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Antifungal Activity of *Eucalyptus urophylla* Oil Against *Aspergillus niger* and *Fusarium oxysporum*

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ABSTRACT

Essential oils obtained from Eucalyptus posses many bioactivities as fungicidal, antimicrobial, insecticidal and other activities. This study elucidated chemical compounds and antifungal activity of *Eucalyptus urophylla* leaves oil. Effectiveness of *E. urophylla* leaves oil were evaluated as antifungal against pathogenic fungi of *A. niger* and *F. oxysporum*. *Eucalyptus urophylla* oil was obtained by hydrodistillation method from fresh leaves of *E. urophylla*. Gas chromatography-mass spectrometry (GC-MS) analysis was used to analyze the chemical compounds of *E. urophylla* oil. Antifungal activity of *E. urophylla* oil was tested with *in-vitro* assay against *A. niger* and *F. oxysporum* strains with five levels of oil concentration (1 mg ml^{-1}; 5 mg ml^{-1}; 10 mg ml^{-1}; 15 mg ml^{-1}; 20 mg ml^{-1}). GC-MS analysis showed the most abundant compounds of *E. urophylla* oil were 1,8-cineole (66.31 %), α-pinene (16.92 %), α-terpinyl acetate (6.00 %) and γ-terpinene (5.13 %). Antifungal assay showed inhibitory effects of *E. urophylla* against *F. oxysporum* with IC_{50} = 1.61 mg ml^{-1} and *A. niger* with IC_{50} = 13.56 mg ml^{-1}. Antifungal activity of *E. urophylla* oil in this study was probably due to the presence of 1,8-cineole. Results indicated the possibility of *E. urophylla* as antifungal against *F. oxysporum* and *A. niger*.

INTRODUCTION

Plant pathogens such as fungi, nematode, bacteria, and virus are responsible to plant diseases or damages. Among those patogens, fungus is considered as the most important plant pathogen, causing various diseases in plants (Fletcher et al., 2006). In developing countries, the loss of food crop production and after harvest were resulted by pathogenic fungi. Fungal species, such as *Aspergillus* and *Fusarium,* caused rotting of seeds, stems and plant wilt. *Fusarium oxysporum* is a plant pathogen that causes death in seeds, seedlings, damping-off, and the reduction of growth in various horticultural crops. *Fusarium oxysporum* is a fungus that responsible for many diseases in horticultural crops that have high economic value such as what was found in bitter gourd and bottle gourd (Cumagun, Aguirre, Relevante, & Balatero, 2010), while *Aspergillus niger* is rot diseases on mango and other agricultural products such as citrus, cocoa, snake fruit, and corn. *Aspergillus niger* causes a black mold disease on some vegetables and fruits such as onions, peanuts, and grapes. This fungus can also contaminate some foods (Sharma, 2012).

Various efforts involving the use of synthetic fungicides have been conducted to prevent, control or eliminate fungal plant diseases, especially in agriculture. Despite its effectiveness in controlling plant diseases, the use of synthetic fungicides has been discouraged due to its negative impacts on environment and health. Excessive used of synthetic fungicides can upset ecosystem balance and induce pathogen resistance. Therefore, it is necessary to find environmentally friendly fungicides such as essential oils. Essential oils possess fungicide activities which have a rich in bioactive content (Shaaban, El-Ghorab, & Shibamoto, 2012).

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Several studies have isolated hundreds of chemicals in the essential oils, and show that they have the activity as antifungal, antibacterial, anti-parasitic, and other bioactivities. Essential oils can heal respiratory system problems and infections such as reducing asthma, flu, colds, sinus infections, and earache. In addition, essential oil can also be used in skin to treat acne, skin burns, insect bites, and warts, also in scalp as anti dandruff. Essential oils have some effects to the nerve system to treat depression conditions, anxiety, and fatigue. In digestive system, essential oils can repair some problems and improve hormonal balance (Cooksley, 1996). Essential oils are volatile material composed of hydrocarbons (sesquiterpenes and other terpenes) and oxygenated components (esters, alcohols, ethers, aldehydes, phenols, ketones, phenol ether, and lactones). Antifungal activity in essential oils related to its primary alcohol, α, β-saturated aliphatic aldehydes, tertiary alcohol, and hydrocarbons (Kocić-Tanackov & Dimić, 2013).

