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Research Article

Cinnamon Leaf Extract to Control Anthracnose Disease on Chilli Plants in Bali: A Novel and New Potential

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ABSTRACT
Industrial synthetic fungicides, as a means of controlling plant diseases, can negatively affect the environment. Biofungicides are implemented in environmentally friendly agriculture. The implementation of biofungicides entails the use of plant extracts containing antifungal compounds to control diseases in plants. This research is conducted to determine the potential of the Cinnamomum burmannii (C. burmannii) leaf extract as a biofungicide as a means of controlling the anthracnose disease in chilli plants in Bali in vitro. The anthracnose disease is caused by the fungus Colletotrichum capsici (C. capsici). The method used in this study is the extraction of C. burmannii leaves. Then, the potential of the C. burmannii leaf extract against C. capsici with diffusion wells is tested, the minimum inhibitory concentration (MIC) is determined and scanning electron microscopy is performed. The crude extract of the C. burmannii leaves can inhibit the growth of the test fungi with an inhibition zone diameter of 2.2 cm and MIC of 0.5%. The acetone extract of the C. burmannii leaves can inhibit the growth of colonies, biomass and fungal spore germination. Some treatments, such as T2, T3 and T4, inhibit the growth of colonies in rows by 17%, 30% and 46%. At the same concentrations of T2, T3 and T4, the growth of the biomass and spores is inhibited by 14%, 24% and 87% and 51%, 69% and 86%, respectively. At the T5 and T6 treatments of the colonies, fungi biomass and spores cannot grow. The mechanism of the C. burmannii leaf extract can control fungi development by destroying the structure of fungal cell walls.

Keywords: Antifungal, biofungicide, anthracnose, Colletotrichum capsici fungus

INTRODUCTION

One of the diseases that attack large chilli plants (Capsicum annuum L.) or cayenne pepper (C. frutescens) is anthracnose, in which the disruption of the growth and production of chilli is usually effectual. The incidence and intensity of anthracnose in Bali is 63% and 68% (Khalimi et al., 2019). Decreased chilli production due to anthracnose in Thailand ranges from 10% to 80% (Poonpolgul and Kumphai 2007). Anthracnose is caused by the Colletotrichum capsici (C. capsici) fungi (Sila and Sopialena, 2016). Its symptoms include the initial formation of blackish brown spots on chillies that gradually expand into a soft rot. In the middle of the spots are black spots consisting of seta and conidium. The attacked fruit changes colour from red to dry brown (Semangun, 2007).

The use of industrial chemical fungicides for the disease control in plants attacked by fungi can harm the environment because residues of industrial chemical fungicides are extremely difficult to decipher by microorganisms (Suprapta, 2014). Industrial chemical fungicide residues can poison the consumers, both animals and humans. For this reason, this research on the control of plant diseases by using biofungicides is conducted, particularly because the use of biofungicides has several advantages from the economic, biodegradability and environmental friendliness viewpoint (Shivanna and Garampalli, 2014).

Plant extracts used in biofungicides can be obtained from various parts of the plant, such as roots, bark, leaves, flowers, fruit, seeds or buds. Plant extracts contain secondary metabolites that can be antimicrobial. Moreover, some researchers have reported plant extracts as antifungal. Saman tree (Samanea saman) leaf extracts can control the Fusarium solani fungus, which causes a stem-

rotting disease in dragon fruit plants in Bali (Rita et al., 2013). Combined extracts of forest chilli (Piper caninum) and Tricoderma harzianum can control the blast disease, which attacks local rice plants in Bali. Blast disease is caused by the fungus Pyricularia oryzae (Suriani et al., 2019). Rachma (2012) reported that Cinnamomum burmannii (C. burmannii) has antifungal properties against Candida albicans in vitro.

The acetone extract of the C. burmannii leaf from the Belok Sidan Village in Petang District, Badung Regency, Bali Province, Indonesia inhibits mycelia and biomass and the formation of fungal spores that cause wilting in tomato plants. Treatments with 1%, 1.25%, 1.50%, 1.75% and 2% concentrations can inhibit fungal mycelia compared with the controls, with successive inhibitions of 41.66%, 78.11%, 88.33%, 91.11% and 100% (Darmadi et al. 2015). The research on the inhibition of the C. burmannii leaf extract and the mechanism of inhibition of the extract on tested fungi is extremely important. The present work is related to the utilisation of the C. burmannii leaf extract as an environmentally friendly biofungicide.

