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Rutaceae: conservation at Eka Karya Bali Botanic Garden and its *in vitro* antifungal activity screening

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Abstract

Several species of Rutaceae have been widely used and commercialized in all regions in Indonesia. Some species of Rutaceae are consumed as fresh fruit and traditional medicine for various kinds of diseases, as well as to add aroma to various Indonesian culinary. Since 1959, Eka Karya Bali Botanic Garden (Eka Karya BBG) has successfully collected dozens of Rutaceae species with unknown potential. In addition to reporting the conservation of Rutaceae in Eka Karya BBG, this study aimed to screen the anti-fungal activity of Rutaceae methanolic extract toward *Aspergillus niger*, *Cladosporium* sp., and F*usarium solani*. Leaves of 13 species of Rutaceae (*Boenninghausenia* sp., *Citrus aurantifolia*, *C. maxima*, *C. medica*, *Clausena* sp., *Melicope* sp., *Micromelum* sp., *Murraya paniculata*, *Toddalia* sp., *Zanthoxylum* sp., *Z. alatum*, *Z. limonella*, and *Z. ovalifolium*) were collected, cleaned, air-dried, soaked in methanol for three days, then evaporated using a rotary evaporator to obtain the plant crude extract. The *in vitro* inhibitory assay was conducted by the diffusion method. As a result, only *C. medica*, *Clausena* sp., and *Z. limonella* exhibited antifungal activity against those tested fungi. Their antifungal activity increased on day 2 post-treatment but slowly decreased on day 3. Thus, the result of this experiment can be used as preliminary data to research Rutaceae plant extracts as an alternative method to control pathogenic fungi. However, further research is needed to maintain and increase its inhibitory effect.

INTRODUCTION

Rutaceae, a family of the order Rutales, are shrubs and woody plant with infrequently thorny stems. Rutaceae has single leaves without stipula, entire leaf margin, glabrous leaf surface, and has oil glands. Rutaceae has a single flower with 4 to 5 white sepals, facing inward anthers, and the number of stamens is similar to the number of the sepals. The fruit of Rutaceae is hesperidium type with round shape, and the skin of the fruit has 3 layers, in which the inner layer is insulated to form a space fulfilled with water bubbles (Weiss,1997).

Several species of Rutaceae have been widely used and commercialized in almost all regions in Indonesia. The leaves of *Citrus aurantifolia* and *C. hystrix*, as well as the fruit of *C. aurantifolia*, are used to eliminate the fishy odors and add aroma to various Indonesian culinary (Apriliani et al., 2014; Hulyati et al. 2014). Traditionally, the fruit of *C. aurantifolia* is also used to relieves cough (Meliki et al., 2013; Hulyati et al., 2014; Slamet and Andarias, 2018), while the fruit of *C. hystrix* is usually used to treat varicella disease (Yassir and Asnah, 2019). Moreover, the fruit of *C. aurantifolia* and its skin are used to treat heart disease, hypertension, and

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rheumatism (Meliki et al., 2013). Additionally, Novitasari et al. (2014) mentioned that the essential oil of *C. microcarpa* had a strong anti-termite activity, while the leaves of *Aegle marmelos* and *C. maxima* were used to regrow the hair-loss as the chemotherapy effect and to treat toothache, respectively (Slamet and Andarias, 2018).

Recent studies exhibit the anti-microbial activity of some Rutaceae species. However, the study about the antifungal activity of Rutaceae is still limited. The essential oil of *C. aurantiifolia*, *C. aurantium*, and *C. limetta* strongly inhibits *Escherichia coli*, *Staphylococcus aureus*, *S. mutans*, *Lactobacillus acidophilus*, and *Candida albicans* (Miller et al., 2014). *C. albicans* is also inhibited by the presence of *Zanthoxylum zanthoxyloides* root extract and *Z. macrophylum* leaf extract (Kosh-Komba et al., 2017).

