

PHYTOCHEMICAL ANALYSIS OF *Mucuna pruriens* L. LEAVES EXTRACT AND ITS INHIBITION TEST AGAINST *Curvularia lunata* (WAKK). BOED. THAT CAUSED LETTUCE LEAF SPOT

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ABSTRACT

Curvularia lunata is a pathogenic fungi that attack various plant species including lettuce. Its control needs to be carried out in an environmentally friendly manner, namely using botanical fungicides. *M. pruriens* is a legume plant that is widespread in Indonesia that has a potential as a botanical fungicide, beside as a traditional medicine. The purpose of this study was to study the chemical content of *M. pruriens* leaves and its potency to inhibit the growth of *C. lunata*. The determination of the compounds was carried out by Gas Chromatography-Mass Spectrophotometry and the inhibition test was carried out using diffusion well, and agar dillution method. The result showed that the crude extract of *M. pruriens* leaves contained ethyl isothiocyanate that act as an antifungal with AUC of 20,78%. The MIC of the extract was 0,9%. The extract at 5% concentration was capable to inhibit *C. lunata* growth, mycelium mass, and conidia production respectively 38,33%; 67,14%; 99,88% compared to control.

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INTRODUCTION

Curvularia lunata is a pathogenic fungus that attacks various types of plants (Garcia-Aroca et al., 2018), including lettuce. The highlands climatic conditions that have high humidity are optimal conditions for

pathogenic fungi to grow. *C. lunata* is one of the important pathogens that cause leaf spots in lettuce especially in nurseries and causes extensive losses (Baiyee et al., 2018). The first report on lettuce was in Thailand in 2018 (Pornsuriya et al., 2018). *C. lunata* spreads

through the soil and seeds (Akinbode, 2010; Watanabe, 2010). The application of synthetic fungicides is the most effective control technique for controlling *C. lunata*. Mancozeb-based fungicides are the most effective fungicides (Abrar et al., 2020).

Long-term application of mancozeb-based fungicides will cause *C. lunata* to be resistant against this fungicide (Susanto and Prasetyo, 2013). Therefore, environmentally friendly control is needed, namely with botanical fungicides. *Mucuna pruriens* is a legume plant that is widespread in Indonesia (Mulyani et al., 2016). *M. pruriens* is often used as a traditional medicine traditional (Sathiyarayanan and Arulmozhi, 2007), in addition, *M. pruriens* also has the potential to be a botanical fungicide because it has antifungal properties (Nidiry et al., 2011). Nidiry et al. (2011) in their study state that *M. pruriens* seeds extractive was able to inhibit *Colletotrichum* sp. and *Fusarium solani*. Khan et al. (2008) also state that *M. pruriens* seed extract from various solvent showed antifungal activity against *Aspergillus niger*.

Study conducted by Rayavarapu et al. (2011) found that the whole plant of *M. pruriens* extract was able to inhibit the growth of *Curvularia lunata*, *Fusarium oxysporum*, *Pencillium expansum*, *Rhizoctonia solani*,

Tiarosporella phaseolina, *Ustilago maydis*. The aim of this study was to determine the content of compounds in the leaf extract of *M. pruriens* and its potential in inhibiting the growth of *C. lunata*.

MATERIAL AND METHOD

The equipments used in this study were autoclave, laminar air flow cabinet, vacuum rotary evaporator, gas chromatography-mass spectrophotometry, haemocytometer, microscope, blender, test tubes, petri dishes, erlemeyers, beaker glass, measuring cups, sprayers, micropipette, tweezers, scalpels, ose needles, cork borer, calipers, stationery, and camera. The materials used in this study were *M. pruriens* leaves and lettuce leaves that were symptomatic of *C. lunata* attacks. Other materials used are aquades, PDA (Potato Dextrose Agar) media, alcohol (70%) Pro Analyst Methanol MERCK™, HCl (10%), aluminum foils, plastic wraps, Whatman No.2 filter papers, tissue papers, and labels.