MATERIALS AND METHODS

C. burmannii leaf extraction

The C. burmannii leaves used in this study was sourced from the village of Bedugul in Baturiti District, Tabanan Regency, Bali Province, Indonesia. The selected C. burmannii leaves were healthy, and the undamaged leaves were green. The leaves were chopped into small pieces and dried for three days at room temperature. Then, the leaves were blended until they were in powder form. The leaf powder was macerated in methanol at the ratio of 1:10 (w/v) for 48 hours. The filtrate was obtained by filtering through a gauze and the Whatman no. 2 filter paper. The crude extract was obtained by evaporating the filtrate using a vacuum rotary evaporator at 40 °C. The crude extract was used for further testing.

Antifungal activity test

The antifungal activity test of the C. burmannii leaf extract against the pathogenic fungus causing the anthracnose disease in chilli plants was carried out by the diffusion well method. Several tests were conducted in this experiment, including the determination of the minimum inhibitory concentration (MIC) of crude extracts, the effect of the extracts on the growth of fungal colonies on potato dextrose agar (PDA), the effect of the extracts on the growth of the fungal biomass and the effect of the extracts on the sporulation in liquid media (Darmadi et al., 2019b). The data were analysed using a completely randomised design with six treatments and four replications. The concentration of 0% (control), abbreviated as T1, was used as the treatment, followed by the successive concentrations of 0.5% = T2, 0.75% = T3, 1% = T4, 1.25% = T5 and 1.5% = T6.

The percentage calculation of the inhibition of the C. burmannii leaf extract on the radial growth, biomass and spore growth in the fungal test is given by the following equations:

$$\mathrm{IR}(\%) = \frac{(\mathrm{DC} - \mathrm{DT})}{\mathrm{DC}} \times 100\%$$

$$\mathrm{IB}(\%) = \frac{(\mathrm{WC} - \mathrm{WT})}{\mathrm{WC}} \times 100\%$$

where IR is the inhibitory activity against radial growth in percent, DC is the diameter of the fungus colony without extract treatment (control), DT is the diameter of the fungus colony treated with the extract, IB is the inhibitory activity against biomass growth in percent, WC is the weight of the fungus biomass without the extract treatment (control), WT is the weight of the fungus biomass treated with the extract, IS is the inhibitory activity against spore growth in percent, SC is the number of fungus spore without the extract treatment (control) and ST is the number of fungus spore treated with the extract.

Observation of the structure of hifa C. capsici with electron microscopes

The mechanism of the inhibition of the C. burmannii acetone leaf extract against the cell wall of C. capsici was determined by adding 1 mL of the test fungi and 5 g of the crude extract into the Erlenmeyer glass and then adding the potato dextrose broth (PDB) media at 50 mL. The Erlenmeyer glass was shaken horizontally to mix evenly the fungus, extract and PDB media. The Erlenmeyer glass containing test fungi mixed with the extract and PDB media was placed into a shaker incubated at 24 °C for seven days. The fungi biomass was filtered using a tissue paper and placed in an oven, then weighed until the fungi biomass was constant. The same approach was performed without using the extracts (control). Then, the sample was prepared for scanning electron microscopy (SEM) (Darmadi, 2015).

RESULTS AND DISCUSSION

Inhibitory test of C. burmannii leaf crude extract

The crude extract of C. burmannii leaves can inhibit the mycelia fungus (C. capsici) in vitro by using the PDA media with an inhibition zone diameter of 2.2 cm. This inhibition (2.2 cm) could be categorised as strong. According to Shahidi (2004), if the diameter of the inhibition zone measuring ≥20 mm is included in the strong category, then the diameter of the inhibition zone measuring 10–19 mm can be categorised as part of the medium category, whilst that measuring 1–9 mm can be classified as part of the weak category. The smallest inhibitory concentration (i.e. MIC) of the C. burmannii leaf extract was 0.5%. This finding indicates that the C. burmannii leaf extract is an extremely effective biofungicide and can control the chilli rot disease caused by the fungus C. capsici. The smaller the MIC of an extract is, the more effective the extract can be used as a biofungicide (Suprapta, 2014).

