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Inhibition of *Aspergillus niger* by Application of *Punica granatum* Peels and *Cucurbita maxima* Seeds as Bio-fungicide

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ABSTRACT

Infection on plant caused by *Aspergillus niger* leads to the destruction of quantity and quality of crop yields. Normally, this disease is solved by the chemical fungicides. Therefore, this study was carried out to seek a potential natural fungicide from fruit waste which is safer and economical to inhibit *Aspergillus niger*. *Cucurbita maxima* (pumpkin) seeds and *Punica granatum* (pomegaranate) peels were extracted using maceration method with 80 % ethanol. Brine Shrimp Lethality Assay (BLSA) was used to test the presence of bioactive components in the extracts at concentration of 10 µg/mL, 100 µg/mL and 1000 µg/mL and they are expressed in terms of LC_{50} (Median Lethal Concentration) respectively. The study revealed that *Cucurbita maxima* extract was inactive, while *Punica granatum* extract and the mixture of both extracts at ratio 1:1 were active at 1000 µg/mL. Furthermore, the antifungal activity of *Cucurbita maxima* extract, *Punica granatum* extract, and mixture of both extracts were further tested using well-diffusion method against *A. niger* at 25 mg/mL, 50 mg/mL, 75 mg/mL and 100 mg/mL respectively. The findings revealed the mixture of both extracts were exerted effectively against *A. niger* at the lowest concentration with 20.67±2.52 mm and this gave significant zone of inhibition. The result of the study indicates that the mixture extraction of pomegranate peels and pumpkin seeds at 25 mg/mL has a great potential to be formulated as commercial bio-fungicide.

Keywords: M*ixture extraction; Antifungal; Brine shrimp lethality assay; Aspergillus; Pumpkin seeds; Pomegranate peels*

INTRODUCTION

Plant disease is a cause of losses in crop yield that could be a threat to global food security as growing size of human population from time to time [1]. Fungal infection could lead to severe loss of fresh produce, significant indirect economic effects to growers, the increasing price of production to the consumer direct and severe pathological effects on humans and animals [2]. *Aspergillus niger* is a filamentous fungus that naturally causes contaminant in food and it is the causal agent of the black mold [3]. It can even produce ochratoxins which is a group of very harmful secondary metabolites [4]. This fungus has capacity to cause

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decay of varied organic substances including fruits, vegetables, nuts, beans, grains, wood and herbal drugs [3].

There are many ways to treat fungal infection such as synthetic fungicides, biological control, and natural fungicides. Amongst these treatments, chemical fungicide is used heavily to treat plant disease and effectively to kill any plant pathogen [5]. However due to some circumstances, it leads to many side effects towards users and consumers [6]. Despite of their popularity, people nowadays are aware of the side effects of chemical compound in synthetic fungicides. They have slowly shifted to the alternative treatment that is safe and inexpensive. Pomegranate peels and pumpkin seeds are highly discarded fruit waste from the fruit-processing and they have been recognised as economic sources of an active antifungal agent [7].

Pomegranate peels contain varied nutritional values such as gallic acid, flavonols, ellagic tannins, anthocyanin, procyanidins and ellagic acid which demonstrate antimicrobial activity [8]. The active compounds such as punicalagin, castagalagin, granatin, catechin, gallocatechin, kaempferol are isolated from the pomegranate peels which have antimicrobial activity against *Candida albicans*, *Aspergillus niger*, *Penicillium citrinum*, *Rhizopus oryzae* and *Trichoderma reesei* [9]. Meanwhile pumpkin is an edible fruit and mostly consumed. Pumpkin flesh and seeds are known for their proteins and antioxidant vitamins [10]. Peptides found in pumpkin seeds were discovered for having capability to inhibit fungal growth such as *Botrytis cinerea*, *Fusarium oxysporum*, and *Mycosphaerella arachidicola* [11]. Therefore, the combination of pomegranate peels and pumpkin seeds could be an effort to formulate a bio-fungicide that preserves the yield and quality of crops.