Extract preparation

M. pruriens leaves samples were taken in South Denpasar. The leaves then washed and cleaned and then airdried for 10 days. The dried leaves then grinded with a blender to get simplisia. The leaf simplisia then macerated with methanol Pro Analyst MERCK™ in a

ratio of 1:5 for 5x24 hours and stirred periodically. The solution then filtered using Whatman No.2 filter paper. Macerate concentrated with a vacuum rotary evaporator to obtain a crude extract.

Compound analysis

Analysis of *M. pruriens* leaf extract compounds was carried out at the Bidlabfor Forensic Laboratory of the Bali Regional Police Departement. The extract was analyzed with Agilent 7890 MSD 5977B type Chromatography-Mass Spectrophotometry (GC-MS) Gas, with a Wakosil ODS/5C18-200 silica column with a size of 4.6 x 200 mm using N₂ gas as a career. The injection temperature used was 290°C for 27 minutes with an injection rate of 1 ml/min.

Culture media preparation

PDA media is made by boiling 200 g of potatoes in 1 L of aquades for 1 hour. The boiled water of the potatoes then filtered and added 20 g of dextrose, 15 g of agar, and 0.5 g of chloramphenicol, then stirred until dissolved. The media was sterilized using an autoclave at a temperature of 121°C for 15 minutes. The sterile media then allowed to stand so that the temperature becomes 40°C and then poured into a sterile petri dish.

Pathogen isolation and identification

Symptomatic lettuce leaves were taken and sterilized using 70% alcohol on the part between the symptomatic and healthy part. The leaves were cut by 0.5 cm x 0.5 cm and then placed on a solid PDA medium and incubated for 5 days at a temperature of 25°C. The growing fungus then purified. Once pure, isolates were observed under a microscope and identified based on their morphology following the Barnett and Hunter (1998) identification key.

Determination of minimum inhibitory concentration

Determination of Minimum Inhibitory Concentration was carried out by diffusion wells method with triplicate. The tested concentration was 0%; 0,1%; 0,2%; 0,3%; 0,4%; 0,5%; 0,6%; 0,7%; 0,8%; 0,9%; 1%; 1,1%; 1,2%; 1,3%; 1,4%; 1.5% and aquades as control. The extract is dissolved with 80% Tween 10% solution to obtain the test concentration. A total of 0.5 ml of spore suspension was poured in 20 ml of diluted PDA and shaken until evenly distributed in a petri dish. Wells are made using a cork borer with 5 mm diameter. Each well was filled with 20 µl of extract solution. The media then stored in a refrigerator at a temperature of 4°C

for 2 hours, then incubated at a temperature of 25°C for 2 days.

Inhibition test assay

The inhibition test against the growth of *C. lunata* was carried out by agar dilution method. The design used was a Complete Randomized Design with four replicate. The observed variables were colony growth, mycelium mass, and the number of conidia. The concentration of the extract tested was 0.1%; 0,2%; 0,5%; 1%; 2%; 5%; and 0% as a

control. A colony of *C. lunata* with 5 mm in diameter was placed in the center of a solid PDA medium that already contains the extract. Observations were made as the control fungus fully grew to the petri. For colony growth assay, the diameter of the colony was measured, then the percentage of colony growth inhibition of the extract is calculated by equation (1).

$$\text{Inhibiton rate (\%)} = \frac{\text{control diameter} - \text{treatment diameter}}{\text{control diameter}} \times 100\% \dots \dots \dots (1)$$

For the mass of mycelium assay, the mycelium of *C. lunata* was cleaned from the media by pouring heated 10% HCl, then filtered using filter paper. The mycelium then dried at a temperature of 80°C until its weight constant, then weighed. For the amount of conidia formed, conidia were harvested by pouring 20 mL of aquades, then dredged with an ose needle so that a conidia suspension

obtained. The number of conidia was calculated using a haemocytometer.

Data analysis

The data obtained were quantitatively analyzed using analysis of variance (ANOVA) at a level of 5% and then analyzed using the Least Significant Difference (LSD) test with a level of 5%.

RESULT AND DISCUSSION

Maceration of 0.5 kg of simplisia leaves of *M. pruriens* produced a rendement of 4.13%, which is 20.63 g of green-yellow-

black crude extract. The crude extract obtained then analyzed using GC-MS and tested its antifungal activity. The results of the

analysis of the content of compounds in the leaf extract of *M. pruriens* using GC-MS

showed that there were 22 compounds contained in the extract shown in Figure 1.