Antifungal activity of the C. burmannii leaf acetone extract against fungi colonies

The C. burmannii leaf acetone extract can inhibit the growth of test fungi colonies in vitro with the PDA media. Moreover, the C. burmannii leaf extract can inhibit the growth of test mushroom colonies. The T2, T3 and T4 treatments from the C. burmannii leaf acetone extract can inhibit the radial growth of fungus colonies at 17%, 30% and 46% relative to the controls, respectively. The T5 and T6 treatments of C. burmannii leaf extract can kill fungal colonies (Figure 1). The mushroom colonies killed with the T5 and T6 concentrations were re-grown on the control media, and they were unable to grow or die (Figure 2). This finding indicates that the C. burmannii leaf extract is fungistatic at the 0.5%, 0.75% and 1% concentrations, but it is fungicidal at 1.25% and 1.5% concentrations. The results of this study indicate the close relationship between the concentration of the C. burmannii leaf extract and the inhibitory power of the C. burmannii leaf extract against the test fungus. The higher the concentration is, the greater the inhibitory power becomes. This trend is depicted by the equation $y = 52.88x + 0.423$. $R^2 = 0.947$. The extract concentration affects inhibitory activity at 94.7% (Figure 3).

Table 1. Inhibitory activity of the C. burmannii leaf extract on the growth of the C. capsici fungi colony 21 HSI (days after inoculation)

Extract concentration (%)	Fungi colony diameter (mm)	Percentage of inhibitory compared to control
T1	92a*	-
T2	76b	17
T3	64c	30
T4	50d	46
T5	0e	100
T6	0e	100

*Mean values followed by the same letter within columns do not differ significantly on the basis of Duncan's multiple range test at $p \leq 0.05$.

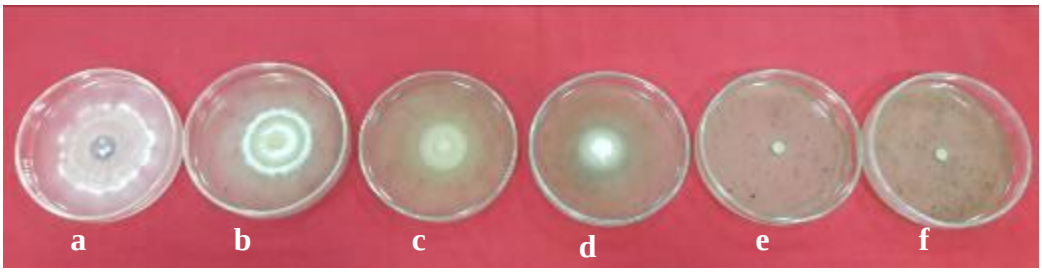


Fig.1: Test of inhibition of the C. burmannii leaf extract under different concentrations on the growth of C. capsici: a. control, b. 0.5% concentration, c. 0.75% concentration, d. 1% concentration, e. 1.25% concentration and f. 1.5% concentration

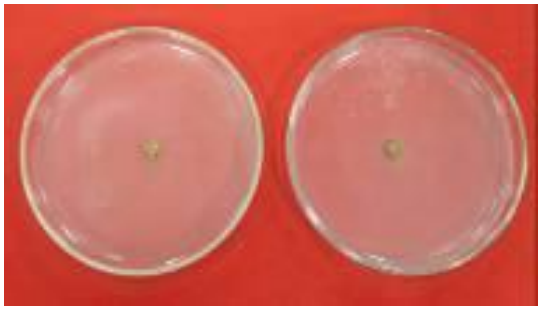


Fig.2: Test fungi colonies that cannot grow with the C. burmannii leaf extract at 1.25% and 1.5% concentrations (Figure 1, e–f). Colonies grown on the control media were also unable to grow.

The C. burmannii leaf extract can be regarded fungistatic against the test fungus if the extract can inhibit mould growth and can be regarded fungicidal if the extract can cause death of the test fungus. These fungistatic and fungicidal properties can be determined by increasing or decreasing the concentration of the extract; thus, an effectiveness test on the test fungi was performed accordingly. Singh and Tripathi (2015) reported that cinnamon (Cinnamomum zeylanicum) extracts can be fungistatic at the concentration of 100 ppm and fungicidal at the concentration of 200 ppm in terms of inhibiting the growth of the antracnose fungi of bananas (Colletotrichum musae). Hong et al. (2015) reported that cinnamon can also inhibit the growth of Colletotrichum gloeosporioides fungal colonies causing the chilli disease in Korea. Treatments with additional doses lead to a decrease in the diameter of fungal colonies. The different treatments at 0, 2, 5, 8 μL on individual petri dishes showed a decrease in the diameter size of the test fungal colonies by 51.8, 33.4, 10.8 and 1.6 mm, respectively.

