Antifungal Activity of Methanolic Extract of *Calliandra calothyrsus* against Storage Fungi Isolated from Dried-stored Spices

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Keywords: Antifungal, *Calliandra calothyrsus,* Postharvest Fungi, Spices.

Abstract: Methanolic extract of *Calliandra calothyrsus* (Leguminoceae) was conducted. The aim was to investigate the presence of flavonoid and the ability of the crude extract to inhibit mycelial growth and conidial germination of storage fungi. Eight fungal species were isolated from ten kinds of dried spices obtained from traditional markets in Medan North Sumatera. Fungal isolation was conducted using dilution method in dichloran 18% glycerol agar (DG18) medium. Plant methanolic extract obtained from 4 kg fresh leaves of *Calliandra calothyrsus*. The leaves were dried under shade to constant weight and ground using electrical grinder to a 1.2 kg fine powder and dissolved in ±15 l of methanol (MeOH) for 48 hours. and dried using rotary evaporator. For qualitative flavonoid determination, 10 g of fine powder of the leaves was dissolved 100 ml methanol and suspended for 12 hours, then acidified with 6% hydrochloric acid (HCl). The presence of flavonol was identified using Ultra Violet Visible Spectroscopy (UV-Vis). Results showed methanolic extract of *Calliandra calothyrsus* contain flavonoids group of flavonol. The crude extract have no antifungal activity againts storage fingi. However, all of the concentration inhibit conidial germination to all fungi tested.

1 INTRODUCTION

Spices are one of commodities that susceptible infected by fungi (Škrinjar et al. 2012; Toma and Abdulla 2013). Most genera of storage fungi are able to colonize stored spices at low water activity (a_w) (≤ 0.85) (Pitt and Hocking 2009). The infection occurred during preharvest, harvesting and postharvest handling. Field fungi such as *Fusarium*, *Alternaria*, *Helmintosporium, Cladosporium* cause damage on crops during harvesting while moisture content of the commodities still high, while storage fungi such as *Aspergillus* and *Penicillium* infect during storage with low a_w (Nurtjahja et al. 2017).

The damage of spices by storage fungi cause moldiness, color change, aroma, taste and contaminated by mycotoxins. Flavonoids are secondary metabolite derivatives of 2-phenyl-benzo[α]pyrane synthesized in all parts of the plants (Seleem et al. 2017). They play a role to provide color to attract pollinator in flower, fragrance and taste of the fruit and protect plant from UV radiation and pathogenic fungi Mierziak et al. (2014).

The toxicity of flavonoids inhibit mycelial growth and spore germination is an alternative to control fungal infection and mycotoxin contamination on dried-stored spices. Molds control using chemicals such as fumigant such as ethylen oxide dan sulphur dioxide commonly used, however, the chemicals leave residue that are toxic to human. Fungal biological control using phytochemical is potential as an alternative to prevent fungal infection on spices during storage. The purpose of the study was to investigate antifungal activity crude methanolic extract of *Calliandra calothyrsus* in inhibiting mycelial growth and fungal population of storage fungi isolated from dried spices.

2 MATERIALS AND METHOD

2.1 Isolation of Fungi

Ten kinds of dried spices were obtained from traditional markets in Medan, North Sumatera. Each fungal species on each kind of spice was isolated by

Nurtjahja, K. and Pasaribu, A.
Anti-fungal Activity Methanolic Extract of Calliandra calothyrsus against Storage Fungi Isolated from Dried-stored Spices.
DOI: 10.5220/0010612600002775
In *Proceedings of the 1st International MIPAnet Conference on Science and Mathematics (IMC-SciMath 2019)*, pages 538-541
ISBN: 978-989-758-556-2

dilution method followed by pour plate method in dichloran 18% glycerol agar (DG18 medium) (5 g/l peptone, 10 g/l glucose, 1 g/l KH_2PO_4, 220 g/l glycerol, 0.5 g/l $MgSO_4.7H_2O$, 15 g/l bacto agar, 2.0 mg/l dichloran and 100 mg/l chloramphenicol (Pitt and Hocking 2009). Twenty five gram of each sample in 500 ml erlenmeyer was diluted in 250 ml sterilized distilled water. The suspension was homogenized using shaker (Gallenkamp, orbital shaker SG92, England) 100 rpm for 2 minutes. Four dilutions, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were made. From the 10^{-3} or 10^{-4} dilution, 1 ml was transfered onto petridish (diameter 9 cm) and pour plated in DG18 medium. The plates were incubated at ambient temperature (29±2ºC) for 6 days. All colonies were counted as colony forming unit per gram (cfu/g) of the sample. Each single separate colony was isolated and cultured on czapex yeast extract agar (CYA) or CYA+20% sucrose (CYA20S) and identified. Macroscopic and microscopic identification of each fungal species were conducted according to Pitt and Hocking (2009).. All of the storage fungi are culture collection of Microbiology Laboratory, Biology Department, Sumatera Utara University. Each fungal isolates was kept on potato dextrose agar (PDA) slants and subcultured before used.