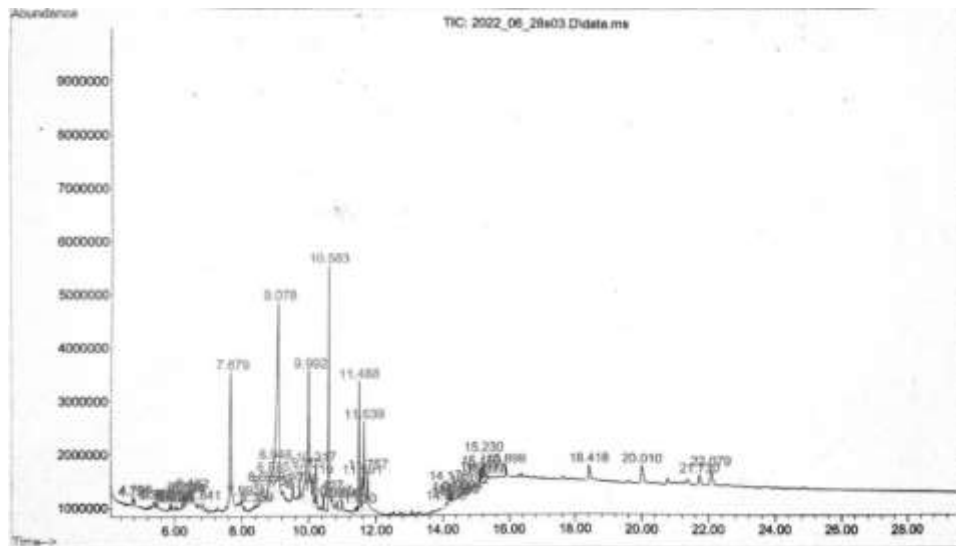


Figure 1 Chromatogram of *M. pruriens* leaves extract

Compounds contained in the leaf extract of *M. pruriens* were, 3-propoxypropan-1-amine; N-butylacetamide; 2-oxobutanoic acid; D-galactonic acid, γ -lactone; phenylephrine; β -D-glucosyloxyazoxymethane; 3-(1-isopropyl-but-3-enyloxy)-butyric acid; 9,11-octadecadiyonic acid,8-hydroxy-, methyl ester; ethyl isothiocyanate; inositol; D-galactose; heptadecanal; 1,2-15,16-

diepoxyhexadecane; hexadecanoic acid, methyl ester; N-hexadecanoic acid; tetraacetyl-d-xylonic nitrile; phytol; 9,12-octadecadienoic acid (Z,Z)-; linoelaidic acid; 9,12,15-octadecatrien-1-ol; 9,12,15-octadecatrienoic acid, (Z,Z,Z)-; and octadecanoic acid. The main compounds contained in the of *M. pruriens* leaves crude extract are presented in Table 1.

Table 1. Primary compounds found in *M. pruriens* leaves extract

No.	Compound Name	AUC (%)	Rf
1	N-butylacetamide	11,31	7.679
2	2-oxobutanoic acid	11,31	7.679
3	Ethyl isothiocyanate	20,78	9.078
4	Inositol	10,51	9.992
5	D-galactose	10,51	9.992
6	N-hexadecanoic acid	8,33	10.583

The main component contained in the *M. pruriens* leaves crude extract was ethyl isothiocyanate with an AUC value of 20.78% at a retention time of 9,078, this compound has an antifungal property (Smolinska et al., 2003; Kurt et al., 2011; Wu et al., 2011; Kara and Soyly, 2020). N-butylacetamide and 2-oxobutanoic acid are the second most constituent compounds found in the extract with an AUC ratio of 11.31% at a retention time of 7,679. Successively these compounds function as insect repellents (Debboun et al., 2015) and flavorings (PubChem, 2004). The third highest content were inositol and D-galactose at a time of 9,992 with an AUC value of 10.51%. Inositol often used in the treatment of Polycytic ovary syndrome

(PCOS) (Holub, 1986; Levine, 1997; Nestler et al., 1999; Sacchinelli et al., 2014; Unfer et al., 2012), whereas D-galactose used as a senescent inducer in the study of aging models in animals (Bo-Htay et al., 2018; Shwe et al., 2018). N-hexadecanoic acid was also a compound that contained in the *M. pruriens* leaves crude extract with an AUC value of 8.33% at a retention time of 10,583.