Stem extract of *Toddalia asiatica* exhibits strong inhibition toward *E. coli* and *S. aureus* (Maarisit and Lawani, 2020). Moreover, *Aegle marmelos* leaf extract has significantly inhibited the growth of dermatophytic fungi, such as *Trichophyton mentagrophytes*, *T. rubrum*, *Microsporum canis*, *M. gypseum*, and *Epidermophyton floccusum* (Balakumar et al., 2011).

Plants exploitation without conservation can lead to plant extinction in the future. Therefore, it is necessary to conserve, protect, and preserve it. Among all the plant species included in Rutaceae, Eka Karya Bali Botanic Garden (Eka Karya BBG) has successfully collected dozens of them since 1959. However, there are still many species collected with unknown potential. In addition to reporting the conservation of Rutaceae at Eka Karya BBG, this study aimed to screen the antifungal activity of Rutaceae. Methanol leaves extracts of 13 species of Rutaceae plant collection at Eka Karya BBG were expected to inhibit the growth of causal agents of major diseases in horticultural crops, namely *Aspergillus niger*, *Cladosporium* sp., and *Fusarium solani*.

MATERIALS AND METHODS

This study was conducted at the Laboratory of Applied Botany, Eka Karya BBG, from June to July 2018. Thirteen species of Rutaceae plant collections of Eka Karya BBG were used in this study, including *Boenninghausenia* sp., *Citrus aurantifolia*, C. *maxima*, *C. medica*, *Clausena* sp., *Melicope* sp., *Micromelum* sp., *Murraya paniculata*, *Toddalia* sp., *Zanthoxylum* sp., *Z. alatum*, *Z. limonella*, and *Z. ovalifolium*. Leaves were cleaned by running water then cut into small pieces to enlarge the surface area and speed up the drying process. Leaves were then air-dried for 3 to 6 days. Dried leaves were then soaked in the sealed Erlenmeyer filled with methanol for 3 days, and this process is called maceration, intended to soften and destroy plant cell walls to release the soluble phytochemicals. The soaked leaves were stirred once a day by slowly shaking the Erlenmeyer. After 3 days, the soaked leaves were filtered using filter paper to obtain the maceration products. The maceration products were then evaporated in IKA RV10 rotary evaporator to obtain the plant crude extract.

Aspergillus niger, *Cladosporium* sp., and *Fusarium solani* were used as the pathogen in this study. All fungal isolates were the collections of Faculty of Biology, Udayana University. Each fungus was cultured on potato dextrose agar (PDA). Ten milliliters of sterile distilled water were poured into the cultured fungi. Spores were rubbed using ose to mix it with sterile distilled water to obtain the fungal suspension, then poured into a sterile bottle. To assay the inhibitory activity of Rutaceae crude extracts against fungi, 100 µL of each fungal suspension was added in the molten PDA, then plated in a Petri dish. After the PDA was set, a sterile filter paper was put in the center. Each 50 µL of plant crude extracts was dripped on the filter paper, then incubated at 22°C for 3 days. Meanwhile, 50 µL of methanol was dripped on filter paper as a control. The diameter of the clear zone was measured using a caliper.

RESULTS AND DISCUSSION

Up to September 2019, Eka Karya BBG, has collected 139 plant numbers from 16 genera of Rutaceae (Figure 1). Above all, species from some genera, such as *Citrus*, *Zanthoxylum*, and *Melicope* were the most collected in Eka Karya BBG (Figure 1a). *Citrus* collection consists of 28 plant numbers, including *C. aurantifolia* (3 plant numbers), *C. aurantium* (1), *C. amblycarpa* (2), *C. hystrix* (1), *C. maxima* (10), *C. medica* (3), *C. japonica* (1), *C. nobilis* var. *microcarpa* (1), *Citrus* × *aurantium* (1), and *Citrus* sp. (5). Comparatively, *Melicope* collection consists of 18 plant numbers, comprising 1 plant number of *M. confusa*, 9 *M. latifolia*, 1 *M. lunu-akenda*, and 7 *Melicope* sp. Meanwhile, *Zanthoxylum* collection consists of 15 plant numbers,