2.2 Plant Material

Calliandra calothyrsus was obtained from abandoned area 1300 meter above sea level at Berastagi, Karo Regency, North Sumatera Province, Indonesia.

2.3 Plant Extraction

Methanol extract of *Calliandra calothyrsus* was prepared according to Larson et al. (2016). Four kilogram of the fresh leaves were air dried to constant weight and ground using electrical grinder to 1.2 kg fine powder and dissolved in 15 l 96% methanol (MeOH) for 48 hours. The simplisia was then filtered using Whatman's No.1 filter paper, removed to clean vessel and concentrated using rotary evaporator (Heidolph, Instruments GmbH and Co, KG Walpersdorfer Str, Germany). This was repeated three times. The extract appears as semi-solid greenish paste.

2.4 Flavonoid and Flavonol Test

For test flavonoid, 10 g fine powder of the leaves was extracted with 100 ml 96% methanol and suspended for 12 hours, the extract was filtered using Whatman's No.1 filter paper and hydrolized by hydrochloric acid (HCL) 6% in warm condition for 1 hour. Immediate development of red color indicates the presence of flavonoids. The flavonol in methanol extract was identified both using Ultraviolet-Visible spectroscopy (UV-Vis) (200 to 500 nm) and nuclear magnetic resonance proton spectrum (^{1}H-NMR).

2.5 Antifungal Test

Paper disc diffusion inhibition method was used to determine antifungal activity of the extract in petri dish 9 cm diameter. Agar plugs (5.0 mm in diameter) of potato dextrose agar plate containing 5 days fungal mycelia were made. Plant extract was dissolved in DMSO to make concentration 0, 20, 40, 60, 80 and 100%. Antifungal ketoconazole (10 µl/mL) were used as the reference standard. Steril paper discs (5.0 mm) were impregnated with each extract concentration and allowed to dry before being placed in PDA plate. The agar plugs and discs were placed in petridishes (9 cm diameter) containing PDA plates. This was repeated three times. All plates were incubated at 29°C for 6 days and observed for zone inhibition. The diameter of the zones were determine in millimeter (mm).

2.6 Germination Inhibition Test

Ten mL of semi-solid potato dextrose agar medium containing plant extract at concentration 0, 20, 40, 60 and 80% and ketoconazole (10 µl/mL) were made. Conidial suspension of each storage fungi were prepared by washing 7 days old PDA cultures with 10 mL steril distilled water containing 0.05% Tween 80. The concentration of conidia was adjusted to 1×10^7 and 1 mL of the conidial suspension was mixed thoroughly with the semi-solid PDA containing plant extract. Three drops of conidial suspension of each fungal species were cultured on sterilized microscope glass slide. All of the glass slides were covered with cover glass then were incubated for 20 h at ambient temperature (29°C). Each was repeated three times. The percentage of germinating conidia was observed microscopically after 20 h by counting 100 conidia. Germinating conidia were considered based on the presence of germ tubes at the germ pore.

3 RESULTS AND DISCUSSION

3.1 Inhibition Zone of Plant Extract against Postharvest Fungi

The presence of the secondary metabolites *C. calothyrsus* showed no inhibition on the growing mycelia except for mycelia of *Aspergillus candidus* that inhibited begin at 40% extract concentration (Table 1). The highest crude leaves extract (80%) have no effect on the mass growing mycelia of the other fungal species. In contast to ketoconazole, all mycelia of storage fungi were inhibited and *Aspergillus tamarii* was the most inhibited. However, the effect of the extract reduced fungal population particularly on *A. chevalieri*, *A. flavus*, *A. niger* and *A. tamarii* (Table 2).

