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BIOLOGICAL CONTROL OF ANTHRACNOSE DISEASE (*Colletotrichum acutatum)* IN CHILI PEPPERS BY CRUDE LEAF EXTRACT OF FIG (*Ficus septica* Brum.f.)

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SUMMARY

Fig (*Ficus septica* Brum.f.) leaf extract has bioactive compounds with antifungal properties due to alkaloid and phenolic compounds which are antioxidants. Fig is widely distributed and *F. septica* accessions have large genetic variability, which may be separated based upon morphological traits. *Colletotrichum acutatum* is a plant pathogen, which causes the most destructive fungal disease (called anthracnose) in the Solanaceae family, and is triggered by post-bloom fruit drop. The fungus *C. acutatum* is also the causal agent of the anthracnose disease of chili peppers. The present study aimed to study the mode of action of *F. septica* leaf extract in controlling the growth of fungus *C. acutatum*. Experiments were conducted to measure antifungal activity as well as diffusion to identify the mode of action of fig leaf extract, including a control treatment using sterile distilled water. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used. The minimum concentration inhibition (MIC) to inhibit the growth of *C. acutatum* on potato dextrose agar (PDA) was 0.9% with inhibition zone diameter (7.25 mm) on the third day of incubation. Based on the *in vitro* test on PDA and based on the antifungal activity test, the leaf extract of *F. septica* inhibited the growth of *C. acutatum* with inhibition zone diameter of 30 mm. However, inhibitory action of the *C. acutatum* leaf extract is not widely known. The SEM and TEM analyses and mode of action of *F. septica* leaf extract confirmed that the growth of fungus *C. acutatum* was inhibited and controlled through diffusion process into the fungal cells, and then interfered with the structure of the cell organelles. Consequently the cells suffered from lysis and become empty, and eventually the fungal growth was inhibited resulting in cell death. We conclude that crude leaf extract of *F. septica* contains bioactive compounds with the antifungal substances which can be safely used as an alternative measure to control anthracnose disease of chili peppers.

Keywords: *Ficus septica*, bioactive compounds, antifungal components, *Colletotrichum acutatum*, anthracnose disease, chili peppers

Key findings: This study confirmed the antifungal properties of the leaf extract of *F. septica* and its prominent role in inhibiting and controlling the growth of fungal pathogen *C. acutatum* causing Anthracnose disease in chili peppers.

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INTRODUCTION

In chili peppers, the Anthracnose disease is a major disease caused by the genus *Colletotrhricum* which includes five different species: *C. gloeosporioides*, *C. Capsici*, *C. acutatum*, *C. demantium* and *C. cocodes* (Kim *et al*., 1999). The symptom of anthracnose disease in chili peppers begins with a shiny rind followed by softening of the tissue, and then the surface of the fruit becomes sunken and brownish known as a lesion (Kim *et al*., 1984). Anthracnose is the most common disease of chili peppers and infects almost the entire plant (Figure 1B). According to Sudiarta and Sumiartha (2012) and Sudirga (2016), the anthracnose disease is mostly caused by *C. acutatum* which infect the chili peppers in Bali, Indonesia.

Anthracnose disease can damage the aesthetic value of the chili and reduce yield by up to 50% or even more (Semangun, 2007). However, the Anthracnose disease is generally controlled by the use of synthetic fungicides. Continuous use of synthetic fungicides may lead to the emergence of pathogen resistance, pollute the environment and may also be harmful to consumers. Based on this, it is necessary to find an alternative control method of anthracnose disease by utilizing natural / botanical fungicides which are also not hazardous or toxic to end users and the environment.

A preliminary study of the 20 different plants species revealed that in terms of antifungal activity against *C. acutatum* which causes anthracnose disease on chili pepper, and identified six different plants that can inhibit the growth of *C. acutatum*. These six plants were *F. septica*, *Albizia saman*, *Piper nigrum*, *Piper crocatum*, *Piper retrofectum* and *Thitonia difersifolia*. Among the six species compared to other plant species, *F. septica* leaf extract has the highest inhibitory activity with an inhibition zone of 30 mm. Some other plant species are also reported to have antifungal activity such as *Ageratum conyzoides* has antifungal activity against *Penicillium italicum* (blue mold) the cause of fruit rot disease in Mandarin orange (Dixit *et al*., 1995); *Origanum manjorona* has antifungal activity against *C. gloeosporioides* the cause of anthracnose disease in coffee (Silva *et al*., 2008); *Albizia saman* has antifungal activity against *Fusarium* sp. the cause of wilt disease on chili plants (Suprapta and Khalimi, 2012).