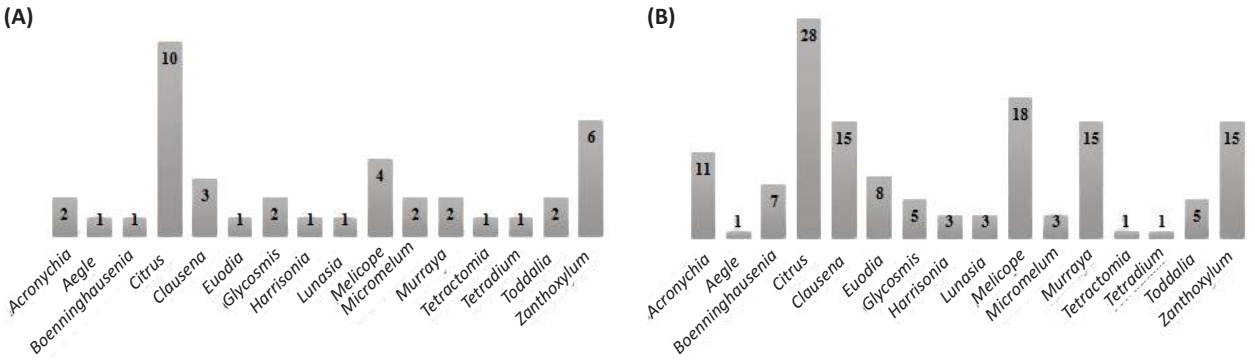


Figure 1. Genus of Rutaceae plants collected in Eka Karya Bali Botanic Garden A: total species collected in each genus, B: total number of the plant collected

which are 2 *Z. armatum*, 3 *Z. limonella*, 2 *Z. ovalifolium*, 2 *Z. paniculatum*, 1 *Z. rhetsa*, and 5 *Zanthoxylum* sp. Similarly, each *Clausena* and *Murraya* collection consists of 15 plant numbers as follows *C. excavata* (3), *C. harmandiana* (1), *Clausena* sp. (11), *Murraya* sp. (2), and *M. paniculata* (13) (Figure 1b).

Apart from the living plant collection, Eka Karya BBG collects plants in the form of herbarium as well. Herbarium is part of a plant that has been preserved by certain techniques and is generally used for scientific purposes (Mertha et al., 2018). As an ex-situ plant conservation site, the herbarium of Eka Karya BBG has a role to store and preserve plant diversity, as well as facilitate the identification or re-identification process. Among 40 species of Rutaceae collected in Eka Karya BBG, only 24 species have been collected as herbarium, such as *Citrus medica*, *C. maxima*, *Zanthoxylum armatum*, *Z. ovalifolium*, *Murraya paniculata*, and *Boenninghausenia albiflora*.

The present study was designed with the attention of safer and effective bioactive agents to investigate the activity of plant extract against fungi. Plant sources used in this study were the collection of Eka Karya BBG, which were selected for the following steps including extraction and screening methods. However, collecting plant extract still becomes a challenge since the plant is rich in bioactive compound. Since the extraction method is an important part of collecting plant extracts, methanolic crude extracts were used in this study to minimize the analogous compounds of the yield. The polarity of methanol organic solvent has been confirmed to provide the ability to extract a variety of bioactive molecules and influence both the qualitative and quantitative composition of the active compounds into plant extract (Njeru et al., 2015). Antifungal activity of plant extracts was evaluated by measuring the inhibitory effect on different species of Rutaceae against *Aspergilus niger*, *Cladosporium* sp., and *Fusarium solani*, as shown in Table 1. Those three fungi are widely known as phytopathogens in such crops. *Fusarium* sp. was found as a disease causative agent on dragon fruit and sugar cane, (Pinaria and Assa, 2017; Andriastini et al., 2018), while *Fusarium solani* was reported as a disease causative agent on lychee and bean (Pinaria and Assa, 2017). Based on the finding of Soesanto et al. (2012), F*usarium oxysporum* and *Cladosporium musae* were included in 2 of 5 important disease-causing pathogens in bananas. Widodo and Wiyono (2012) reported a new plant disease known as leaf curling on papaya caused by *Cladosporium cladosporioides*. In addition, *Aspergillus niger* was reported to be the causal agent of crown rot, pod rot, and seed rot in peanuts, as well as black rot in onions (Pinaria and Assa, 2017).

