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# PRELIMINARY STUDY ON ANTIFUNGAL ACTIVITY OF PYROLIGNEOUS ACID FROM *Rhizophora apiculata*

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## ABSTRACT

*Pyroligneous acid is the name of the crude condensate produced from the distillation of smoke generated in the process of charcoal making. The antifungal activity of Rhizophora apiculata pyroligneous acid was evaluated. Pathogenic fungi was firstly taken from the infected leaves of untreated chilli plant and cultured on Potato Dextrose Agar (PDA). Purification of the culture was done by using pour plate method. The fungi morphology was further determined by using methylene blue under light microscope and was identified as Leveillula species. Leveillula sp. was successfully isolated and further cultured. Four different concentrations of pyroligneous acid (25, 50, 75 and 100 %) was mixed thoroughly with the molten agar using food poisoned technique. The antifungal activity was left for 72hours before the mycelial growth was measured. The results revealed that 50, 75 and 100% of pyroligneous acid showed significant antifungal activity towards Leveillula species with 100% of percentage inhibition. Hence, this study has proven that pyroligneous acid can be an alternative in agriculture as organic fungicide to combat L. taurica on chilli plants.*

**Keywords**: Antifungal activity, *Rhizophora apiculata*, pyroligneous acid, *Leveillula taurica*

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## 1. INTRODUCTION

Genus *Rhizophora* is one of the genus with medicinal benefit since they are sources of well-known and medically useful secondary products (Halim *et al.*, 2013).The most common species representatives are *Rhizophora apiculata*, *Rizophora mucronata and Rhizophora mangle*. In Malaysia, *Rhizophora apiculata* or locally known as 'bakau minyak' is a famous mangrove plant that widely used in charcoal industry. These charcoal will be marketed locally and exported mainly to Japan for various purposes such as cosmetics and pharmaceutical usage.

Pyroligneous acid (PA) is a crude condensate produced from distillation of smoke generated in the process of wood carbonization and charcoal making. Various study on the pyroligneous acid of *R. apiculata* such as antibacterial, antioxidant and radical scavenging activities has been done previously, which reported interesting results, respectively. Recent study on the antibacterial effects of *R. apiculata* pyroligneous acid towards 16 pathogenic bacteria showed that the dichloromethane extracts possess the most potential antibacterial agent with minimum inhibitory concentration (MIC) values between 1.56-3.12mg/mL. Scanning electron micrographs (SEM) on the treated

*Bacillus subtilis* confirmed the damaged cells caused by the extract (Ibrahim *et al.*, 2014). Study on antioxidant and radical scavenging activities by Loo *et al.* discovered the dichloromethane extracts of pyroligneous acid exhibited superior free radical scavenging activities with $EC_{50}$ value = 0.1235 mg/ml, whereas in phosphomolybdenum assay, the extract also showed the greatest antioxidant efficacy (Loo *et al.*, 2007).

Pathogenic and toxinogenic fungi, which constantly developing resistance against commonly used fungiside, have becoming a critical problem in many areas such as agriculture, human and animal health, as well as storage of foods. (Howard *et al.*, 2010 ; Ahmad *et al.*, 2011). Besides their potential to cause yield losses and food decay, many of them produce dangerous secondary metabolite which are extremely hazardous to consumers. Therefore, few studies on antifungal activity of pyroligneous acid against several plant pathogenic fungi has been reported. Investigation on the inhibition effect of pyroligneous acid towards the pathogenic fungus, *Alternaria mali*, which is known to be the agent of alternaria blotch of apple plants has revealed that the growth of *A. mali* was completely inhibited using pyroligneous acid at a dilution of 1:32 (Jung, 2007). However, its antifungal activity against *Leveilulla taurica* which causes powdery mildew disease on a broad range of plants such as chilli, pepper, tomato, eggplant, onion ,cotton and other crops, specifically in Malaysia, has not yet been studied in detail. For instance, the powdery mildew disease can cause a serious fungal threat to agricultural production (Zheng *et al.*, 2013). The higher the level of powdery mildew infections, the higher the loss of production of crops to the farmers. Thus, the discovery of new antifungal agents with cost-effective and chemical-free from natural resources is a fundamental challenge for scientific community.