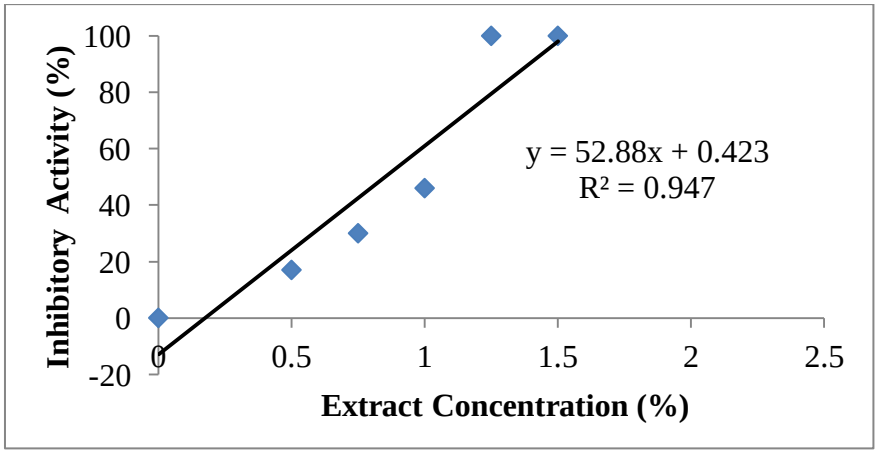


Fig.3: Effect of extract concentration on inhibitory activity

Several other plants besides cinnamon can inhibit the growth of the fungus C. capsici causing anthracnose disease in chilli plants, including the Callistemon lanceolatus plant Pongamia pinnata. Plant extracts of C. lanceolatus and P. pinnata can be used together or separately. The 10% concentrations of five types of solvents (acetic acid, acetone, ethanol, petroleum ether and chloroform)

can inhibit the growth of the test fungus mycelia (More et al., 2010).

Inhibitory test of the C. burmannii leaf extract against fungi biomass

The acetone extract of C. burmannii leaves can inhibit the growth of the C. capsici fungi biomass in vitro with the PDB media. The C. burmannii leaf acetone extract can significantly ($p > 0.05$) inhibit the growth of fungal biomass. The T2 (0.5%) treatment of the C. burmannii leaf acetone extract inhibited the growth of the test fungal biomass with an inhibitor activity percentage of 14%. The T3 and T4 treatments of the C. burmannii leaf extract inhibited the growth of the fungal biomass by 24% and 87%, respectively. Even the T5 and T6 treatments of the C. burmannii leaf extract inhibited the growth of the test fungal biomass by 100%; in other words, at these concentrations, the mushroom biomass cannot grow or die (Table 2). This finding indicates that the inhibition of C. burmannii leaves on the fungal biomass can be determined by the extract concentration, as expressed by the equation $y = 79.65x - 12.21$, $R^2 = 0.861$ (Figure 4).

Table 2. Inhibition activity of the C. burmannii leaf extract on the growth of the C. capsici fungi biomass

Extract concentration (%)	Fungi biomass dry weight (mg)	Percentage of inhibitory compared to control
T1	210a*	-
T2	125b	14
T3	75c	24
T4	27.5d	87
T5	0e	100
T6	0e	100

*Mean values followed by the same letter within columns do not differ significantly on the basis of Duncan's multiple range test at $p \leq 0.05$.

