therefore, this study revealed that at the ratio 2:8 of pineapple crown to pomegranate peel mixture has a great potential to be formulated as commercial biofungicide by utilizing the fruit waste.

Keywords: *Fruit Waste; Antifungal; Aspergillus; Manggo*

1. Introduction

Mangifera indica (*M. indica*) is one of the vital fruits that have been cultivated in Malaysia for several years starting for export sector to other countries. However post-harvest mango has faced several threats including prone to several diseases that caused by fungi makes the demand is hard to be fulfilled [1]. *Aspergillus niger* (*A. niger*) is the predominant fungus found on post-harvest mango which caused several diseases including Aspergillus rot and Black rot [1]. *A. niger* has contributed in crops loss because its ability to secrete ochratoxin A (OTA) and fumonisin B2 [2]. Moreover, OTA affect several types of fruits including dried or high moisture content fruits [3; 4]. These secretions are harmful to human and will cause decaying process occur rapidly on the affected fruits [5].

With regards to susceptibility of mango toward fungi, most of the mango growers used synthetic fungicide such as iprodione and mancozeb on post-harvest mango [6]. These synthetic fungicides indirectly affect human health [7]. Besides synthetic fungicides, world nowadays has slowly shifted to organic or natural fungicides as healthy lifestyle has evolved widely. In lien with this, enormous numbers of people are likely to consume a lot of fruits that lead to the accumulation of agriculture waste. Pineapple and pomegranate are worldwide fruits that can be found almost in every parts of the world. Pineapple crown has become one of the agriculture wastes in the industry even though it has the capability to regenerate into new plants. Besides its richness of cellulose that possesses great tensile strength that can be used to formulate antifungal paper, it also contains bromelain and phenolic acids (hydroxycinnamolydiglycerides and minor flavonoids) [8; 9; 10]. The ability of bromelain as antifungal agent at low concentration, 0.3 µmol/L of the bromelain which able to suppress 90% of *Fusarium proliferatum*, *Fusarium oxysporum* and *Fusarium verticillioides* growth was discovered [11]. Besides, ferulic acid and p-coumaric acid were found for having the capability to inhibit several *Colletotrichum* sp. [12]. While pomegranate peel consists of diverse form of phenolic acids content which helps in formulating biofungicide. Pomegranate peel consist of 198.90+3.92 GAE mg/g DW and 96.20+1.19 GAE mg/g DW of phenolic acids and flavonoids respectively [13]. Phenolic acids found in pomegranate peel were discovered for having the capability to inhibit *Colletotrichum* sp., *Alternaria* sp., *Rhizoctnia* sp., *Aspergillus* sp., and *Fusarium* sp. [14; 13]. Thus by the combination of the pineapple crown and pomegranate peel could help in formulating a biofungicide to increase the crops quality and quantity.

2. Methodology

2.1. Fruit waste extraction

Pineapple crown and pomegranate peel were washed with sterile water and allowed to drain [15]. An amount of 901 g of pineapple

crown and 560 g of pomegranate peel were dried at 40°C for five days.
Soxhlet extraction process was used to extract 40 g and 60 g of pineapple crown and pomegranate peel's powder with 1600 mL of 75% acetone and 800 mL absolute 80% methanol respectively and continued with rotary evaporation method at 55ºC (80 rpm) [16]; [17]. The crude extracts were stored at 4ºC.

2.2. Medium preparation

Potato dextrose broth was prepared by mixing 20 g of dextrose and 4 g of potato starch. The mixture was dissolved in 1000 mL of distilled water and boiled until mixed homogenously [18]. Mixture of dextrose and potato starch resulting Potato Dextrose Broth (PDB) and continued with alteration of PDB pH using 30% acetic acid until the pH of PDB was 5.1±0.3 at 25°C. Next, 39 g of Potato Dextrose Agar (PDA) were dissolved in 1000 ml distilled water and boiled to mix the mixture homogenously. Both PDB and PDA were autoclaved at 121°C for 15 minutes and stored at 4ºC until further used [19].

2.3. Antifungal test

Five days old *A. niger's* spore were scraped or dislodged using sterile hockey stick after been flooded with 2000 µL of PDB [20]. Sterile hockey stick was used to scrape the spore and directly dip into boiling tube containing 2000 µL PBD and the hockey stick was repeatedly washed throughout the re-suspended method [21]. The spore suspension used was at $1.70x10^4$ spores/mL that counted by using hemocytometer [22]. The crude extracts and commercial fungicides were diluted to 100 mg/mL using sterile distilled water.
Well-diffusion method was used in the study. Sterile cork borer was used to punch the 8 mm well in the middle of PDA agar plate and 100 µL of spore suspension was spread on the agar plate. The 100 µL treatment was pipetted into the well. The treatments consist of 100 mg/mL of initial ratio, further ratio, positive and negative controls. First, the initial ratios used were 100 mg/mL of 2:1, 1:1 and 1:2 of pineapple crown to pomegranate peel. Second, further tested ratios were 100 mg/mL of 4:6, 3:7, 2:8 and 1:9 of pineapple crown to pomegranate peel respectively [23]. Lastly, the commercial fungicides were used as positive control which consists of 100 mg/mL of Brand X, Brand Y and Brand Z. While negative controls used were 100% of acetone, methanol and sterile distilled water. Each of the treatment was carried out in triplicate and incubated for 24 hours at 28ºC [19].