## 2. MATERIALS AND METHODS

### 2.1 Pyroligneous acid

The *R. apiculata* pyroligneous acid was obtained from charcoal factory in Kuala Sepetang, Taiping, Perak.

The smoke that escape from the chimneys of the charcoal's kiln at temperature of 240-500 °C were passed through a 30 meter extension of air cooled stainless steel pipe for condensation purpose. The condensed smokes or known as pyroligneous acid or wood vinegar was collected in a polyethylene container and stored at room temperature (30±2 °C) and was used in the present study. The pyroligneous acid has a clear reddish brown color with smoky odor.

### 2.2 Preparation different concentration of Pyroligneous Acid

The pyroligneous acid was filtered through a Whatman No. 1 filter paper to eliminate any debris and oily phase. It was then diluted with sterilized distilled water to give different concentration (25, 50, 75 and 100%).

### 2.3 FTIR screening

The functional group present in pyroligneous acid were identified using PerkinElmer Frontier FTIR model.

### 2.4 Isolation and identification of fungal

The fungi used in this study were obtained from untreated chilli plant. The dominant populations of fungal that were successfully grown in media were chosen to be isolated. The fungal was isolated under aseptic techniques. Single spore of each fungal from the growth of mycelium was transferred into fresh PDA that was amended with streptomycin to prevent bacteria growth. This is for sub-culture by using sterilized cork-borer and was sealed with parafilm to prevent contamination. The plate was then incubated at 30°C for three days. The fungus isolated from the leaves of chilli plant was then stained using methylene blue to determine the morphology of fungi under light microscope. The identification was performed at genus level on the basis of microscopic characteristics (septation in mycelium, presence of specific reproductive structures, shape and structure of conodia). The fungal strains were grown on potato dextrose agar (PDA) and were incubated at 30ºC.

### 2.5 Antifungal activity of pyroligneous acid

The antifungal activity of the pyroligneous acid was tested against *Leveillula* sp. using the poisoned food technique in triplicates at all concentrations (Kumar & Kaushik, 2013). Series of dilution of pyroligneous acid with concentration of 25, 50, 75 and 100% were added into molten potato dextrose agar (PDA) in a test tube. The solution was mixed thoroughly and then poured into sterilized petri plate to solidify. With the aid of a cork borer, 6mm mycelium block of *Leveillula* sp. was inoculated at the centre of each petri plate in an inverted position for greater contact of the mycelium with the agar (Ibrahim *et al.*, 2016). The plate was then sealed by using parafilm. The diameter of fungi growth in each plates were observed after 72 hours. The control plates were kept where the *Leveillula* sp. were grown in same condition on PDA without pyroligneous acid. The antifungal activity of the *R. apiculata* pyroligneous acid represented by percentage inhibition of mycelia growth which calculated by using formula;

$$\text{Percentage inhibition (\%)} = \frac{dc-dt}{dc} \times 100$$

where *dc* = average of mycelial growth in control, *dt* = average of mycelial growth in treatment (Satish *et al*., 2007)

### 2.6 Statistical Analysis

Data were analyzed by using Statistical Analysis Software (SAS) Version 9.1 and subjected to one-way Analysis of Varience (ANOVA). The mean values were compared by using Tukey test with significant value of $P \leq 0.05$.

## 3. RESULT AND DISCUSSION

### 3.1 FTIR Screening

Analysis on chemical constituents from *R. apiculata* revealed that alcohols compound were predominant constituents (Abidin *et al*., 2013) in this species. The FTIR spectrum of pyroligneous acid gave a broad band peak present at 3398.75 $cm^{-1}$ arises from the hydroxyl group. Another absorption band at 1421.58 $cm^{-1}$ and 1615.79 $cm^{-1}$ strongly indicated the

presence of the C=C of aromatic ring, whereas a strong absorption band at 1701.42cm$^{-1}$ was attributed to the carbonyl group C=O. The presence of C-O bond was also confirmed from the band peak observed at 1361.87 cm$^{-1}$.

