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SCREENING OF ANTIFUNGAL ACTIVITY ON RAW RUBBER SHEET BY NATURAL EXTRACTS

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Abstract: Fungal contamination on raw rubber sheets causes a low selling price. Therefore, it is very important to control the growth of these fungi. The objective of this study is to search for natural extracts that can be used to prevent fungal growth on raw rubber sheets. Six fungal isolates; P1, A1, A2, A3, A4, and A5 were found on studied raw rubber sheets. They were morphologically classified into two genera, *Penicillium* and *Aspergillus*. These isolates were tested with 7 herbal extracts; tumeric, garlic, pomelo, lime, kaffir lime, neem, and large leaf neem by agar disk diffusion method. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were also determined by broth dilution method. The results showed that distilled water garlic extract was the most effective agent against the growth of the tested isolates. Minimal inhibitory concentrations (MICs) of garlic extract for P1, A1, A2, A3, A4, and A5 were 0.31, 0.31, 0.31, 0.63, 0.63, and 0.31 g% while minimal fungicidal concentrations (MFCs) were 0.63, 0.63, 0.63, 1.25, 1.25, and 1.25 g%, respectively. The garlic extract at 10 times MIC could kill all isolates within 5 hours. In conclusion, the garlic extract is potentially effective as fungistasis and fungicide used to prevent or kill fungal contamination on raw rubber sheets.

Keywords: raw rubber sheet, antifungal activity, natural extracts

INTRODUCTION

Raw rubber sheets from *Hevea brasiliensis* are the economically important products of Thailand. In Southern region, the major cultivation area, fungal contamination on raw rubber causes a low selling price. The common fungi found on raw rubber are *Aspergillus* sp., *Pennicillium* sp., *Fusarium* sp. *Paecilomyces* sp. and *Trichoderma* sp. (Premphet *et al*., 2012). Enormous efforts are made to inhibit the fungal growth on rubber sheets, for example solar energy house for air dried sheet**.** In addition, certain chemical substances such as paradinitrophenol, acetic acid, potassium sorbate, and potassium benzoate, have been used as potential antifungal agents (Samiran and Dutta, 2003**)**; **.** However, using these chemical substances involves safety concern on chemical remains. Alternatively, solar energy house is used in some areas such as North or North East regions of Thailand but it is costly. Therefore, among the potential new agents, natural products could be an interesting alternative because natural products usually are less toxic and cheaper than some chemical substances.

An important source of natural products is plants or herbs which can be cultivated in many regions of Thailand. These natural products have been found in vitro to have antifungal properties; for example, rhizome of tumeric, clover of garlic, leaves of kaffir lime, and leaves of neem could inhibit *Aspergillus* growth whereas peel of pomelo and leaves of lime could inhibit *Penicillium* growth (Martins *et al*., 2009; Reddy *et al*., 2009). In addition, Chatkaew *et*

al., (2009) demonstrated that crude extract from *Cassia siamea* leaves at the concentration of 10,000 mg/L suppressed the spore germination of *Fusarium* sp. and *Lasiodiplodia theobromae*, causative agents of Banana Crown Rot, by 100 and 64.36% respectively but there was no effect on mycelial growth. Furthermore, Elsayed, Y and Shabana, Y. (2018) demonstrated that essential oils extracted from clove and camphor have fungal activities against *Aspergillus niger* and A*lternaria alternata,* the most common fungi infesting the heritage oil paintings and other heritage objects. Clove and camphor oils suppressed *Aspergillus niger* and A*lternaria alternata* cultivated on simulated canvas painting model. In addition, camphor oil at concentration 80% (v/v) showed a better antifungal activity than clove for both fungi.

Therefore, the aim of this study was to investigate the efficiency of natural product extracts in inhibiting fungal growth on raw rubber sheet. Minimal inhibitory concentration (MIC), the minimal fungicidal concentration (MFC) and the time killing of the selected natural products were studied.

MATERIALS AND METHODS

Isolation and Characterization of fungi from raw rubber sheets

Raw rubber sheets contaminated with fungi were collected from para rubber farms in Suratthani and Nakhonsi- thammarat province. The contaminated fingi were cultivated on Sabouraud Dextrose Agar (SDA), and they were characterized by using cell morphology and colony morphology.