Flavonoids are group of polyphenolic compounds having a benzo-γ-pyrone that commonly found on plants. They are synthesized in response microbial infection (Dixon et al. 1983). Flavonoids consist of chalcone, flavon, isoflavon, flavonol, flavanon, flavonol, and antocyanidin (Andrae-Marobela et al. 2013) The ability of flavonoid as antimicrobe was reported by Yigit et al. (2009). Flavonoids namely 3.7.5'-trihidroksi anthocyanidin and 3.5-dihidroksi-7-metoksi antocyanidin isolated from *Monanthotaxis littoralis* (Annonaceae) inhibit mycotoxigenic fungi such as *Aspergillus*, *Fusarium* and *Penicillium* isolated from maize (Clara et al. 2014).

Based on analysis using UV-Vis spectroscopy and ^{1}H-NMR, we assumed the methanolic extract of *C. calothyrsus* contain flavonoid group of flavonol. Flavonoids including flavanones, flavones and flavans has antifungal activity. Purified flavon inhibit mycelial growth and conidial germination was reported by Cotoras et al. (2011). The used of the crude extract and low concentration the flavonoid compounds in the plant might the antifungal have no effect on growing mycelia.

Our results is in corcordance to Steinkellner and Mammerler (2007), they report that low flavonoid concentration exhibit slight antifungi properties againts *Fusarium oxysporum f.* sp. *lycopersici.* The effect of purified flavon on mycelial growth and conidial germination of *Botrytis cinerea* was studied by Cotoras et al. (2011), they found that the mycelia of *B. cinerea* stop growing after six days incubation without reaching maximum radial growth. Dorta et al. (2005) reported that the presence of flavonoid surrounding fungal mycelia and conidia interact with the plasma membrane and it affects mitochondrial respiratory chain. Low concentration of the secondary metabolites was indicated with no inhibitory effect on mycelial growth of storage fungi,

However, both of the compounds inhibit fungal population to most of all storage fungal tested. The use of the crude extract prevent fungal population, therefore, spoilage and mycotoxin contamination of foodstuffs during storage can be prevented. Basil leave with concentration lower than 85 % has no effect on all fungi tested. The minimum inhibition of the extract begin to inhibit fungal mycelia at concentration 85 % particularly on *Aspergillus candidus* and *Aspergillus tamarii*. Some species of fungi such as *A. oryzae*, *A. niger*, *A. fumigatus* and *Penicillium* have no effect at the highest concentration (100%).

Table 1: Antifungi activity of crude methanol extract of *Calliandra calothyrsus* on mycelial growth of storage fungi.

Fungal species	Extract concentration / fungal population (cfu/ml)					
	0	20	40	60	80	Ketoconazole
Aspergillus candidus	0-	0-	1.5	2.3	3.5	3.13
A. chevalieri	0-	0-	0-	0-	0-	0.30
A. flavus	0-	0-	0-	0-	0-	3.40
A. fumigatus	0-	0-	0-	0-	0-	5.13
A. niger	0-	0-	0-	0-	0-	5.43
A. oryzae	0-	0-	0-	0-	0-	5.23
A. tamarii	0-	0-	0-	0-	0-	7.23
Penicillium sp.	0-	0-	0-	0-	0-	2.86

Table 2: Antifungi activity of crude methanolic extract of *Calliandra calothyrsus* on conidial germination.

Fungal species	Extract concentration / fungal population (cfu/ml)					
	0	20	40	60	80	Ketoconazole
Aspergillus candidus	0	2.8×10^6	2.7×10^6	2.2×10^6	2.5×10^6	0
A. chevalieri	4.2×10^6	13.2×10^6	4.5×10^6	6.6×10^6	4.1×10^6	0
A. flavus	1.0×10^6	1.0×10^6	3.5×10^6	4.2×10^6	2.5×10^6	0
A. fumigatus	3.2×10^6	2.6×10^6	4.5×10^6	4.8×10^6	6.1×10^6	0
A. niger	0	2.2×10^7	2.7×10^6	2.4×10^6	1.9×10^6	0
A. oryzae	0.5×10^7	8.3×10^7	3.4×10^7	4.5×10^7	4.6×10^7	0
A. tamarii	1.1×10^6	0.4×10^7	1.8×10^6	0.9×10^6	0.6×10^6	0
Penicillium sp.	31.6×10^6	27.2×10^6	14.2×10^6	5.1×10^6	7.0×10^6	0

4 CONCLUSION

The presence of secondary metabolites in methanolic extract of *C. calothyrsus* reduce fungal population of storage fungi.

ACKNOWLEDGEMENT

The research was supported by TALENTA-Sumatera Utara University, Grant no. 322/UN5.2.3.1/ PPM/KP-TALENTA USU/2019.

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