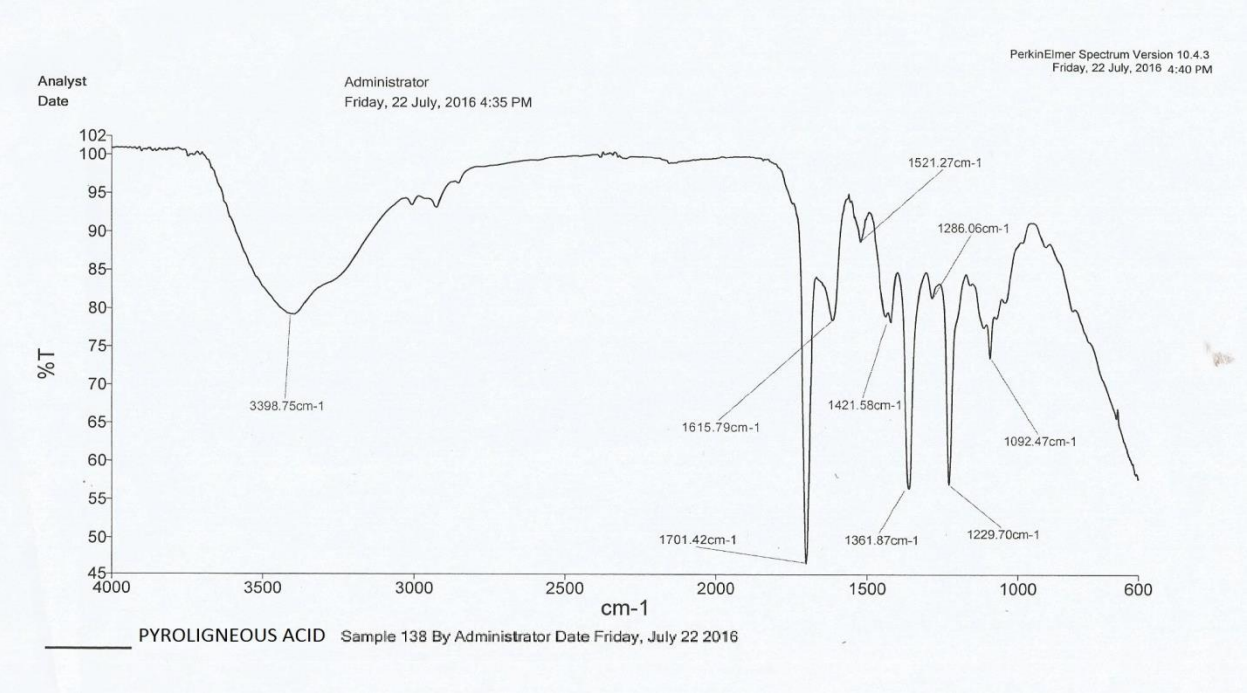


Figure 1: The IR spectrum of *Rhizophora apiculata* pyroligneous acid

### 3.2 Identification of fungi

*Leveilulla* sp. was isolated from untreated chilli plant and cultivated on Potato Dextrose Agar and was identified using methylene blue. The hypae of *Leveilulla* sp. is confirmed based on its conidial state reported on *Peganum harmala* (Ahmed & Abdel-Azeem, 2015). The pure colony obtained from the 3 days culture is shown in Figure 2.