Essential oils from the family of Myrtaceae also have fungicide activities (Avasthi, Gautam, & Bhadauria, 2010; Baptista, Zimmermann-Franco, Lataliza, & Raposo, 2015; Siddique, Perveen, Nawaz, Shahzad, & Ali, 2015). *Eucalyptus urophylla* is one of 700 species of Myrtaceae. It is an indigenous plant from Indonesia (Hendraswari & Bhumibhamon, 2009). Essential oils of eucalyptus and the main compound toxicity against a variety of microbes, including bacteria and fungi (Fiori et al., 2000). Essential oils which are extracted from this tree species contain various bioactive substances that can be used as anti-bacteria, antifungals, analgesic, anti-inflammation, antioxidants, and mosquito repellants (Cheng et al., 2009). Previous studies also showed the effectiveness of these essential oils as antifungals. Gakuubi, Maina, & Wagacha (2017) showed the effectiveness of *E. camadulensis* oil as antifungal of *Fusarium spp.* López-Meneses et al. (2015) evaluated the effectiveness of *E.globulus* oil against *F. moniliforme* and *A. parasiticus.*

Bioactivity of essential oil depends on its chemical constituents. The main compound found in eucalyptus essential oil is 1,8-cineole (Somda, Leth, & Sérémé, 2007; Damjanovic-Vratnica, Dakov, Sukovic, & Damjanovic, 2011). Several studies explained that 1,8-cineole is one of the active compounds that has activity as an antifungal agent (Vilela et al., 2009; Kim & Park, 2012; Mousavi & Raftos, 2012). However, there was lack of information available regarding to antifungal activity of *E. urophylla* oil against plant pathogenic fungi.

This study was conducted to determine the chemical compounds and the effectiveness of *E. urophylla* leaves oil as antifungal agent against plants pathogenic fungi of *F. oxysporum* and *A. niger*. The chemical composition of *E. urophylla* in this study was analyzed by GC-MS and *in-vitro* assay as antifungal activity analysis. The inhibitory concentration 50 % (IC_{50}) of *E. urophylla* oil against *F. oxysporum* and *A. niger* was also determined.

MATERIALS AND METHODS

Sample of *E. urophylla* Oil

Sample of *E. urophylla* oil used in this study was obtained by water-steam distillation of fresh *E. urophylla* leaves harvested from the Forest Research and Education of Wanagama I, Gunung Kidul District, Yogyakarta, Indonesia. The anhydrous sodium sulfate was used to dry over *E. urophylla* oil from water content, and oil in sealed bottle was maintained at approximately 0 ºC until used.

GC-MS Analisys

Gas Chromatography – Mass Spectrometry (GC-MS) QP 2010 S (Shimadzu Co. Ltd, Kyoto, Japan) was used to analyze chemical composition of *E. urophylla* oil with capilar colum Agilent HP 5 MS (30 m length). Mobile phase used helium gas in 60 ml per minute flow rate with injection split, injection temperatur of 310 ºC and injection volume of 1.0 µl. EI (electron-impact ionization) at 70 eV was used for ionization. The identification of *E. urophylla* oil compounds was obtained from chromatogram result and compared retention time with database library or literatur data.

Antifungal Activity

Fungal strains of *F. oxysporum* and *A. niger* in this study were obtained from Inter University Center, Universitas Gadjah Mada, Yogyakarta. The cultures were transferred to medium as stock cultures and used as inoculum. Antifungal activity of *E. urophylla* oil was examined by Wang, Chen, & Chang (2005) method with slight modification.