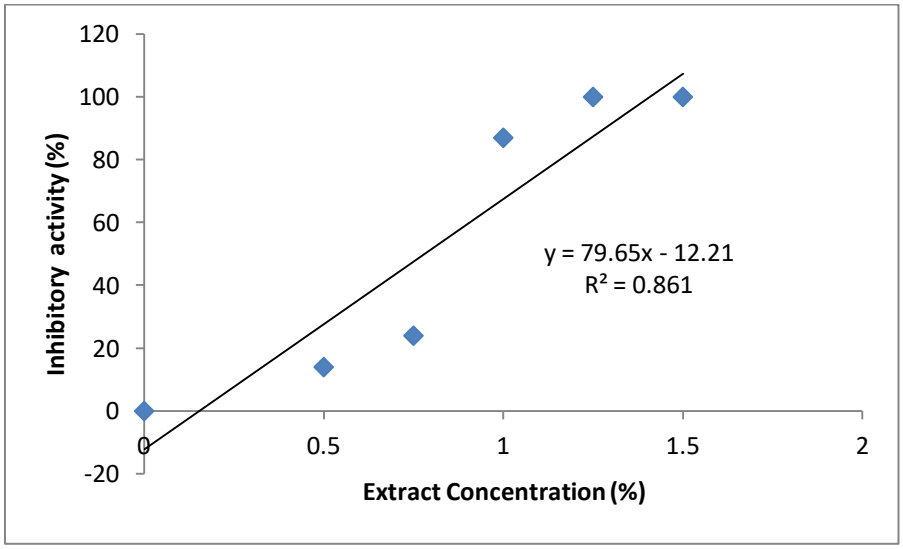


Fig.4: Effect of extract concentration on inhibitory activity

The research on cinnamon extracts inhibiting the growth of the Fusarium oxysporum fungi, further causing a wilting disease in large chilli plants (Capsicum annum L.), has been reported in Egypt. The C. burmannii extract at 0.5%, 1%, 2%, 4% and 6% concentrations can inhibit the growth of the fungus F. oxysporum (1) and (2) by 18.5%, 29.6%, 35.1%, 64.8% and 100% and 14.8%, 24.8%, 31.1%, 61.8% and 100%, respectively (Ragab et al., 2012). The utilisation of cinnamon to inhibit the growth of the anthracnose fungi has been reported by Maqbool et al. (2010). Cinnamon oil is used to inhibit the growth of mycelia and fungal spore germination on bananas. The 0.1%, 0.2%, 0.3% and 0.4% treatments of cinnamon oil in bananas stored at 13°C±1°C with 80% –90% humidity for

28 days were reported. All treatments significantly ($p > 0.05$) inhibited the growth of fungal mycelia and the germination of Colletotrichum musae spores incubated for seven days at room temperature. The best treatment was at 0.4%, as this concentration can inhibit the growth of mycelia and fungal spore germination by 83.2%.

Inhibitory test of C. burmannii leaf extract on the formation of fungi spores

The acetone extract of C. burmannii leaves significantly ($p > 0.05$) inhibited the formation of the test fungus spores in vitro on the PDB media. The greater the concentration of the extract is, the greater the inhibition of the formation of fungal spores becomes. The T2 treatment of the C. burmannii leaf extract inhibited the formation of fungal spores with inhibitory prosentsae by 51%. The T3, T4, T5 and T6 treatments inhibited the formation of fungal spores with successive concentrations at 69%, 86%, 100% and 100% (Table 3). The inhibitory power of the extracts from the fungal spore formation was strongly influenced by the concentration of the C. burmannii leaf extract, as expressed by the equation $y = 68.51x + 10.57$, $R^2 = 0.939$ (Figure 5).

Table 3. Inhibitory activity of C. burmannii leaf extract on the formation of C. capsici fungi spores

Extract concentration (%)	Number of fungi spores/ mL ($\times 10^4$)	Percentage of inhibitory compared to control
T1	13.8a*	-
T2	6.8b	51
T3	4.3c	69
T4	2d	86
T5	0e	100
T6	0e	100

*Mean values followed by the same letter within columns do not differ significantly on the basis of Duncan's multiple range test at ($p \leq 0.05$).