Extraction of natural products

Seven local herbs, rhizome of tumeric, clover of garlic, peel of pomelo, leaves of lime, leaves of kaffir lime, leaves of neem, and leaves of large leaf neem, were collected from Nakhon Pathom and Suratthani province. Each herb was extracted with two solvents, distilled water and 95% ethanol, in separatory funnel for 2 h (10 g herb : 100 ml solvent). The distilled water extracts were kept at 4 ℃, while ethanolic extracts were evaporated by rotary evaporator and crude extracts were suspended in 1ml of 5% DMSO for further study.

Screening of antimicrobial activity of natural extracts

Antimicrobial activity was determined by disk diffusion method. A suspension of any tested fungi containing 10^5 cells/ml fungal spores was spread on Sabouraud Dextrose Agar by three-way swab technique using sterile cotton swabs. 10 µl of the extract solution was dropped on 6 mm filter papers placed on the agar surface. Cycloheximide, was used as a positive control and 5% DMSO and sterile distilled water were negative controls. All plates were incubated at 30˚C for 24-48 h. The diameter of the inhibition zones was measured. Triplicates of each plate have been done.

Examination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

The MIC of natural products was determined by broth dilution method. The suspension of each crude natural product extract (10% w/v) was serially diluted in 100 µl of Sabouraud Dextrose Broth (SDB) in 96 well plates. 100 µl of 10^4 cells/ml fungal spore suspension in SDB were added to each well. The plate was incubated at 30˚C for 18-24 h. Each test was repeated in duplicates. The MIC was noted as the minimal concentration of extracts that inhibited the growth of fungi. All suspensions that did not show turbidity of the fungal growth were stransfered to SDA and incubated at 30˚C for 24-48 h. The minimal concentration of the suspensions that did not show single colony on SDA was noted as MFC.

Time-kill study

One mL of the test culture was added to 19 mL of fresh SDB. Kinetics of kill testing was conducted by exposing $5x10^4$ cells/ml of fungal spore to the garlic aqueous extract at 10 times of MIC for various periods of time. At the end of the exposure time (i.e. 1, 3, 5, and 24 h.), the "treated" cells were serially diluted in the neutralizing medium. The diluted fungal suspensions were spread on to SDA and incubated at 30 °C for 24 h. to determine the surviving colony forming unit (CFU) per mL. Time kill was recorded when viable cells were reduced at least 10^3 fold from the initial concentration.

RESULTS

Isolation and characterization of fungi from raw rubber sheets

Six fungal isolates; P1, A1, A2, A3, A4, and A5 were isolated. They were morphologically classified into two genera, *Penicillium*; P1 and *Aspergillus*; A1, A2, A3, A4, and A5 as shown in Figure 1.

Screening of antimicrobial activity of natural extracts

The aqueous crude extracts of natural products of rhizome of tumeric, peel of pomelo, leaves of lime, leaves of kaffir lime, leaves of neem, and leaves of large leaf neem showed no antifungal effect against all fungi tested. Both distilled water and ethanolic extracts of garlic showed the highest antimicrobial effect against all isolates determined by disk diffusion method as shown in Table 1. Based on this result, these extracts were selected for further testing.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

The distilled water extract of garlic showed more effective against all the isolates than the ethanolic extract of garlic as shown in Table 2.