Potato Dextrose Agar (PDA) media (20 ml) was poured into a Petri dish and 1 ml *E. urophylla* oil solution (prepared by mixing the oil with methanol solvent to obtain various level of oil concentration) was introduced onto petri dish. *Eucalyptus urophylla* oil concentrations assessed were 1, 5, 10, 15, and 20 mg ml^{-1}. Then the dishes were left in a laminar airflow for 12 hours to allow for the methanol to evaporate. Inoculation with the fungi was carried out by using agar mycelium plug (5 mm diameter) from the stock culture. The Petri dishes were sealed with a parafilm and incubated in an incubator at 25 ˚C. Growth of mycelium was measured every 2 days for 14 days or until the colony covered the entire media. PDA media added with methanol without essential oil solutions was used as control. Each treatment was replicated 3 times and then averaged. The percentage of fungal growth inhibition was calculated by the following equation (Siramon, Ohtani, & Ichiura, 2013):

% Inhibitory = [1-(Sa/Sb)] x 100 ……..…1)

where Sa is the surface area of mycelium growth of treatment (cm^2) and Sb is the surface area of mycelium growth of control (cm^2). The IC_{50} of the *E. urophylla* oil was obtained using probit analysis for each fungus.

Statistical Analysis

The data were analyzed by Completely Random Design (CRD) statistical analysis with three replications using SPSS software. Statistical analysis differences between average values were analyzed using ANOVA and Honestly Significant Difference (HSD) tests. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical Compounds of *E. urophylla* Oil

The chemical compounds of *E. urophylla* oil are presented Table 1. The GC-MS analisys showed that *E. urophylla* oil consisted of 8 chemical compounds belong to monoterpene hydrocarbons, oxygenated monoterpene, ester, and sesquiterpene. The components of *E. urophylla* oil were mainly belong to oxygenated monoterpene and monoterpene hydrocarbons. The chemical components of *E. urophylla* oil that belong to monoterpene hydrocarbons were α-pinene (16.92 %), β-pinene (2.42 %), β-ocimene (0.45 %), γ-terpinene (5.13 %), and terpinolene (1.05 %). The component that belong to oxygenated monoterpene was 1,8-cineole (66.31 %), sesquiterpene was β-caryophyllene (1.75 %) and the ester compound was α-terpinyl acetate (6.00 %).

In this study the main compound of *E. urophylla* oil is 1,8-cineole (66.31 %). Other major compounds of *E. urophylla* oil in with percentage > 5 % are α-pinene (16.92 %), α-terpinyl acetate (6.00 %), γ-terpinene (5.13 %), respectively. The major compounds structure of *E. urophylla* oil can be seen in Fig. 1. Compare to the other studies this result relatively similar although vary in the percentage. The main compound of *E. urophylla* oil from Taiwan was 1,8-cineole (58.3 %) (Cheng et al., 2009) and from Brazil was also 1,8-cineole (65.4 %) (Filomeno et al., 2016). Variation in the percentage of 1,8-cineole content was probably due to the variation of chemotype, site, rainfall, and nutrition content of the soil that could affect plant metabolism. Results from other study on several species of eucalyptus also revealed that the main compound of eucalyptus leaves oil was 1,8-cineole (Damjanovic-Vratnica, Dakov, Sukovic, & Damjanovic, 2011; Sebei, Sakouhi, Herchi, Khouja, & Boukhchina, 2015).