Among 13 plant extracts, only *Citrus medica*, *Clausena* sp., and *Zanthoxylum limonella* confirmed activities against three tested fungi, while other species were unable to inhibit the mycelial growth of the fungi. It must be remembered that just because the activity cannot be detected from these species, it doesn't mean that there is no activity showed in this condition, and the reasons should be varied. Crude extracts are a complex mixture of many plant metabolites synthesized by plants which variations in the chemical properties modify their antimicrobial activity (Osman et al., 2012; Azwanida, 2015). Additionally, solvents play an important role in the natural product. Different use of each solvent resulted in different chemical compositions of the crude extracts (Gopalasatheeskumar et al., 2017; Truong, 2019). Nandan and Vangalpati (2015) found

Table 1. Inhibitory effects of Rutaceae plant extracts against fungal pathogens on the day 2 post-treatment

Plant extracts	Clear zone diameter (cm)		
	Aspergillus niger	*Cladosporium sp.*	*Fusarium solani*
Control	0.00	0.00	0.00
Boeninghausenia sp.	0.00	0.00	0.00
Citrus aurantifolia	0.00	0.00	0.00
C. maxima	0.00	0.00	0.00
C. medica	1.45	1.70	1.58
Clausena sp.	1.66	1.48	1.73
Melicope sp.	0.00	0.00	0.00
Micromelum sp.	0.00	0.00	0.00
Murayya paniculata	0.00	0.00	0.00
Toddalia sp.	0.00	0.00	0.00
Zanthoxylum sp.	0.00	0.00	0.00
Z. alatum	0.00	0.00	0.00
Z. limonella	2.45	2.08	2.60
Z. ovalifolium	0.00	0.00	0.00

that quercetin, rutin, and kaempferol in *C. medica* methanolic extract were higher than other solvents such as ethanol, ethyl acetate, and water. In diffusion assay, the appearance of the inhibition zone can be detected as a clear zone formed between the disk and hypha, which indicates that there is no mycelial growth between them (Horváth et al., 2016).

In this study, the biological activity of *C. medica*, *Clausena* sp., and *Z. limonella* was exhibited as potent antifungal agents. This was proved by the formation of clear zones of inhibition around the filter paper on the agar plate containing plant crude extract. On the other hand, there was no clear zone formed in the control treatment. This indicated that the clear zone formed in *C. medica*, *Clausena* sp., and *Z. limonella* were caused by plant extracts, not the solvent of the extracts. The qualitative phytochemical investigation by Panara et al., (2012), Adham (2015), and Kalariya et al. (2019) exhibited the presence of carbohydrates, amino acids, alkaloids, flavonoids, phenols, tannins, steroids, and glycosides in *C. medica*. Similarly, alkaloids, flavonoids, glycosides, phenols, and tannins were also found in the methanolic extract of *Clausena excavata* (Elumalai and Kasinathan, 2016). Additionally, Negi et al. (2011) and Devi et al. (2015) reported the presence of alkaloids, coumarins, proteins, phenols, tannins, flavonoids, glycosides, steroids, and terpenoids in *Z. limonella* methanolic extracts. Those chemical compounds have been widely reported to have a broad spectrum of biological activities, including their antifungal properties.