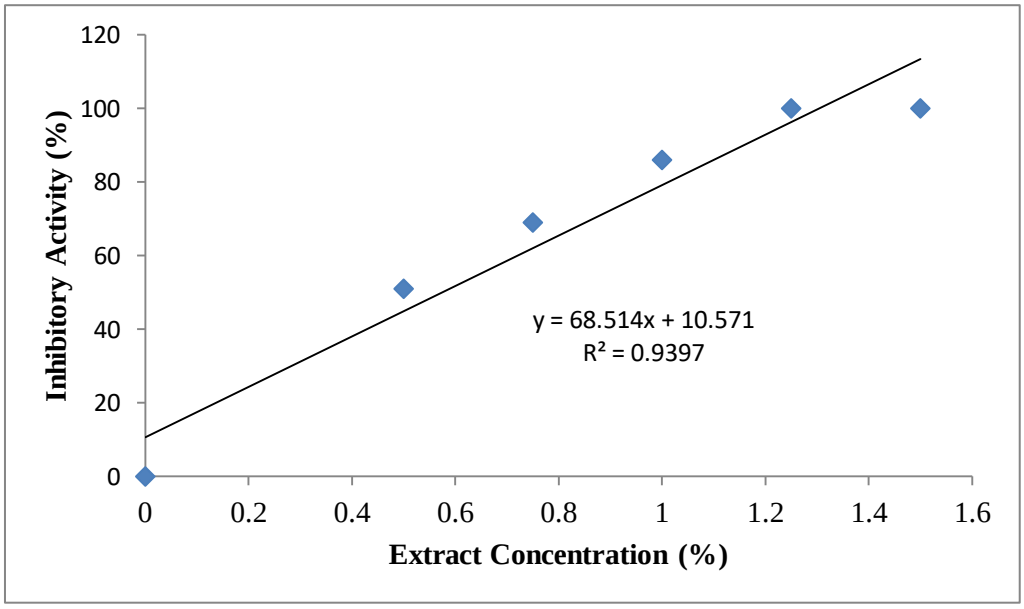


Fig.5: Effect of extract concentration on inhibitory activity

The inhibition of fungal spore formation is extremely important in terms of controlling plant diseases caused by fungi. Spores are a fungus breeding tool. Cowan (1999) reported that the ability of plant extracts can be antimicrobial, i.e. it can inhibit the germination of pathogenic fungal spores. Gupta et al. (2008) found that cinnamon extracts can inhibit the growth of the fungus Alternatia sp., Aspergilus fumigatus, Penicillium sp. and Rhizomucor sp. with inhibition zone diameters

of 25, 15, 35 and 10 mm, respectively. The ability of C. burmannii leaf extracts can inhibit the colonies, biomass and germination of test fungi spores, as these extracts contain secondary metabolites or phytochemical compounds that are antifungal compounds. The phytochemical compounds of C. burmannii leaves growing in the Bedugul Tabanan area of Bali are alkaloids, steroids, phenolics and saponins (Table 4).

Table 4. Phytochemical tests of C. burmannii leaves

No	Phytochemical test	Reagent	Discoloration	Information
1	Alkaloids	Na_2 OH +$CHCl_3$+$H_2SO_4$2N+ Meyer's reagent	There are no white deposite	+alkaloid
2	Flavonoids	Mg-Hcl	Green to pink	- flavonoid
3	Steroids	Liberman Burchad	Yellow to light green	+ steroid
4	Triterpenoid	Liberman Burchad	Yellow to light green	- triterpenoid
5	Phenolate	$FeCl_3$	Dark green to purple	+ fenolat
6	Saponin	The heated Aquades shake	Embossed stable foam	+saponin
7	Tannin	NaCl +gelatin	There are deposits	- tannin

Note: + refers to 'contain' (+, ++ and +++ represent the colour intensity/number of deposits)
– refers to 'does not contain'

Darmadi et al. (2015) reported the secondary metabolite content of C. burmannii leaf extracts obtained from different areas, namely, the village of Petang, Badung Regency, Bali. C. burmannii leaf extracts contain phytochemical compounds consisting of flavonoids, steroids, phenolics and tannins. The content of a chemical compound or a secondary metabolite of the same type of plant can differ due to varying soil conditions, climate and seasons. Balakumar et al. (2011) and Gahukar (2012) reported that the contents of secondary metabolite compounds from plant extracts of the same species differ due to environmental and phyto-system effects. Gillitzer et al. (2012) and Talibi et al. (2012) reported that the varying bio-compositions of chemical components of plant extracts (secondary plant metabolites) obtained from the same plant species can produce different responses due to extract solubility, pH, and extract-type volatility, which are influenced by soil conditions and climate, and the growth occurs depending on plants and seasons.