Table 1. Chemical composition of *E. urophylla* oil

No.	Retention time	Component	Group	Formula	Percentage (%)
1.	9.714	α-Pinene	Monoterpene hydrocarbons	$C_{10}H_{16}$	16.92
2.	11.185	β-Pinene	Monoterpene hydrocarbons	$C_{10}H_{16}$	2.42
3.	13.583	1,8-Cineole	Oxygenated monoterpene	$C_{10}H_{18}O$	66.31
4.	13.942	β-Ocimene	Monoterpene hydrocarbons	$C_{10}H_{16}$	0.45
5.	14.349	γ-Terpinene	Monoterpene hydrocarbons	$C_{10}H_{16}$	5.13
6.	15.290	Terpinolene	Monoterpene hydrocarbons	$C_{10}H_{16}$	1.05
7.	23.630	α-Terpinyl acetate	Ester	$C_{12}H_{20}O_2$	6.00
8.	25.597	β-Caryophyllene	Sesquiterpene	$C_{15}H_{24}$	1.75
	Total				100.00

Remarks: The chemical compounds were identified based on their retention time compared to Wiley 229 database library

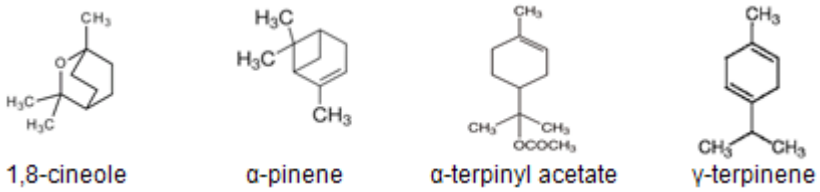

Fig. 1. Structure of major compounds of *E. urophylla* oil

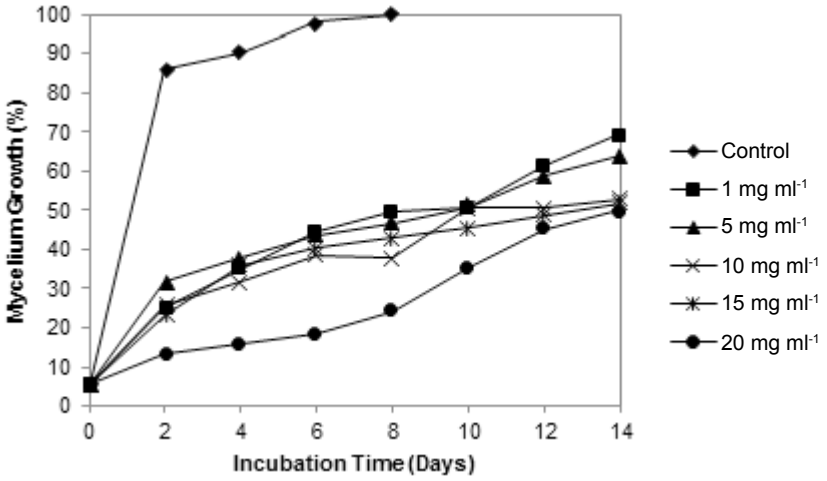

Fig. 2. Growth of *F. oxysporum* mycelium on medium added with several concentrations of *E. urophylla* oil

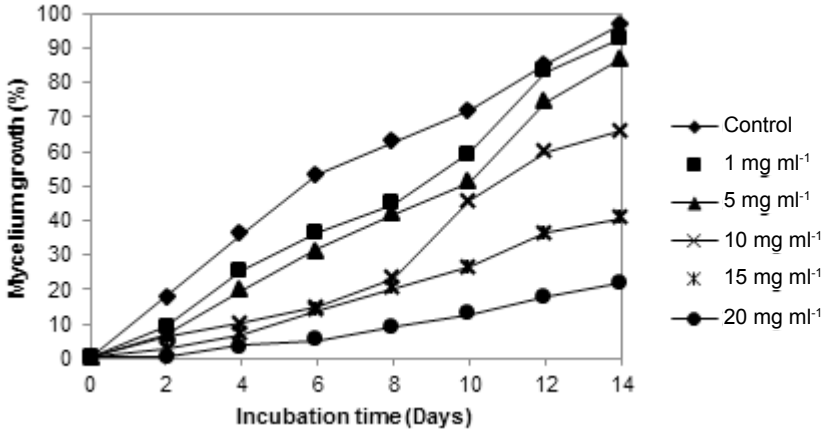

Fig. 3. Growth of *A. niger* mycelium on medium added with several concentrations of *E. urophylla* oil