This compound has anti-inflammatory activity (Aparna et al., 2012). Minimum inhibitory concentration (MIC) was determined to determine the minimum concentration of *M. pruriens* leaves crude extract that were able to inhibit fungal growth. The results of the MIC test are presented in Table 2.

Table 2. MIC of *M. pruriens* leaves crude extract against *C. lunata*

No	Extract concentration (%)	Inhibition zone diameter (mm)
1	0	0
2	0,1	0
3	0,2	0
4	0,3	0
5	0,4	0
6	0,5	0
7	0,6	0
8	0,7	0
9	0,8	0
10	0,9*	0,57
11	1	1,33
12	1,1	1,63
13	1,2	1,83
14	1,3	2,27
15	1,4	2,63
16	1,5	3,03
17	Aquades	0

Description: *MIC

The test results of *M. pruriens* leaves crude extract showed that the minimum concentration of the extract to be able to inhibit the growth of *C. lunata* was at a concentration of 0.9%. This is shown in Figure 2, namely with the formation of an inhibitory zone at a concentration of 0.9% after 2 days of incubation.

The *M. pruriens* leaves crude extract had a weak inhibitory power against the growth of *C. lunata* due to the diameter of the inhibition zone formed less than 5 mm (Davis and Stout, 1971). This could be because the concentration of antifungal compounds

contained in the crude extract of the leaves of *M. pruriens* was quite low. According to Suprpta (2014), plant extracts that had a minimum inhibitory concentration above 0.5% are less suitable to be used as botanical fungicides. This is because the content of active ingredients contained in the extract is quite low so that it requires a lot of ingredients to make it and is considered less practical and economical, as well as the use in high concentrations of plants has the potential to cause phytotoxic. The diffusion power of the extract also thought to be a factor in the

lack of strength of the extract in inhibiting *C. lunata* (Rios et al., 1988).

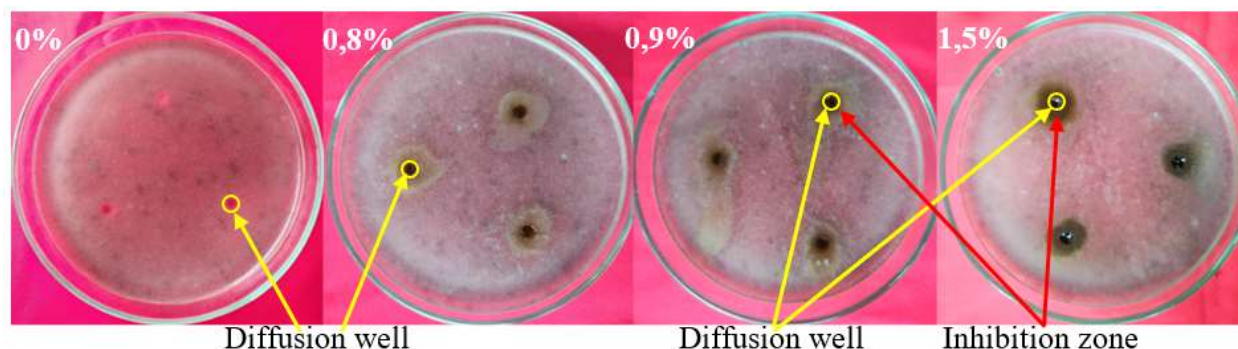


Figure 2 MIC of *M. pruriens* leaves crude extract against *C. lunata*

The ANOVA analysis of the inhibition test showed that the treatment of differences in the concentration of *M. pruriens* leaves crude extract had a significant effect on the average colony diameter, the percentage of

inhibitory power, the average weight of the mycelium, and the density of spores. The results of the test of inhibitory power of the *M. pruriens* leaves crude extract against *C. lunata* are presented in Table 3.