The screening method for antifungal activity was evaluated quantitatively, observed through the diameter of the inhibition zone on the agar plate two days post-treatment, which ranged from 1.45 cm to 2.60 cm, created by three plant species mentioned before (Table 2). Based on the diameter of the inhibitory zone formed by *C. medica* against *A. niger*, *Clodosporium* sp., and *F. solani*, the inhibition of *C. medica* on *Cladosporium* sp. was higher than other tested fungi. Chen et al. (2019) revealed that antifungal properties called D-limonene and γ-terpinene contained in *C. medica* were effective to inhibit the growth of *F. solani* in *Panax notoginseng*. Interestingly, among three *Citrus* species assayed in this study, only *C. medica* showed inhibitory effects against fungal colonies (Table 1). It demonstrates that the crude extract from each species has characteristic and component to offer bioactivity against fungi. A previous study evaluated the effects of *Citrus* plant extract, including *C. bergamia*, *C. aurantium*, *C. sinensis*, *C. deliciosa*, and *C. limon* against fungal colonies and resulted that only *C. limon* was unable to inhibit the mycelial growth of *A. flavus* (Restuccia et al., 2019).

This study displayed the ability of *Clausena* sp. and *Z. limonella* to restrict the growth of *F. solani* compared with other fungi. The maximum inhibitory zone on *Clausena* sp. was recorded up to 1.73 cm, followed by 1.66 cm on *A. niger* and 1.48 cm on *Cladosporium* sp. This finding was supported by Kumar et al. (2012), which demonstrated that a new γ-lactone coumarin, named excavarin-A contained in leaves of *Clausena*, had been proven to be able to inhibit the pathogenic fungi in plants and human. Among the tested extracts, *Z. limonella* exhibited the greatest inhibitory effect (Table 1). The inhibitory zone caused by *Z. limonella* extracts against *F. solani, A. niger*, and *Cladosporium* sp. were 2.6 cm, 2.45 cm, and 2.08 cm, respectively. On the contrary, the lowest activity of these three potent extracts was recorded in *C. medica* against *A. niger* with a diameter of 1.45 cm. The range between both the highest and the lowest activity explained that the presence of anti-fungal properties in *C. medica*, *Clausena* sp., dan *Z. limonella* were considered to provide defense mechanisms against fungi. These varied diameters are correlated with a combination of phytochemical compounds, fungal tolerance in the environment (Sah et al., 2011), and other genetic characteristics of the fungal (Walker dan White, 2018). Correspondingly, Supabphol and Tangjitjareonkun (2014) reported that dictamine derived from *Z. limonella* was effectively suppressed the growth of *Cladosporium cucumerinum* and *Pyricularia oryzae*. Besides, the essential oil of *Z. limonella* possessed inhibitory activity against *A. flavus*, *A. ochraceus*, *A. parasiticus*, and *F. moniliforme* (Nanasombat and Wimuttigosol, 2011). Another study described an antifungal activity of pinocembrocide in *Z. limonella* possessed toxic activity against *Penicillium italicum* by suppressing mycelial growth via cell membrane/wall-targeting mechanism (Chen et al., 2020).

For further analysis, the activities of selected plant crude extracts were observed to evaluate the persistence effect against tested fungi. As shown in Figure 2, the inhibitory activity of methanolic extract