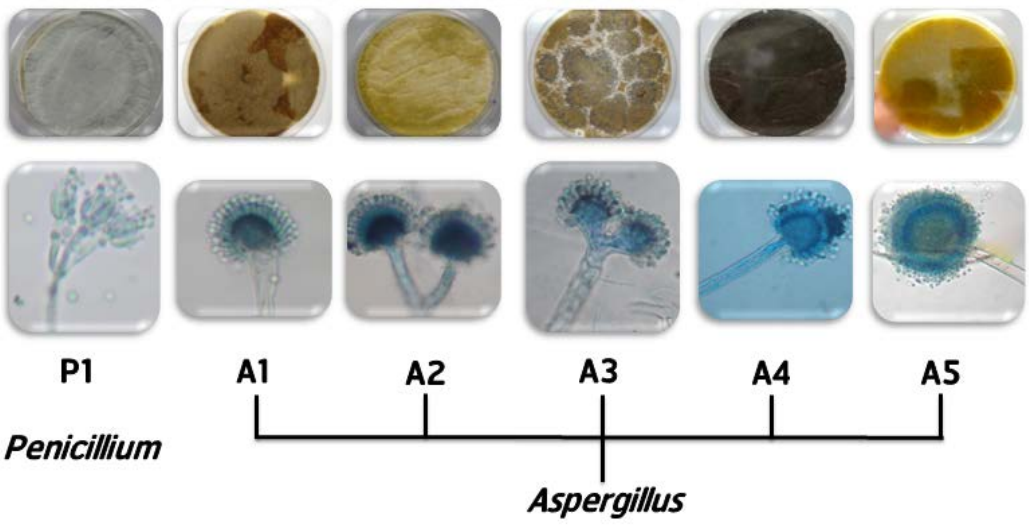


Figure 1. The morphology of six fungal isolates

Table 1. The inhibition zone of natural extracts against fungal isolates

Natural extracts	Extract	Diameter of inhibition zone (mm)					
		Isolate					
		P1	A1	A2	A3	A4	A5
rhizome of tumeric (*Curcuma longa*)	Aqueous extract	-	-	-	-	-	-
	Ethanol extract		12.7±2.5	10.7±2.1	10.3±0.6	-	10.0±1.0
Garlic (*Allium sativum*)	Aqueousextract	27.7±2.1	19.7±4.5	16.5±2.1	7.3±1.4	23.5±4.9	16.5±0.7
	Ethanol extract	22.3±2.5	11.7±2.3	13.0±2.0	12.0±2.7	12.3±2.5	10.3±0.6
leaves of lime (*Citrus aurantifolia*)	Aqueous extract	-	-	-	-	-	-
	Ethanol extract	8.5±0.7	10.7±1.5	8.5±0.7	12.0±0.0	11.6±1.5	8.7±0.6
Peel of pomelo (*Citrus maxima*)	Aqueous extract	-	-	-	-	-	-
	Ethanol extract	9.0±1.0	10.7±2.1	9.7±0.6	9.3±0.6	9.0±0.0	9.0±0.0
leaves of kaffir lime (*Citrus hystrix*)	Aqueous extract						
	Ethanol extract	12.5±3.5	-	9.6±0.2	-	12.0±0.0	-
leaves of neem (*Azadirachta indica*)	Aqueous extract	-	-	-	-	-	-
	Ethanol extract			9.0±0.0			
leaves of large neem (*Azadirachta excelsa*)	Aqueous extract	-	-	-	-	-	-
	Ethanol extract	9.5±0.7	10.0±0.0	9.0±1.4		8.5±2.1	
cycloheximide (positive control)		15.4±1.2	17.3±1.3	16.7±1.2	16.3±1.3	19.2±1.1	16.4±2.1

Table 2. MIC and MFC of the garlic extracts

Strains	Aqueous extract		Ethanol extract	
	MIC (g%)	MFC (g%)	MIC (g%)	MFC (g%)
P1	0.31	0.63	62.50	250.00
A1	0.31	0.63	62.50	250.00
A2	0.31	0.63	15.60	31.25
A3	0.63	1.25	31.25	31.25
A4	0.63	1.25	31.25	31.25
A5	0.31	1.25	15.60	31.25

Time kill assay

The garlic extract at 10-fold MIC concentration could kill all isolates ($5x10^4$ cells/ml) within 5 hours as shown in Figure 3.