Table 3 Inhibitory power of *M. pruriens* leaves crude extract against *C. lunata*

No	Extract concentration (%)	Average diameter of colony (cm)	Colony growth inhibition rate (%)	Average mycelium mass (mg)	Mycelium mass shrinkage rate (%)	Conidia density (10^5 CFU/ml)	Conidia inhibition rate (%)
1	0%	9,00a	0,00a	225,78a	0,00a	2,138a	0,00a
2	0,1%	9,00a	0,00a	202,43b	10,17b	1,382b	25,54b
3	0,2%	8,95a	0,56a	184,20bc	18,40c	1,075b	44,41b
4	0,5%	8,75a	2,78b	164,03c	27,45d	0,557c	70,03c
5	1%	8,58a	4,72b	131,00d	42,15e	0,223cd	88,18cd
6	2%	6,40b	28,89c	93,95e	58,38f	0,090cd	94,98d
7	5%	5,55c	38,33d	74,30e	67,14g	0,003d	99,88d

Information: Numbers followed by the same letter in the same column are not significant based on the LSD test at the level of 5%

Based on Table 2, the extract concentration of 0.1% still had not inhibited the growth of *C. lunata* colonies. Growth inhibition of *C. lunata* colonies occurred at an extract concentration of 0.2%; 0.5%; 1%; 2%; and 5% with a successive inhibitory power

value of 0.56%; 2.78%; 4.72%; 28.89%; and 38.33%. The results of the test of the inhibitory power of *M. pruriens* leaves crude extract against the growth of *C. lunata* colonies are shown in Figure 3.

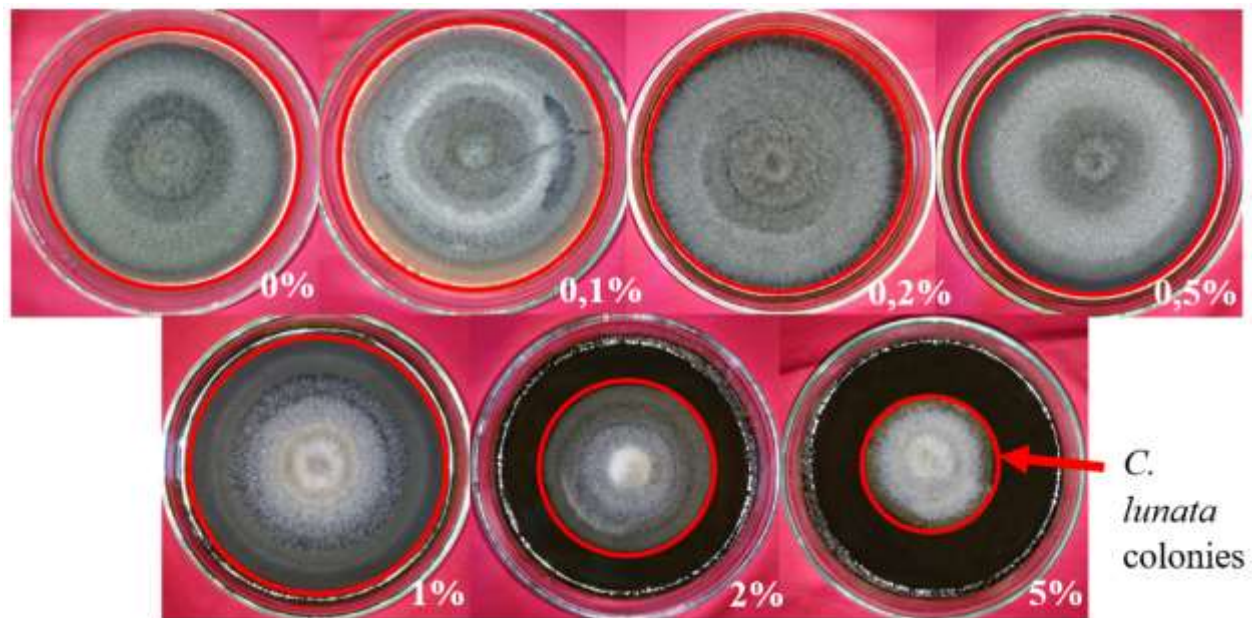


Figure. 3 Inhibitory power of *M. pruriens* leaves crude extract against *C. lunata* colonies growth

In addition to inhibiting the growth of *C. lunata* colonies, the extract was able to reduce the weight of the mycelium and inhibit the formation of *C. lunata* conidia. Based on Table 2, the extract was able to reduce the weight of *C. lunata* mycelium ranging from 0.1% to 5% concentrations with values ranging from 10.17% to 67.14% and inhibiting the formation of *C. lunata* conidia

ranging from 0.1% to 5% concentrations with values ranging from 25.54% to 99.88%.