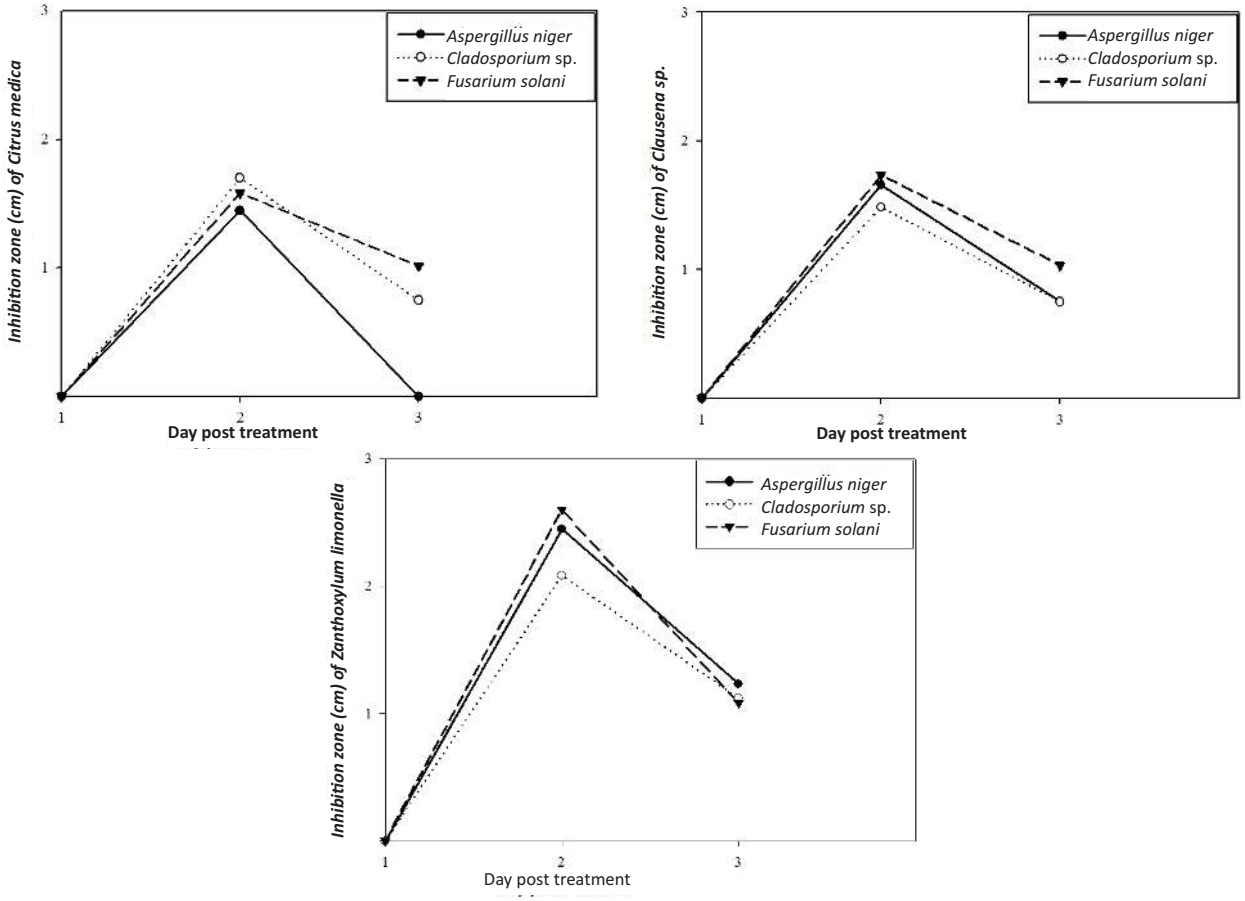


Figure 2. Inhibitory zone of *Citrus medica*, *Clausena* sp., and *Zanthoxylum limonella* extract against *Aspergillus niger*, *Cladosporium* sp., and *Fusarium solani* on day 2 and 3 post-treatment

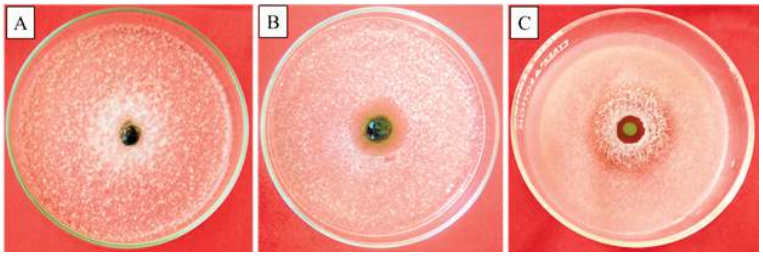


Figure 3. Antifungal activity of *Citrus media* methanolic extract against A: *Aspergillus niger*, B: *Cladosporium* sp., and C: *Fusarium solani* on day 3 post-treatment

of *C. medica*, *Clausena* sp., and *Z. limonella* on PDA increased on day 2 post-treatment, and became lower on day 3. *Z. limonella* showed the strongest activity against all three fungi (Table 1, Figure 2), but the antifungal effect decreased on three species with different degrees. As presented in Figure 3, the inhibitory activity of *C. medica* against *A. niger* was completely suppressed on day 3 post-treatment, while others still showed their ability to restrict the mycelial growth during the same period of incubation (Figure 3). Two possibilities could be described that either persistency of active compound contained in *C. medica* was low, or the type of *A. niger* strain assayed here had high pathogenicity. It is not a surprise since the natural product has been known as biodegradable and non-persistent compounds. Wibawa et al. (2019) mentioned that the inhibition of the methanolic extract only lasted until the second day and faded on the third day, which is probably because the active ingredients of the extract are volatile compounds.