The effect of secondary metabolite compounds from plant extracts with antifungal properties was reported by Roshan et al. (2012). The plant roots of Glycyrrhiza glabra entail antifungal compounds because they contain phytochemical compounds consisting of saponins (glycyrrhizin or glycyrrhizic acid), flavonoid, coumarin and essential oils. Results from GC-MS analysis found that C. burmannii leaf extracts contain cinnamaldehyde compounds. This compound is anti-microbial and antifungal (Figure 6). The content of cinnamaldehyde compounds in cinnamon was also reported by Wang et al. (2005). Taiwan's local cinnamon (Cinnamomum osmophloeum Kaneh) contain antifungal compounds. The MIC results of cinnamaldehyde compounds, namely, Coriolus versicolor and Laetiporus sulphurous, against the test fungi were 50 and 75ppm.

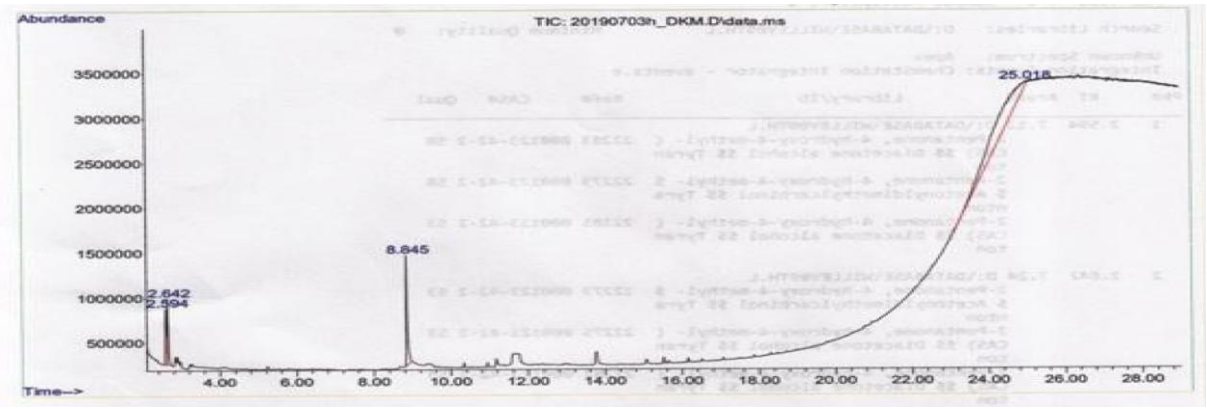
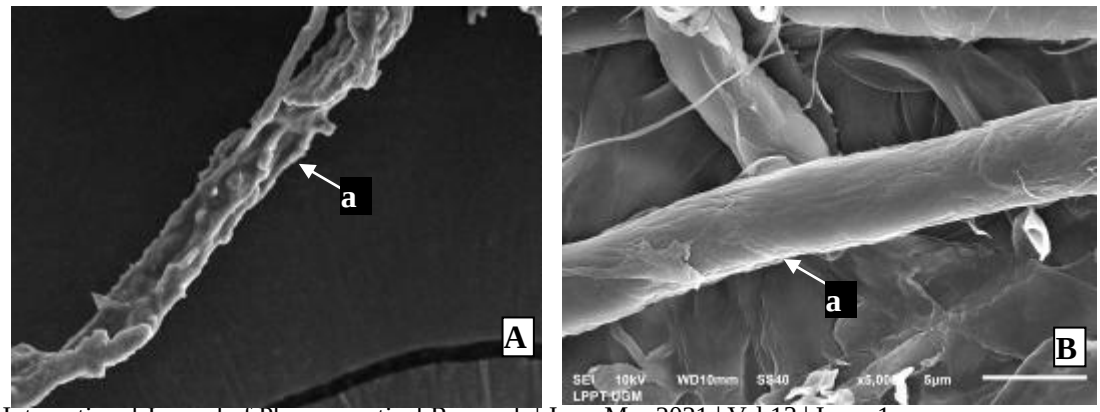


Fig.6: Chromatograms from the GC-MS analysis of the C. burmannii extract consisting of four peaks of successive compounds (diacetone alcohol, acetonyldimethycarbinol, cinnamaldehyde and 2-methyl-5H-dibenz [bf] azepine)

Mechanism of C. burmannii leaf extracts in inhibiting the fungus growth C. capsici