The inhibition test results showed that the *M. pruriens* leaves crude extract was able to inhibit the growth of *C. lunata* which was shown by decreasing the diameter of the colony, the weight of the mycelium, and also the conidia produced by the fungus. Crude extract of *M. pruriens* leaves was able to

inhibit the growth of *C. lunata* allegedly because the extract contains ethyl isothiocyanate. Based on the results of the GC-MS that had been carried out (Table 1), the crude leaf extract of *M. pruriens* contains ethyl isothiocyanate. Ethyl isothiocyanate is a compound that has antifungal activity (Kara and Soylu, 2020; Kurt et al., 2011; Smolinska et al., 2003; Wu et al., 2011)

The most effective extract concentration in inhibiting the growth of *C. lunata* was the extract concentration of 5% with the inhibitory power of the colony reaching 38.33%. This is supported by research conducted by Kara and Soylu, (2020), Kurt et al. (2011), and Yang et al. (2021). In addition, at the same concentration, the mycelium of *C. lunata* shrank by 67.14%. This is thought to be due to morphological alterations in *C. lunata*. Compounds of the isothiocyanate group are able to damage the morphology of fungi (Kara & Soylu, 2020; Yang et al., 2021). Crude extract of *M. pruriens* leaves with a concentration of 5% also caused *C. lunata* to produced almost no conidia. This suggests that the crude extract of the leaves of *M. pruriens* not only inhibits the growth of fungi, but was also able to inhibit the formation of conidia. This also reported by Otoni et al. (2014) and Yang et al.

(2021). According to Sudana (1997), the formation of conidia in pathogenic fungi is one of the important factors in the spread and infectious rate of plant diseases. Therefore, compounds capable of inhibiting the formation of conidia are very well used as the active ingredients of fungicides.

Crude extract of *M. pruriens* leaves was able to inhibit the growth of *C. lunata*, this in line with Rayavarapu et al. (2011). Thanks to the ethyl isothiocyanate that contained in the extract. Ethyl isothiocyanate is a compound that belongs to the group of isothiocyanate. Isothiocyanate is a volatile compound that is one of the derivatives of glucosinolate which is generally contained in plants of the family Brassicaceae (Plaszko et al., 2021). Compounds from the isothiocyanate group have been widely studied for their antifungal activity (Kara & Soylu, 2020; Kurt et al., 2011; Otoni et al., 2014; Park et al., 2013; Smolinska et al., 2003; Wu et al., 2011; Yang et al., 2021).

Gluthatione, one of natural antioxidant that produced by living organism (Aguirre et al., 2006; Pócsi et al., 2004), often become the target of isothiocyanate group compounds. (Plaszko et al., 2021). This also stated by Schreiner and Koide (1993). It can be said that isothiocyanate compounds can cause oxidative stress in fungi because the amount

of glutathione is reduced resulting in accumulation Reactive Oxygen Species ROS, this is in line with research conducted by Bertóti et al. (2016). Oxidative stress can lead

to the occurrence of protein dysfunction, protein oxidation, enzyme inhibition, lipid peroxidation, as well as DNA damage (Aguirre et al., 2006; Pócsi et al., 2004).

CONCLUSION

Crude extract of *M. pruriens* leaves contains 22 compounds with the most compounds, namely ethyl isothiocyanate which is antifungal. The inhibitory power of the *M. pruriens* leaves crude extract against

C. lunata was weak with a MIC value of 0.9% and was able to inhibit colony growth, reduce the weight of the mycelium, and inhibit the formation of *C. lunata* conidia by 38,33%; 67,14%; and 99,88% respectively compared to control.

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5