Fig (*F. septica* Brum.f.) was first described by the Dutch Botanist Nicolaas Laurens Burman in 1768 (Brummitt, 2001). Fig, also called 'Hauli tree' in the Philippines and Taiwan, is a wild shrub of the family Moraceae living at low altitudes from India to North Australia, and throughout Malaysia. It lives on the edge of the vegetation, often in degraded environments and by the community it is only used as a traditional medicine (Figure 1A). Vital *et al*. (2010) reported in his studies that a crude extract of *F. septica* leaves can inhibit the growth of *Staphylococcus aureus*, *Canida albicans* and *Escerechia coli* with inhibition zones of 14, 18 and 13 mm, respectively. Suspected chemical compound contained in the leaves, fruits and roots of *F. septica* in the form of alkaloids, saponins, flavonoids, tannins and polyphenols (de-Padua *et al*., 1999). According to Damu *et al*. (2005), the extracts of *F. septica* stem bark contain phenanthro-indolizine alkaloids which are cytotoxic. Previous studies revealed that fig contains active compounds like antofine and ficuseptine (Castillo *et al*., 2012). Antofine compounds are considered as anticancer

Figure 1. A). Leaf of *Ficus septica*, B) Anthracnose disease on chili pepper (Source: private collection, 2014).

while ficuseptine compounds are considered as antibacterial and antifungal compounds.

Past studies reported the extract of the root bark of *F. septica* contains flavonoid compounds (which belong to the flavanones class) and these compounds can inhibit the growth of bacteria such as *Vibrio cholerae* and *Escherichea coli* (Sukadana, 2010). The extract of *F. septica* stems also containing alkaloids compounds belongs to the class of alkaloids phenanthro-indolizidine consisting of ficuseptines BD (1-3), 10R, 13aR-tylophorine N-oxide (4), 10R, 13aR-ylocrebrine N-oxide (5), 10S, 13aR-tylocrebrine N-oxide (6), 10S, 13aR-isotylocrebrine N-oxide (7), and 10S, 13aS-isotylocrebrine N-oxide (8). The alkaloid class of these biological compounds are cytotoxic. According to findings of Nugroho *et al*. (2011), the fractionation of ethanol and hexane obtained in leaf extract of fig has potential as an anticancer compound. Besides that, the leaves and roots of *F. septica* also contains saponins and flavonoids, fruits contain alkaloids and tannins, and roots also contain polyphenols (de-Padua *et al*., 1999).

Based on a preliminary *in vitro* test on PDA, leaf extract of *F. septica* can inhibit the growth of *C. acutatum* with an inhibition zone of 30 mm diameter. Several previous studies have not reported the inhibitory mechanism of the bioactive compounds of *F. septica* leaf extracts against the growth of pathogen *C. acutatum*, and it is uncertain mode of action of its extract. Therefore, the aim of the present research was to find out the mode of action of leaf extracts of *F. septica* to control the growth of the pathogen i.e., *C. acutatum*.

MATERIALS AND METHODS

Methods of leaf extraction

For extraction of leaf extract, the *F. septica* leaves were chopped, then dried at room temperature, and after that the dry material was made into powder by means of a blender. *F. septica* leaf powder (100 grams) was then macerated with

1000 ml of methanol PA (Pro-Analysis) for 72 hours at room temperature and dark place. The filtrate was obtained by filtering and the residue obtained was then macerated again with 1000 ml of methanol as much as two times. The filtrate obtained are combined and then evaporated using a vacuum rotary evaporator (Iwaki, Japan) at 40°C, to obtain a crude extract that was used for further testing. To find out whether the active compound of *F. septica* leaf extract is polar or non-polar, then partition using counter-current distribution method with two types of solvents i.e., hexan and methanol phase.