EXPERIMENTAL

Preparation of pomegranate peel and pumpkin seed extracts

The pomegranate peels and pumpkin seeds were washed using sterile water and allowed to be air-dried. Both raw peels and seeds were pre-frozen at -80^{o}C for 24 hours and continued with freeze-dried at -40^{o}C for 28 hours [12]. After the process has completed, peels and seeds were ground to powder form. The powder of pomegranate peels and pumpkin seeds was extracted by using maceration technique. 20 g of the powder was mixed with 200 mL of 80% ethanol at ratio 1:10 and incubated in incubator shaker at 40^{o}C for five days at 200 rpm and continued with rotary evaporation at 40^{o}C [13].

Toxicity test

Brine Shrimp Lethality Assay (BLSA) was used to test active compounds in pomegranate peels and pumpkin seeds at different concentration which are 10 µg/mL, 100 µg/mL and 1000 µg/mL. Artificial seawater powder was dissolved in beaker containing 1000 mL distilled water [14] and 10 g shrimp eggs was added into the solution [15]. The hatching process took for almost 48 hours. When the eggs hatched, 10 active nauplii were placed in specimen bottle containing 9 mL of seawater [16]. Each bottle was added with pomegranate peel and pumpkin seed extracts respectively. The result was observed after 24 hours incubation and the dead nauplii was counted [14-16].

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Aspergillus niger culture preparation

Potato Dextrose Agar (PDA) was prepared by dissolving 39g in 1000 mL distilled water and autoclaved at 120^{o}C for 15 minutes. The PDA was poured into petri dish and was allowed to solidify at room temperature. Fungal mycelium of *A. niger* was sub-cultured on PDA and incubated at 28 ^{o}C for seven days [17]. After seven days, the spore was rubbed from colonies using a sterile glass slide [18]. Sterile water was added in petri dish and spore suspension was filtered using sterile double-layered muslin cloth [18]. The collected filtrate was preceded with spore counting. The number of spore was counted using haemocytometer and adjusted using serial dilution to obtain 10^6 spore/mL [19]. The size of fungal inoculum was verified using spectrophotometer in which the absorbance reading was in range 0.08 to 0.1 at 625 nm [20].

Antifungal test

Well-diffusion method was used to determine the antifungal activity of crude extracts. A sterile cork borer was used to make well in the middle of PDA agar plate [21]. *A. niger* spore suspension was used at 100 µL of 10^6 spore/mL and it was spread on the surface of PDA agar [4, 21]. The individual extraction of pomegranate peels and pumpkin seeds were diluted using sterile distilled water to obtain concentration at 25 mg/mL, 50 mg/mL, 75 mg/mL and 100 mg/mL [18]. The ratio used for mixture extraction of pomegranate peels and pumpkin seeds was set at 1:1. Fluconazole and 80% ethanol were used as positive and negative control respectively. All the prepared extraction was transferred into each well of PDA agar plate. Each treatment against *A. niger* was incubated at 28 ^{o}C for 48 hours and all treatments were carried out in triplicates [21].

Data analysis

Statistical analyses were carried out using Statistical Package for the Social Science (SPSS) software 20.0. All values obtained from this study were shown in mean $\pm$ SD. The P-value used in this study was less than 0.05 and it was considered as significant. The comparison of the treatment at the different concentration of crude extract against *A. niger* was analysed using one-way analysis of variance (ANOVA). Then, the Tukey's test was used in determining the significant concentration of *Cucurbita maxima* seeds and *Punica granatum* peels. Probit regression analysis was used to determine toxicity of pumpkin seeds, Pomegranate peels and the mixture of both were compared using Meyer's toxicity index at LC_{50} Values.

RESULTS AND DISCUSSION

Screening toxicity test of crude extracts

Toxicity test was carried out by using Brine Shrimp Lethality Assay (BSLA) in order to determine the presence of bioactive components in the crude extracts. In this study, toxicity of pumpkin seeds, pomegranate peels and mixture of both extracts were analysed using regression analysis at LC_{50} values. The value indicates at which concentration can kill half of nauplii population. Based on Meyer's toxicity index, crude extract is toxic (active) if LC_{50} values are less than 1000 µg/mL and if LC_{50} values are greater than 1000 µg/mL, the crude extract is non-toxic (inactive) [22].