This study was carried out to find the potency of Rutaceae as antifungal agents in supporting awareness of the negative effects of synthetic preservatives. The results (Figure 2) indicate that selected crude extracts show a short time ability to inhibit the mycelial growth of fungi. Of all the tested extracts, only *C. medica*, *Clausena* sp., and *Z. limonella* effectively inhibited the fungal growth. This indicates that only those three plant species contain chemical compounds with antifungal properties. The content of compounds in plants can be varied depending on some factors, including genetics, season, and habitat (You et al., 2011; Wibawa et al., 2019a). Cantín et al. (2012) mentioned that there were large differences in phytochemical profiles between genotypes and/or cultivars of the same species. Recent studies have also shown that environmental conditions and seasons have an impact on the content of plant chemical compounds (Howard et al., 2003; Crespo et al., 2010; Kruger et al., 2011; Wibawa and Lugrayasa, 2020). Phytochemical properties contained in plant sources are expected to contribute to this inhibitory activity. Pavela (2014) and Jiménez-Reyes et al. (2019) declared that the persistent effect of phytochemical property is a relatively short time. This is because the compounds are released in the surrounding environment, thereby limiting the efficacy. On the other hand, synthetic preservatives could be long-term persistent and potentially toxic to organisms (Lushchak et al., 2018).

Rutaceae is one of the plant families that comprise approximately 160 genera, known as potent antimicrobial agent and used traditionally as medicine for some diseases. These roles should be correlated with the properties contained in each plant. Generally, the secondary metabolites are non-essential properties in plant development that contribute to plant survival (Pagare et al., 2015). Referring to the recent topic about plant extract, many plant species offer as antifungal agents in food source and public health. These cases have been a major concern since the spread of plant pathogens can influence the deterioration of food throughout the world (Costa et al., 2013). In this case, some mycotoxins are secreted by fungus from the genera of *Fusarium*, *Aspergilus* (Jimenez-Garcia et al., 2018), and *Cladosporium* (Alwatban, 2014). Fortunately, Rutaceae is one of the solutions. Furthermore, this investigation of the antifungal activity of Rutaceae could be an important step towards the possibilities of using the natural product in plant protection and food safety. This may imply that its ability as an antifungal agent is undoubtedly, but it needs more attention to carry out understandings and future research. So, further analysis is needed to investigate its potential and activity such as chemical compounds, efficiency,

resistance to pathogens, and environmental risks. Supported by any previous studies, the basis of this study allows promising probability to explore another potency of Rutaceae not only as antifungal agents but also other biological effects.

CONCLUSIONS

As a plant conservation site in eastern Indonesia, Eka Karya BBG has collected 139 plant numbers from 16 genera of Rutaceae. Most collections are from Bali, West Nusa Tenggara, and East Nusa Tenggara. In addition to the living collection, Eka Karya BBG also collects plants in the form of herbarium. Among 40 species of Rutaceae collected, only 24 species have been collected as herbarium.

The assay of *in vitro* antifungal activity of Rutaceae exhibited that among 13 plant extracts, only *C. medica*, *Clausena* sp., and *Z. limonella* inhibited the growth of *A. niger*, *Cladosporium* sp., and *F. solani*. The strongest inhibitory effect was shown by *Z. limonella* methanolic extract. Generally, the antifungal activity of *C. medica*, *Clausena* sp., and *Z. limonella* increased on day 2 post-treatment, but slowly decreased on day 3. Thus, the result of this experiment can be used as the preliminary data to research Rutaceae plant extracts as an alternative method to control pathogenic fungi. However, further research is needed to maintain and increase its inhibitory effect.

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