Antifungal activity test

Antifungal activity test of crude extract of the leaves of *F. septica* against *C. acutatum* was done in well diffusion method. The minimum inhibition concentration (MIC) to the growth of *C. acutatum* on PDA was 0.9% with inhibition zone diameter of 7.25 mm on the third day of incubation. While on treatment with a concentration of 1, 2, 3, 4 and 5% could inhibit the growth of fungal pathogen with inhibition zone diameter i.e., 29.27, 38.89, 46,95, 57.23 and 81.39 mm, respectively. According to Ardiansyah (2005), if the diameter of inhibition zone is ≥ 20 mm the inhibitory activity is very strong; 10 to 20 mm the inhibitory activity is strong; 5 to 10 mm the inhibitory activity is moderate; and ≤ 5 mm the inhibitory activity is poor or weak.

Preparation of material for SEM and TEM

For preparations for scanning electron microscopy (SEM), the *C. acutatum* was grown on PDA, the leaf extract of *F. septica* was added for treating while the control was having only sterile distilled water with well diffusion method, and then incubated for three days at 25°C. After three days, the fungal colony was cut on the edge of inhibitory zone with the size of 3 mm and then fixed with a wide range of solutions and samples were observed by SEM using the JSM-6510LA, JEOL, Japan with acceleration voltage of 5 kV (Hall, 1978; Hayat , 1981). Fixation and dehydration processes in the sample for transmission electron microscopy **(**TEM) having the same process as SEM. Samples were cut to ultrathin size (1 µm) using Reichert Ultracut S ultra microtome. Results of ultrathin size pieces were tinged with 6% aqueous uranyl acetate and lead solution Sato. After staining, the ultrathin pieces were observed using a TEM (JEM-1010, JEOL, Japan) with acceleration voltage of 160 kV (Hall, 1978; Hayat, 1981; Kawuri, 2012).

RESULTS AND DISCUSSION

Inhibitory activity of partitioned extract

Based on the results of partition using counter-current distribution method with two types of solvents were hexan and methanol phase which showed that the methanol extract could inhibit the growth of *C. acutatum* with the diameter of inhibition zone of 30 mm, whereas hexane extract phase could not inhibit the growth of this fungus (Figure 2). These results indicated that the active biological compounds in the leaf extract of *F. septica* are antifungal against *C. acutatum* in the phase of methanol and is polar.

Leaf extract of *Aegle marmelos* inhibited the growth of the fungus *C. acutatum* with inhibition zone diameter of 22 mm (Gawade *at al*., 2014). According to Nogodula *et al*. (2012), crude extract of the *F. septica* leaves is able to inhibit the growth of mold *Canida albicans* with inhibition zone diameter of 16.67 mm. However, no such study has been reported on the *F. septica* leaf bioactive compound as a potential botanical fungicide to control anthracnose disease caused by *C. acutatum* on chili peppers. Past studies reported the crude extract of *F. séptica* with strong inhibitory activity against *C. acutatum* (Sudirga *et al*.,

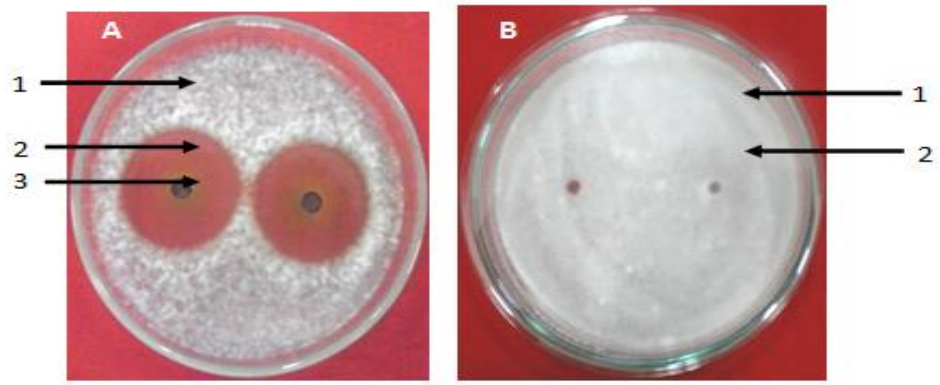

Figure 2. Inhibition zone formed around the well diffusion filled with partitioned leaf extract of *Ficus septica* of methanol phase (A) and hexane phase (B). (1 = mycelium of *C. acutatum*, 2 = well diffusion, and 3 = inhibition zone).