C. burmannii leaf extracts can inhibit the growth of the fungus C. capsici, which attacks the chilli plants by damaging the fungal cell wall or by changing the cell wall structure. The damages on the fungal cell walls cause an imbalance of the components contained within and outside the cell. Cell contents will undergo lysis and eventually cause death of the fungal cells (Figures 7A and B). Darmadi et al. (2019a) reported that the Fusarium wilt disease in tomato plants is caused by Fusarium equiseti. Moreover, C. burmannii leaf extracts can damage the structure of the fungus cell wall of F. equiseti, as shown by the SEM analysis (Figures 7C and D). The C. burmannii leaves used in the test were collected from the village of Belok Sidan in Petang District, Badung Regency, Bali Province (Darmadi et al., 2015). Damages to fungal cell walls due to the administration of plant extracts containing antifungal compounds were also studied by Ahmad et al. (2013). An SEM analysis showed morphological differences in the fungus cells of Candida guilliermondii SN 2006 between the control and the cells treated with cinnamon bark extracts. In the fungus without the treatment of cinnam

on bark extracts, the surface morphology of the cell appeared smooth. By contrast, in the fungus with the extract treatment, the cell surface appeared damaged. Lim et al. (2006) reported the results of the SEM treatment of acetone bark extracts from Rhizophora apiculata tested on Candida albican mushrooms. The treatment with the R. apiculata stem bark acetone extract changed the morphology of Candida albican fungal cells, especially the structures of the cell membranes and the cell walls, compared with those of the controls (without extract treatment). Phongpaichit et al. (2005) carried out an SEM analysis on the methanol extract of the Acorus calamus (A. calamus) rhizome with the Microsporum gypseum test fungus. The treatment with the A. calamus rhizome methanol extract in the test fungi showed shrinking hypha and conidia structures compared with those of the controls.



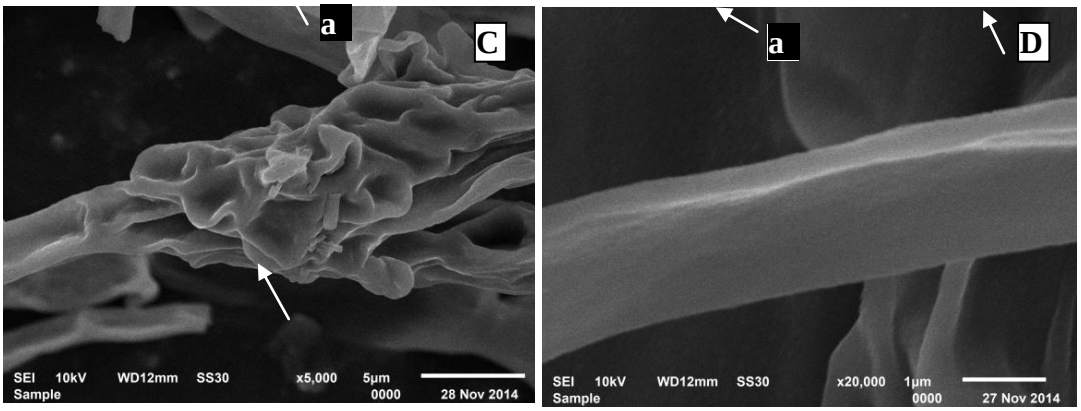


Fig.7: Mechanism of the C. burmannii leaf extract in inhibiting the growth of the test fungi. A. fungi mycelium C. capsici (Treatment): a. damaged hypa; B. fungi mycelium C. capsici (control): a. hypa is good; C. mycelia fungus F. equiseti: a. concave hypha structure, contracted with irregular structure; D. fungus mycelia F. equiseti: a. Smooth hypha structure

CONCLUSION

The acetone extract of C. burmannii leaves obtained from Bedugul in Baturuti Sub-District, Tabanan Regency, Bali Province, Indonesia can inhibit the growth of the C. capsici fungus, which attacks large chilli plants in Bali. The acetone extract of C. burmannii leaves can inhibit the growth of colonies, biomass and fungal spore formation in vitro. The acetone extract of C. burmannii leaves with the T2, T3 and T4 treatments can inhibit the growth of fungal colonies in a row with inhibitory powers of 17%, 30% and 46%, respectively. Treatments with the same concentrations (T2, T3 and T4) can inhibit the growth of biomass and spores by 14%, 24% and 87% and 51%, 69% and 86%, respectively. The T5 and T6 treatments of the colonies, fungi biomass and spores cannot grow. The acetone extract of C. burmannii leaves can inhibit the growth of the test fungi by damaging the structure of the fungal cell wall.

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