2.4. Statistical analysis

Statistical analyses were carried out using Statistical Package for the Social Science (SPSS) software 20.0. All values obtain from this study was showed in mean±Standard Deviation (S.D.). The diameters of inhibition zone of each treatment were analysed by one-way ANOVA and compared by Post Hoc Tukey Test. Then the furthered ratio's data was analysed by Pearson correlation to determine the relationship between the mixture itself. Lastly, the best commercial antifungal was compared with the best crude extract mixture ratio using independent T-test. The P-value used in this study less than 0.05 was considered as significant.

3. Results and discussion

3.1. Initial crude extract ratio against *A. niger*

Antifungal potential of crude extract ratios were assessed in terms of inhibition zone of fungal growth that was illustrated by the clear zone around the treatment provided. Antifungal activity of the initial crude extract mixtures of pineapple crown and pomegranate peel were initially studied in different ratio (2:1, 1:1 and 1:2 respectively). Table 3.1 and Table 3.2 show the comparison of antifungal activities against initial crude extract ratio.

Table 3.1: Comparison of Antifungal Activities of Primary Mixture of Crude Extracts Ratio Against *A. niger*

Mixture of crude extract (100 mg/mL)	Diameter of inhibition zone (mm)	p-value
2:1	23.17±0.76	
1:1	25.50±0.87	0.00*
1:2	30.17±0.76	

The values were presented in mean±S.D., where n=3
*The mean different is significant at p<0.05 by one-way ANOVA
The ratios were subjected to pineapple crown:pomegranate peel

Table 3.2: Comparison of P-Value of the Initial Crude Extracts Mixture on Diameter of Inhibition Zone

The relationship of initial crude extract [Pineapple crown:Pomegranate peel]		p-value
First crude ratio	Second crude ratio	
2:1	1:1	0.03*
	1:2	0.00*
1:1	1:2	0.00*

*The mean different is significant at p<0.05 by Post Hoc Tukey Test.

From Table 3.2, the strongest significant different was exhibited by 2:1 to 1:2 and 1:1 to 1:2 of pineapple crown to pomegranate peel extracts respectively with p=0.00. This indicated that the difference in the ratio in well-diffusion method gave a major impact in the effectiveness of each antifungal activity. Great difference of the inhibition zone helps in differentiate the effectiveness of the antifungal activity [24].

3.2. Extension of antifungal test of pineapple crown and pomegranate peel against *A. niger* by using further ratio of crude extracts mixture

As according to previous data from Table 3.1, the best initial ratio was at 1:2 (30.17±0.76 mm) of pineapple crown to pomegranate peel. The antifungal test was extended by increasing the volume of pomegranate peel extract while reducing pineapple crown volume. Further ratio used were 4:6, 3:7, 2:8 and 1:9 of 100 mg/mL of pineapple crown to pomegranate peel crude extracts mixture respectively. The results were presented in Table 3.3 and Table 3.4.

Table 3.3: Further Ratio of Crude Extracts Mixture to Inhibit *A.niger* Growth

100 mg/mL of crude extract ratio mixture [Pineapple crown: Pomegranate peel]	Diameter of zone of inhibition (mm)	p-value	r-value
4:6	25.67±0.76		
3:7	29.83±1.04	0.00*	0.96
2:8	34.33±1.04		
1:9	35.33±0.58		

The values were presented in mean±S.D., where n=3
*The mean different is significant at p<0.05 by one way ANOVA

From Table 3.3, the inhibition zone showed positive correlation (r=0.96; p=0.00) for the further treatment ratio. This inhibition zone might be contributed by pomegranate peel as it has great and diverse type of phenolic acid compound which helps pineapple crown to disrupt fungal growth and development [13]. Thus, antifungal activities increased with the increased of pomegranate peel volume used in this study to inhibit *A. niger*. The comparison of further treatments effectiveness against *A. niger* was further analysed by using the Post Hoc Tukey Test as presented in Table 3.4.