2014). This treatment effectively reduced and controlled the fungal radial growth on PDA, spore's formation and biomass formation on PD broth medium with the inhibitory activity ranged from 29.72 to 100%. According to Damu *et al*. (2005), the extracts of *F. septica* stem bark contain phenanthro-indolizine alkaloids which are cytotoxic. Overall, the symptom of anthracnose in chili pepper begins with a shiny rind followed by softening of the tissue and then the surface of the fruit becomes sunken and brownish known as lesion (Kim *et al*., 1984).

Inhibition mechanism of *Ficus septica* leaf extract

The observation with SEM on the colonies of *C. acutatum* grown around the zone of inhibition showed a damage *i.e.,* fungal hyphae were observed to have grooves andthin, which indicates that fungal cells loose the contents and cell fluid (Figure 3B), whereas in control, the fungal hyphae appeared intact with hyphae surfaces ungrooved and was smooth (Figure 3A). According to Shalgal *et al*. (2011), the morphological changes were observed in the *Candida albicans* after treating with seed extract of *Swietenia mahogany* at various exposure times. The cells started to shrink and then clumped together before they were completely destroyed by the extract.

Ultra structure observation of hyphae and spores of *C. acutaum* using TEM showed that the cells making hyphae and spores of a fungus that grew around the zone of inhibition underwent lysis i.e., discharge of cell contents and fluid, cell organelles were assumed to be irregular (not organized), cell membranes was perforated, and the cells could be said to suffered from destruction and death (Figures 4B and 5B). However, in the control, it was visible that structures were within cells of hyphae and spores with a thick cell membrane structure and smooth, cell organelles were arranged regularly and looked like the cell organelles such as mitochondria, nucleus, and vacuole (Figures 4A and 5A).

According to Widodo *at al*. (2012), the cell wall became thicker after treatment of coumarin, and this might be due to the accumulation of membranous material in the surrounding of the cell wall surface. The cell wall also became thicker which might be due to increased transmembrane leakage of amino acids and other cytoplasmic content. An abnormal and broader shape of the vacuole was observed than in the normal cell. The cell membrane and cell walls were altered after 24 h to extract