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From Table 1, pomegranate peel extracts shows the highest brine shrimp larvicidal activity with LC_{50} values of 114.23 µg/mL, followed by the mixture extraction with 299.65 µg/mL and the lowest was the pumpkin seed extracts with 3239.09 µg/mL. Thus, pomegranate peel extracts and mixture extraction were considered as toxic (active), while pumpkin seed extracts were non-toxic (inactive) against nauplii based on Meyer's toxicity index. As can be seen from the result, it can be concluded that pomegranate peel extracts have effective bioactive compounds that gave higher lethality percentage compared to the mixture of extracts even when it was tested individually. This might be due to the presence of gallic acid, flavonols, ellagic tannins, anthocyanin, procyanidins and ellagic acid in pomegranate peels that can inhibit the growth of *Candida albicans, Aspergillus niger*, *Penicillium citrinum*, *Rhizopus oryzae* and *Trichoderma reesei* [8]. The shrimp lethality test in Table 1 has shown that % mortality increases gradually with the increase in concentration of the extracts.

Table 1: The number of shrimp nauplii that is survived after treating with pumpkin seeds, pomegranate peels and mixture of both extracts and the percentage mortality

Extracts	**Concentration (µg/mL)**	**Mean Number of Surviving Nauplii After 24h**	**Mortality %**	**LC_{50} (µg/mL)**	**Toxicity**
Pumpkin seeds	10	10	0%	3239.09	Non-toxic
	100	9	10%		
	1000	7	30%		
Pomegranate peels	10	9	10%	114.23	Toxic
	100	7	30%		
	1000	1	90%		
Mixture of pumpkin seeds and pomegranate peels (1:1)	10	10	0%	299.65	Toxic
	100	7	30%		
	1000	3	70%		

*According to Meyer's toxicity index for BSLA

Screening of antifungal activity in crude extracts

Antifungal activity of pumpkin seeds, pomegranate peels and their mixture was performed by the well-diffusion assay against *A. niger*. The combination of pumpkin seed and pomegranate peel extracts against *A. niger* was studied in order to compare between the extracts of pumpkin seed and pomegranate peel when they were tested individually at different concentration.

The findings in Table 2 show that all treatments give inhibition zone against *A. niger*. All treatments have demonstrated the potential of being antifungal agents since *A. niger* was inhibited at intermediate and

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susceptible range. The result showed that *A. niger* was highly susceptible towards the mixture of extracts at all tests concentration. As for pomegranate peel extracts, the result showed that they gave intermediate range at the lowest concentration and susceptible range at higher concentrations. The treatment from pumpkin seed extracts showed intermediate range at all concentrations.

Table 2: Antifungal activity of pumpkin seeds, pomegranate peels and mixture of both extracts against *A. niger* at different concentration (25, 50, 75 and 100 mg/mL)

Treatment	Concentration (mg/mL)	Zone of Inhibition (mm)
Pumpkin seeds	25	12.67±1.53
	50	13.33±1.53
	75	15.33±0.58
	100	15.33±1.53
Pomegranate peels	25	14.00±1.73
	50	21.33±1.53
	75	23.00±2.00
	100	25.33±2.08
Mixture of pumpkin seeds and pomegranate peels (1:1)	25	20.67±2.52
	50	24.33±1.53
	75	25.00±1.00
	100	26.00±2.00
Fluconazole (positive control)	25	19.00±1.00
80% ethanol (negative control)	-	0±0.00

Data are presented in mean±SD; n=3

Comparison on antifungal activity between group treatment against *A. niger* at different concentration

The study findings of pumpkin seeds, pomegranate peels and both mixture against *A. niger* at different concentration are presented in Table 3 using one-way ANOVA analysis. All treatments group revealed significant results, $p<0.05$.