Table 3.4: Comparison of P-Value of the Further Treatment Ratio

The relationship of further crude extract mixture [Pineapple crown:Pomegranate peel]		p-value
First crude ratio	Second crude ratio	
4:6	3:7	0.00*
	2:8	0.00*
	1:9	0.00*

3:7	2:8	0.00*
	1:9	0.00*
2:8	1:9	0.54

*The mean different is significant at p<0.05 by Post Hoc Tukey Test.

Table 3.4 shows the significant different between the further crude ratio in extensive study. Even though at the further ratio of 2:8 (34.33+1.04 mm) and 1:9 (35.33+0.58 mm) of pineapple crown to pomegranate peel respectively inhibit the highest *A. niger* growth but there are no significant different between both ratio with p=0.54. Therefore from this it was found that 2:8 is the best ratio among the further ratio to inhibit *A. niger* growth (pineapple crown to pomegranate peel respectively). By comparing the best initial (1:2) and further (2:8) ratio it shows significant different (p=0.01) that indicate the best ratio mixture among the tested ratio to inhibit *A. niger* was 2:8.

This might be due to the fragility of the cell walls and cytomembrane of *A. niger* that lead to several consequences such as the disruption or inhibition chitin [25]. Chitin is classified as the best microbe-associated molecular patterns (MAMPs) as it becomes a vital component in the composition of fungal cell wall other than β-glucan [26]. The permeability changed was due to unstable K^+ and Na^+ protein channel that lead to unstable action potential occur in the fungal growth mechanism as postulated by [25] in 2014. As the permeability change, it caused several pore formation that weak by the cell wall barrier thus lead the antifungal compound penetrate into the cell to inhibit pyrimidine analog for DNA synthesis in the fungal development [25]. Besides, the consequence of chitin inhibition also caused distruption in hyphal growth as studied by [27]. Therefore, this study showed that the compound present in pineapple crown and pomegranate peel extracts have the ability to caused permeability change in *A. niger*.

3.3 Comparison of positive and negative control with the best antifungal ratio from the pineapple crown and pomegranate peel crude extract mixture

The positive controls used were 100 mg/mL commercial fungicides which are named as Brand X, Brand Y and Brand Z. Whilst negative control involved were 100% of distilled water, acetone and methanol which possess no inhibition zone. The inhibition zone by positive control was illustrated in Table 3.5 and Table 3.6.

Table 3.5: Positive Controls Inhibition Zone against *A. niger*

Type of control	Commercial organic fungicide	Inhibition zone (mm)	p-value
Positive control	Brand X	34.17±1.04	0.00*
	Brand Y	21.33±0.58	
	Brand Z	21.67±0.29	

The values were presented in mean±S.D., where n=3
*The mean different is significant at p<0.05 by one way ANOVA

Table 3.6: The Comparison of P-Value in Commercial Organic Fungicides

Commercial organic fungicides		p-value
First fungicide	Second fungicide	
Brand X	Brand Y	0.00*
	Brand Z	0.00*
Brand Y	Brand Z	0.84

*The mean different is significant at p<0.05 by Post Hoc Tukey Test.

From Table 3.5, the statistical different among all means of commercial fungicide showed significant different with p=0.00 as analysed by one-way ANOVA. Besides, there was great significant different in term of diameter of inhibition zone between Brand X to Brand Y and Brand Z with p=0.00. This showed that Brand X has great antifungal activity against *A. niger*.

Comparison between the best mixture ratio crude extract of pineapple crown and pomegranate peel (2:8 respectively) showed no significant different with p=0.85. This indicates that the ratio mixture between fruit waste from pineapple crown and pomegranate peel at 2:8 has the capability to be utilized for formulating a biofungicide. The antifungal of both samples might be contributed by the present of phenolic acid and bromelain (only for pineapple crown). However, phenolic compound in pomegranate peel is higher and diverse compared to pineapple crown that lead to great inhibition of *A. niger*.

4. Conclusion

This study showed the best initial ratio of pineapple crown to pomegranate peel was 1:2 with 30.17±0.76 mm in inhibiting the growth of *A. niger*. Then, the study was extended to further treatment, where the ratio of 2:8 of pineapple crown to pomegranate peel (100 mg/mL) showed the best ratio mixture inhibitory effect against *A. niger* with 34.33±1.04 mm. Furthermore, the comparison of 2:8 ratio of mixture with the best initial ratio which was at 1:2 of pineapple crown to pomegranate peel (100 mg/mL) also showed the significant result. On the other hand, this 2:8 ratio of pineapple crown to pomegranate peel was compared with the 100 mg/mL of commercial fungicide, Brand X (34.17±1.04 mm) which is the best commercial fungicide to inhibit *A. niger* in this study had showed no significant different at p=0.85. This indicated that the mixture of pineapple crown and pomegranate peel at ratio 2:8 has a great potential to be utilized as potential antifungal agent against *A. niger*.

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