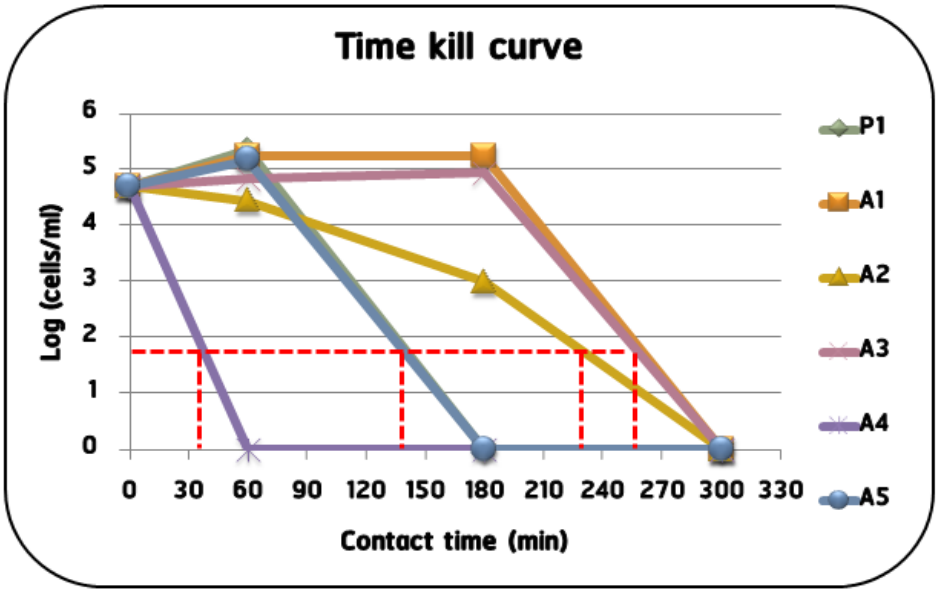


Figure 3. Time kill curve of the garlic aqueous extract

DISCUSSION AND CONCLUSION

Raw rubber sheets are the economically important products of Thailand since they are used in many products including medical devices, surgical gloves and various consumer products. Fungal contamination is a big problem for the production of rubber sheets because it's lower grade and price of the rubber sheets. Various kinds of fungi have been found on raw rubber sheets since fungal spores are abundant in the air and environmental factors such as humidity, temperature and nutrients promote fungal growth on rubber sheets. Chanduaykit (2008) isolated eleven genera of fungi from rubber sheets collected from 14 provinces of the southern part of Thailand. Those fungi included *Aspergillus, Penicillium, Fusarium, Cladosporium, Rhizopus, Mucor, Geotrichum, Trichoderma, Tritirachium, Dalinia* and *Shizophyllum.* In this study, only two genera of fungi, *Penicillium* and *Aspergillus,* were isolated from raw rubber sheets collected from Suratthani and Nakhon- srithammarat province. The isolates were tested against some natural products such as local herbs, rhizome of tumeric, clover of garlic, peel of pomelo, leaves of lime, leaves of kaffir lime, leaves of neem, and leaves of large leaf neem. Distilled water garlic extract showed the highest antifungal effect against all isolates. This result is corresponding to the report of Reddy *et al.*, (2009) who demonstrated that the garlic extract at 10 g/L concentration could inhibit *A.flavus* growth and aflatoxin B1 produced by *A.flavus*. In addition, the garlic distilled water extract was more potent than garlic ethanolic extract because the MICs (0.31-0.63 g%) and MFCs (0.63-1.25 g%) of the garlic aqueous extract were lower than those of the garlic ethanolic extract. One of active compounds in the garlic aqueous extract might be allicin, the major medicinal compound obtained from garlic which is a water soluble compound (Miron, 2000; Yamada, 1977; Block, 1985). It is well documented that an active constituent in a plant is varied on the method of extraction. Kutawa *et al*., (2018) stated that ethanolic garlic extract showed inhibitory activity among *Fusarium* spp and *Rhizopus* spp, where *Rhizopus* spp is more susceptible to aqueous extract while *Fusarium* spp is more susceptible to the ethanolic garlic extract. The antifungal activity of garlic is due to the allicin or ajoene compound which controls the function of some important fungal enzymes.

The time kill results showed that garlic extract at 10-fold MIC concentration could kill all isolates within 5 hours. In addition, the extract stored in 4°C for 3 weeks showed no change in its effectiveness (data not showed). However, further study should be performed to demonstrate the active compounds in this garlic extract.

In conclusion, the garlic aqueous extract can be used as an alternative antifungal agent to inhibit fungal growth on raw rubber sheets.

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