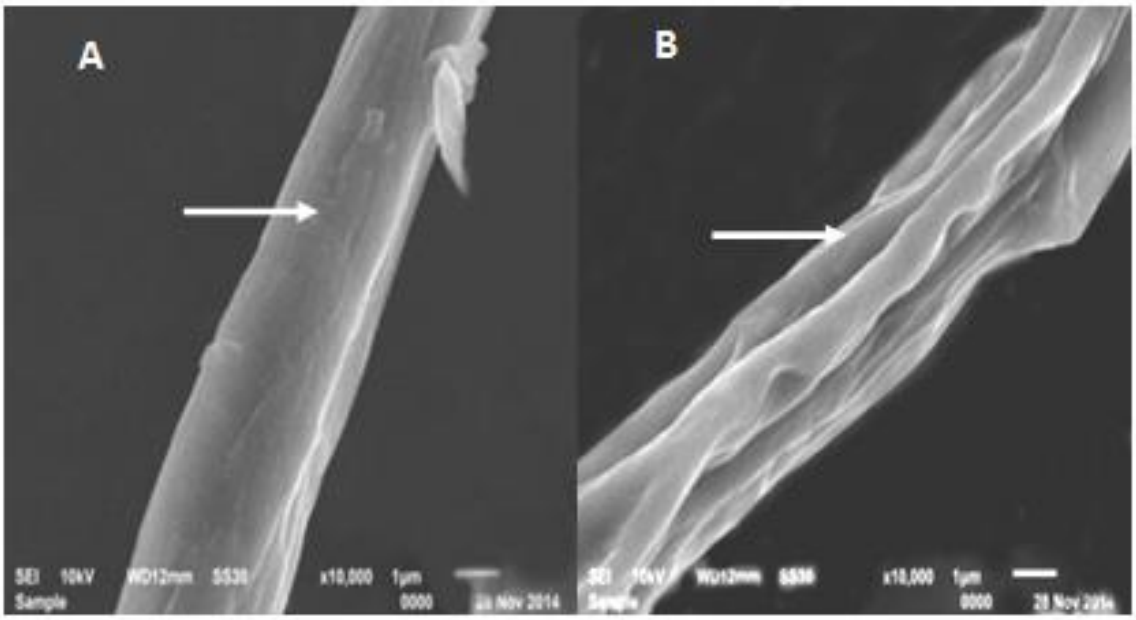

Figure 3. Hypae of *C. acutatum* taken through SEM. A) control, hyphae surface is smooth (arrow), B) treated with leaf extract of *Ficus septica*, grooved surface hyphae (arrow), a magnification of 10,000, bar 1 µm).

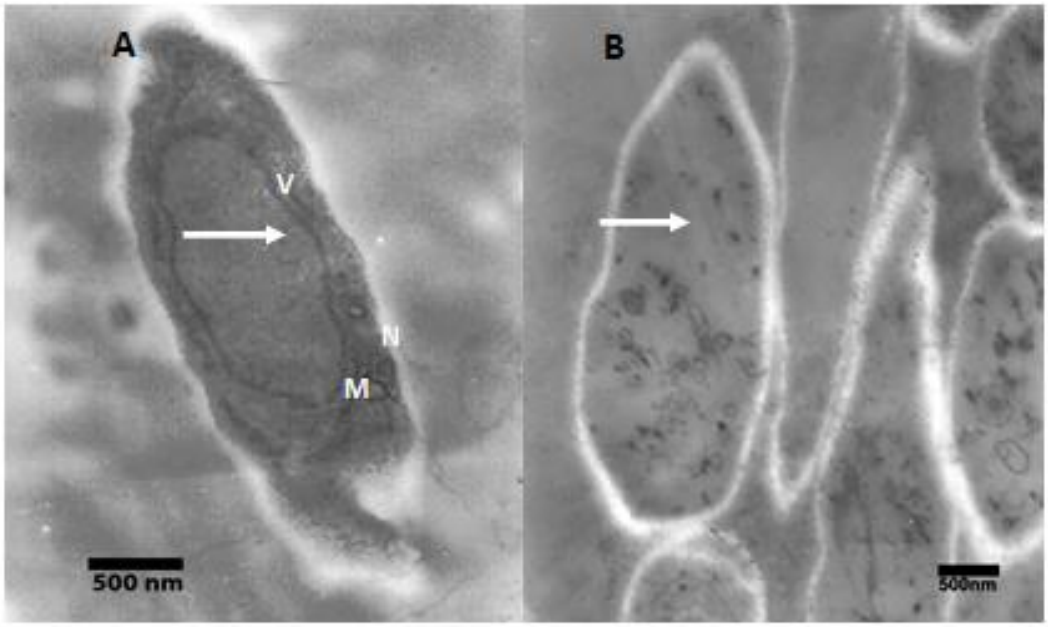

Figure 4. Hyphae ultrastructure of *C. acutatum* taken through TEM. (A) control, cell organelles still arranged regularly (V = vacuoles, N = nucleus, M = mitochondria); (B) treatment with leaf extract of *Ficus septica*, cell organelles were irregular / lysis (arrow), bar 500 nm).