Antifungal Activity of *E. urophylla* Oil

Antifungal activity assay was performed to find out inhibitory concentration (IC) of *E. urophylla* oil against plant pathogenic fungi, namely *F. oxysporum* and *A. niger.* This test was conducted *in vitro* on Potato Dextrose Agar (PDA) media. Result of antifungal activity assay of *E. urophylla* oil against *F. oxysporum* and *A. niger* in an increasing concentration (1 mg ml^{-1}, 5 mg ml^{-1}, 10 mg ml^{-1}, 15 mg ml^{-1}, and 20 mg ml^{-1}) show a tendency of percentage of fungal growth inhibition increased by the increasing of oil concentrations (Fig. 2 and Fig. 3).

The lowest fungal growth inhibition was obtained from oil concentration of 1 mg ml^{-1} and the highest was obtained from the concentration of 20 mg ml^{-1}. Compared to control treatment, the addition of *E. urophylla* oil to the PDA medium exhibited antifungal activity. In the control treatment, *F. oxysporum* has grown to cover the entire medium at 8 days' incubation and for *A. niger* it was achieved at 14 days. In the medium added with the essential oil, even addition with the lowest concentration (1 mg ml^{-1}) has inhibited the mycelium growth. From Table 2, it can be seen that the growth inhibition against *F. oxysporum* is greater than against *A. niger*.

The average of percent inhibitory of *E. urophylla* oil against *F. oxysporum* for oil concentration of 1 mg ml^{-1}, 5 mg ml^{-1}, 10 mg ml^{-1}, 15 mg ml^{-1}, and 20 mg ml^{-1} were 50.37 %, 53.33 %, 56.30 %, 57.22 %, and 75.74 %, respectively. For *A. niger* the average of percent inhibitory were 4.61 %, 10.19 %, 31.90 %, 58.00 %, and 77.29 %, respectively (Table 2). The percentage of inhibitory of *E. urophylla* oil against *Foxysporum* for oil concentration of 1 mg ml^{-1} to15 mg ml^{-1} is not significantly different. It was only significantly different at the concentration of 20 mg ml^{-1}. Against *A. niger*, each oil concentration gave significant results. Result of variance analysis also showed that *E. urophylla* oil possessed the greater activity against *F. oxysporum* than against *A. niger.*

IC_{50} value of *E. urophylla* oil for *F. oxysporum* in this study was 1.61 mg ml^{-1} and 13.56 mg ml^{-1} for *A. niger* (Fig. 4). This study indicated that *E. urophylla* oil was more effective as antifungal against *F. oxysporum* than against *A. niger*. Previous study and several researches have also shown that essential oil possessed greater antifungal activity against *F. oxysporum* than *A. niger* (Dhanasekaran, Thajuddin, & Panneerselvam, 2008; Pujiarti, Yoshito, & Hideaki, 2012). This is probably due to *A. niger* as the dominant fungus.

The histogram shown in Fig. 4 where IC_{50} value of *E. urophylla* oil was compared to authentic compound of 1,8-cineole, it was revealed that the IC_{50} value of authentic compound of 1,8-cineole against *F. oxysorum* (0.35 mg ml^{-1}) and *A. niger* (12.48 %) were smaller than *E. urophylla* oil. This indicated that 1,8-cineole was more effective as antifungal against both fungal species than *E. urophylla* oil. Several researches also showed that the main compound of eucalyptus oil was 1,8-cineole which exhibited capability as antifungal (Vilela et al., 2009; Kim & Park, 2012; Mousavi & Raftos, 2012; Pujiarti, Yoshito, & Hideaki, 2012).