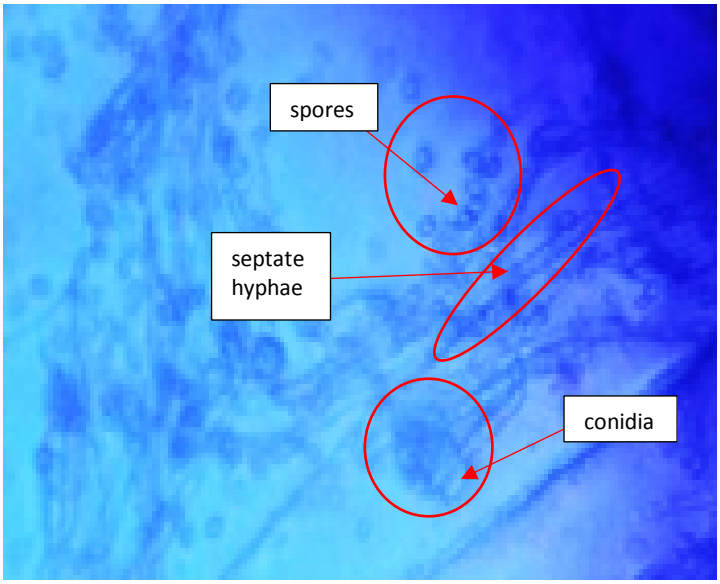


Figure 2: The microscopic observation of *Leveilulla* sp. on chilli plant under 40x10 magnification.

### 3.3 Evaluation of Antifungal Activities

The poisoned food technique was applied to evaluate efficacy of *R. apiculata* pyroligneous acid against *Leveilulla* sp. (Khadse *et. al*, 2015). The pyroligneous acid of *R. apiculata* showed positive result since the fungi only growth on the surface of the PDA agar with 25% pyroligneous acid (PA), instead of the other concentration which are 100%, 75% and 50%. The simplified result of antifungal activities was tabulated in Table 1.

Table 1 : Effect of *R. apiculata* pyroligneous acid against *Leveilulla* sp.

| Concentration of PA | Percentage inhibition (%) |
|---|---|
| 100% | 100.00 ± 0.0 [a] |
| 75% | 100.00 ± 0.0 [a] |
| 50% | 100.00 ± 0.0 [a] |
| 25% | 77.50 ± 0.5 [b] |

*Means followed by the same letters within each bar did not significantly differ by Tukey test at P≥0.05 (n=3)

Helena (2005) stated that lipophilicity or the presence of at least one acidic hydroxyl group considered being a structural feature that essential for a good antifungal activity. Transport

process of molecules through cell membrane in living organism depend on their lipophilicity (Helena *et al.*, 2005). The pyroligneous acid which has been reported to contain a complex mixture of water (10-20%), a mixture of carboxylic acid, several aldehydes and alcohols. It is proved that pyroligneous acid exhibiting strong antifungal activity against *Leveillula* sp. Phenolic derivatives are believed to be one of the component responsible for the antifungal activity of pyroligneous acid. The pH value of pyroligneous acid is low ranging from pH2 - 3, due to its high amounts of volatile acids, mainly acetic acid and formic acid (Loo, 2008). These acids are the main components responsible for the mild corrosive of pyroligneous acid. Pyroligneous acid has long been used as a natural organic pesticide. Pyroligneous acid can inhibit the growth of *Leveillula* sp. thus it helps to increase the growth of plant roots (Loo, 2008).

## 4. CONCLUSION

The present study conclusively demonstrates the antifungal potential of the *R. apiculata* pyroligneous acid against powdery mildew fungi, *Leveilulla* sp. which causes serious fungal threat to agricultural production mostly to the chili plant. *R. apiculata* pyroligneous acid with concentration of 100, 75 and 50% had successfully inhibit the fungal growth using food poisoned technique. The presence of the hydroxyl group appears to be the main structural feature for a good antifungal activity. This is the first report on antifungal activity of *R. apiculata* pyroligneous acid against *Leveilulla* sp. In addition, further study on Minimum Inhibition Concentration below than 50% concentration will be necessary to find the lowest concentration that might inhibit the fungal growth, specifically.

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