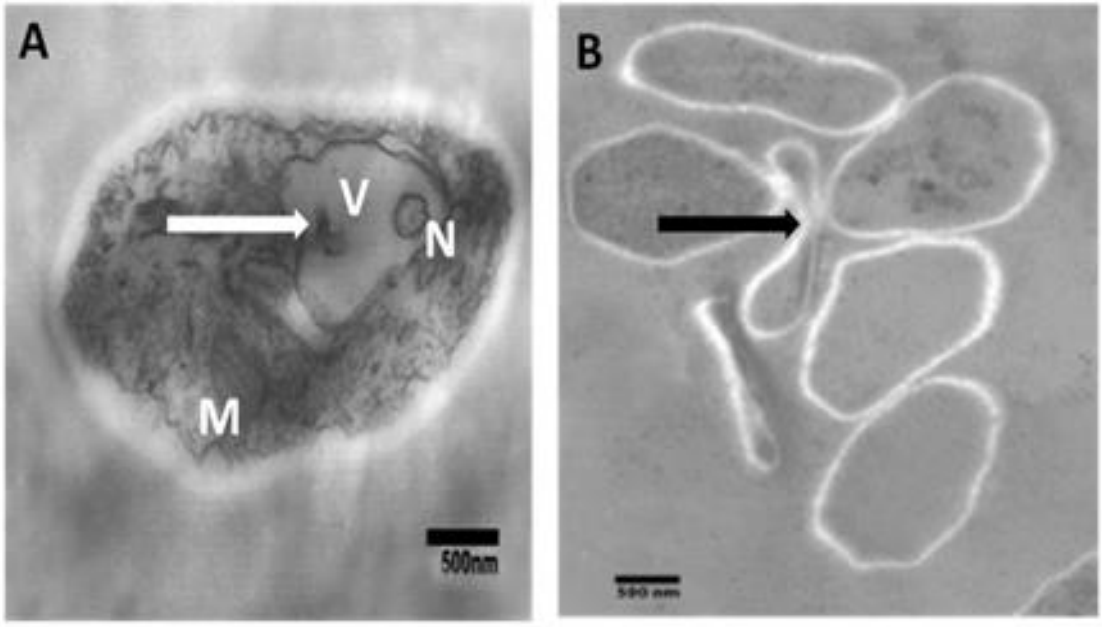

Figure 5. Ultrastructural spore of *C. acutatum* taken through TEM. (A) control, organization of regular cells (V = vacuoles, N = nucleus, M = mitochondria); (B) treatment of leaf extract *Ficus septica*, cell organization is irregular and cell lysis folded (arrow) and the bar 500 nm).

exposure, while the cytoplasmic volume decreased with structural disorganization within the cell cytoplasm (Basama *et al*. 2011). According to Pelczar *et al*. (2003), the mechanism of antimicrobial substances in killing or inhibiting microbial growth is by (a) damaging microbial cell walls, resulting in lysis or inhibiting cell wall formation in growing cells, (b) changing cell membrane permeability which causes cytoplasmic leakage and loss of nutrients, (c) causing cell denaturation, and (d) inhibiting enzymes in cells.

The observation of cell ultrastructure through SEM and TEM showed that the inhibition mechanism of the leaf extract of *F. septica* against *C. acutatum* occurred by affecting the permeability of fungal cell membranes. Ghannoum and Rice (1999) reported that fungal cells have cell walls composed of mannoproteins, β- (1-6) glucans, β- (1-3) glucans and chitin, and plasma membranes contain ergosterol. The mechanism of the action of the antifungal is the ergosterol in the plasma membrane. Ergosterol is the main component of the plasma membrane which functions to maintain the integrity of the fungal cell membrane by regulating the fluidity of the plasma membrane. Compounds that inhibit ergosterol synthesis can result in permeability disturbances in the form of leakage of potassium ions and to cause the cell death. Damage to the cell membrane causing changes in membrane permeability resulted in lysis of the fungal cell contents (Harbone, 1989). According to Amjad *et al*. (2012), the activity of phenolic compounds and flavonoids as antifungal compounds was due to their ability to form complexes with cellular extract proteins, and the protein dissolved, so that the compound was likely to interfere with fungal cell membranes.

CONCLUSION

The role of *F. septica* leaf extract in controlling Anthracnose disease caused by pathogen *C. acutatum* is by affecting the permeability of fungal cell membranes. Disturbance in the permeability of cell membranes caused the cytoplasmic leakage resulting in lysis of the cells, and ultimately the cell growth is inhibited and causes cell death.

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