Previous researches also evaluated the effectiveness of oil extracted from eucalyptus genera as antifungal against several plant pathogenic fungi (Somda, Leth, & Sérémé, 2007; Liu, Chen, Wang, Xie, & Xu, 2008; Kim & Park, 2012; Siramon, Ohtani, & Ichiura, 2013). *Eucalyptus urophylla* oil inhibited the growth of fungi through mechanisms of reduction of mycelium growth, inhibition of spore production and growth of fungal buds. However, antifungal activity possessed by *E. urophylla* oil in this study probably due to the presence of 1,8-cineole which was the main compound (66.31 %). Other compounds of this essential oil probably also have activity as antifungal, but the effectiveness of single or synergic effects for another compounds were not investigated in this study.

Table 2. Percent inhibitory of *E. urophylla* oil and 1,8-cineole against the growth of *F. oxysporum* and *A. niger*

Oil	Concentration (mg ml^{-1})	Fungal Species	
		F. oxysporum	*A. niger*
E. urophylla	1	50.37 ± 6.58 d	4.61 ± 2.86 a
	5	53.33 ± 2.23 d	10.19 ± 2.63 b
	10	56.30 ± 1.16 d	31.90 ± 3.82 c
	15	57.22 ± 1.47 d	58.00 ± 1.78 d
	20	75.74 ± 7.06 e	77.29 ± 2.28 e
1,8-cineole*	1	64.00 ± 6.07 de	12.30 ± 1.64 b
	5	79.73 ± 1.35 e	25.71 ± 1.58 bc
	10	96.25 ± 1.08 f	44.28 ± 1.48 cd
	15	100.00 ± 0.00 fg	51.21 ± 2.21 d
	20	100.00 ± 0.00 fg	61.28 ± 4.67 de

Remarks: * = 1,8-cineole was a main compound in *E. urophylla* and used as comparison [source: Pujiarti, Yoshito, & Hideaki (2012)]. The Average values were followed by different letters means significantly different

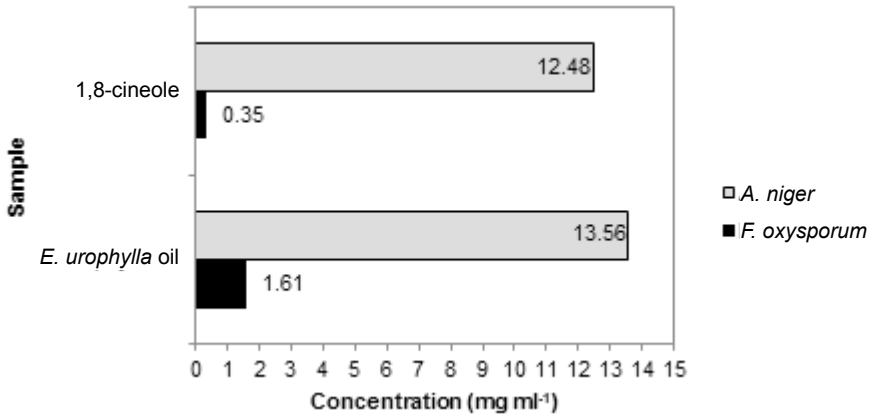

Fig. 4. IC_{50} of *E. urophylla* oil and 1,8-cineole against *F. oxysporum* and *A. niger*

CONCLUSION AND SUGGESTION

This study indicated that *E. urophylla* oil could be used as control agent for pathogenic fungi of *F. oxysporum* and *A. niger*. The effectiveness of *E. urophylla* oil was probably due to the presence of 1,8-cineole compound. This oil more effective against *F. oxysporum* (IC_{50}= 1.61 mg ml^{-1}) than *A. niger* (IC_{50}=13.56 mg ml^{-1}). However, further studies are needed to evaluate the synergic effect of *E. urophylla* oil constituents and its application for other fungi and purposes.

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