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Table 3: Comparison between antifungal activity of crude extracts against *A. niger*

Concentration (mg/mL)	Group	Diameter of inhibition zone (mm)	p-value
25	Seed	12.67±1.53	0.005*
	Peel	14.00±1.73	
	Mixture	20.67±2.52	
50	Seed	13.33±1.53	0.000*
	Peel	21.33±1.53	
	Mixture	24.33±1.53	
75	Seed	15.33±0.58	0.000*
	Peel	23.00±2.00	
	Mixture	25.00±1.00	
100	Seed	15.33±1.53	0.001*
	Peel	25.33±2.08	
	Mixture	26.00±2.00	

Data are presented in mean±SD; n=3
*p<0.05 indicates significant difference by one-way ANOVA test

The comparison of antifungal activity between groups of treatment at different concentration against *A. niger* was further analysed using Post Hoc Tukey Test, as shown in Table 4. Table 4 shows the statistical difference among all treatments, but when it was compared to each treatment between groups, the findings revealed effectiveness for all group treatments at 25 mg/mL against *A. niger* with significant result for peel vs mixture and mixture vs seed and no significant result for seed vs peel. While for higher concentration of treatment at 50 mg/mL, 75 mg/mL and 100 mg/mL, the findings exerted significant antifungal activities for seed vs peel and mixture vs seed and there was no significant antifungal activity for peel vs mixture.

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Table 4: Post-Hoc comparison of antifungal activity of pumpkin seeds, pomegranate peels and mixture of both extracts against *A. niger*

Concentration (mg/mL)	Treatment between group	p-value
25	Seed vs Peel	0.701
	Peel vs Mixture	0.014*
	Mixture vs Seed	0.006*
50	Seed vs Peel	0.002*
	Peel vs Mixture	0.115
	Mixture vs Seed	0.000*
75	Seed vs Peel	0.010*
	Peel vs Mixture	0.237
	Mixture vs Seed	0.000*
100	Seed vs Peel	0.002*
	Peel vs Mixture	0.903
	Mixture vs Seed	0.001*

*The mean difference is significant at $p<0.05$ by Post Hoc Tukey Test

This study found that the mixture demonstrated the highest potential to inhibit the growth of *A. niger* even at the lowest concentration and it gave significant antifungal activities in terms of diameter zone inhibition compared to pomegranate peel and pumpkin seed extracts respectively. It is possible to hypothesize that punicalagin, castagalagin, granatin, catechin, gallocatechin, kaempferol [9, 18] in the pomegranate peels showed synergism with peptide as antifungal properties in pumpkin seeds which resulted in higher antifungal activity of the mixture as compared to pomegranate peel or pumpkin seed alone. This might be the phenolic compound in pomegranate peels contributed to inhibition of *A. niger* since there was a diverse of phenolic compounds in pomegranate peels compared to pumpkin seeds. The findings revealed interaction of bioactive compound in pomegranate peels enhances the antifungal activity of the pumpkin seed extracts A study done by [23] reported that the antimicrobial activity of combination extract of *Aegle marmelos* (stone apple), *Coreopsis auriculuta* (lobed tickseed) and *Cissus quadrangularis* (veld grape) showed higher inhibition zones than alone extract.

CONCLUSIONS

Toxicity screening for selected plant extracts was tested using BLSA against *Artemia* sp nauplii at 10 µg/mL, 100 µg/mL and 1000 µg/mL. The result of BLSA showed that mixture of extracts and pomegranate peel alone extracts exhibited higher mortality rate against *Artemia* sp. The observed median lethal concentration of pomegranate peel alone extracts and mixture of extracts to nauplii indicated the presence

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of potent cytotoxic and probably antifungal components of these fruit wastes. The study was further tested for antifungal activity of pumpkin seeds, pomegranate peels and mixture of both extracts against the growth of *A. niger*. All the treatments were tested at 25 mg/mL, 50 mg/mL, 75 mg/mL and 100 mg/mL and some concentration of extracts had shown remarkable biological activity. The finding showed the highest inhibition of *A. niger* was provided by the mixture of extracts of pumpkin seeds and pomegranate peels in range inhibition zone of 20-26 mm. This study showed that the mixture of extracts has a great potential to be formulated as antifungal agent against the growth of *A. niger*. The antifugal properties of *Punica granatum* peels and *Cucurbita maxima* seeds extract shown in this study might be contributed by the phenolic content. This can be confirmed by working on the standardized phenolic content extract and isolating the phenolic compound in the future. The toxicity properties of the sample can be validated through cytotoxicity test on normal cell line and it is further tested in-vivo.

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