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UTILIZATION OF BETEL LEAVES EXTRACT TO PREVENT THE GROWTH OF *Fusarium oxysporum* f.sp. *capsici* CAUSING FUSARIUM WILT DISEASE IN BELL PEPPER

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ABSTRACT

Utilization of betel leaves extract to prevent the growth of* Fusarium oxysporum *f.sp.* capsici *causing fusarium wilt disease in bell pepper. Betel (*Piper betle* Linn.) is commonly used in Indonesian traditional medicine due to its antimicrobial properties, thus it is often used as an ingredient in natural pesticides. The effectiveness of betel leaf extract as a control for *Fusarium oxysporum* in several plants showed it's potential to control the fungi in bell pepper. *F. oxysporum* f.sp. *capsici* is one of the important pathogens causing Fusarium wilt disease in bell pepper. It is one of the most devastating plant diseases due to its ability to cause a crop failure. The test on the inhibitory capacity of betel leaf extract on the growth of *F. oxysporum* f.sp. *capsici* were carried out by growing the fungi on PDA mixed with various concentrations of betel leaf extracts. The results showed that betel leaf extract treatment at 0.02% concentration was able to inhibit the growth of F. oxysporum f.sp. capsici in PDA at 95.54%. The full inhibition of colony growth (100%) was achieved in the extract treated with the concentration of 0.03%. Meanwhile treatment of 0.17% betel leaf extract was able to inhibit the growth of *F. oxysporum* f.sp. *capsici* on bell pepper stems. Following this result, fractionation of the betel leaf extract by column chromatography was conducted, resulting in 44 fractions. The bioassays of those fractions showed that, there were seven fractions that reveal inhibition capability against *F. oxysporum* f.sp. *capsici.*

Key words: betel, antifungal, *F. oxysporum* f.sp. *capsici*, natural pesticide

INTRODUCTION

The use of betel leaf extract (*Piper betle* L.) as natural fungicide to control several plant pathogenic fungi has been widely used. Several reports have shown that betel leaf extract was effective in suppressing the development of several types of pathogens. Betel leaf extract contains various active ingredients that have antimicrobial properties (Datta *et al*., 2011), such as alkaloids, fatty acids, phenols, alcohol, flavonoids, terpenes, coumarin, and organic acids (Foo *et al*., 2015). Betel leaf extract and galangal rhizome at a concentration of 0.5% were reported to be effective in inhibiting *F. oxysporum* and *Ralstonia solanacearum* bacteria in banana seeds (Phabiola, 2004). While the betel leaf extract of Beleng cultivar at the same concentration was able to inhibit the colony growth of *F. oxysporum* f.sp. *vanillae* as 83.14% (Subrata & Rai, 2019).

Various reports on the effectiveness of betel leaf extract in controlling *F. oxysporum* in several plants indicated that, betel leaf extract was predicted to be able to control the fungus in bell pepper. *F. oxysporum* f.sp. *capsici* is one of the important pathogens causing Fusarium wilt disease in peppers. This disease is one of the most harmful diseases that can cause crop failure (Cahyono, 2003).

A preliminary study was conducted to ensure the consistency of crude extract of betel leaf's ability to inhibit the growth of the colonies of *F. oxysporum* f.sp. *capsici* in vitro on PDA media and bell pepper stems. The result showed that crude extract of betel leaf against *F. oxysporum* f.sp. *capsici* was able to produce an inhibition zone of 34.5 mm. According to Morales *et al*. (2003), such diameter of inhibition zone was categorized into very strong. This research was conducted to ensure the consistency of extract of betel leaf's ability to inhibit

the growth of the colonies of *F. oxysporum* f.sp. *capsici* in vitro on PDA media and bell pepper stems.

MATERIALS AND METHODS

Research Site. This research was conducted from August 2018 to March 2019 at the Biopesticide Laboratory in Agriculture Faculty, Udayana University.

Extraction. The extract of betel leaf was obtained by maceration using methanol as a solvent. The clean betel leaves were chopped into small pieces and dried for 2–3 d. The dried betel leaves were macerated in methanol in the ratio of 1:10 (100 g of betel leaf to 1000 mL of methanol). Maceration was carried out at room temperature for 48 h. The filtrate was obtained by filtering through four layers of gauze and Whatman No. 1 filter paper. The solvent was evaporated using a rotary vacuum evaporator at a temperature of 40 °C, until the solvent separated, and the methanol extract of betel leaf was obtained.

Inhibition Ability of Betel Leaf Extract Against *F. oxysporum* f.sp. *capsici*.

The Minimum Inhibitory Concentration (MIC) Test. The MIC of betel leaf extract against *F. oxysporum* f.sp. *capsici* was carried out on PDA media by placing paper discs that had been previously dipped in crude extract of betel leaf with a serial concentration of 0.1%, 0.25%, 0.5%, 0.75%, 1.0%. The extracts of various concentrations were prepared by diluting the crude extract of betel leaf using acetone as a solvent. The dipped paper disc was placed in the middle of the PDA mixture (10 mL PDA and 200 µL fungal suspension) in petri dish.

The Inhibition Test on PDA Mixture. The inhibition ability of betel leaf extract on the growth of *F. oxysporum* f.sp. *capsici* was carried out by growing the fungi on PDA mixed with various concentrations of betel leaf extracts. The treatments consisted of 0.10%, 0.20%, 0.30%, 0.40%, 0.50% and 0% (negative control). These concentrations were obtained by pouring a 1 mL of extract of each proportions to a 9 mL warm PDA (temperature ± 50 °C) on petri dish, then rotating the petri dish in a circular motion until the media and extract were evenly mixed and solidified. The final concetration of the mixtures became 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, and 0%. Then a 4 days isolate of *F. oxysporum* f.sp. *capsici* were placed into the solidified PDA mixture, using a 5 mm diameter cork borer and a transfer needle.

Inhibition Test on Bell Pepper Stem. The tests to determine the inhibition ability of betel leaf extract on the growth of *F. oxysporum* f.sp. *capsici* on bell pepper stems was carried out *in vitro* in 30 mL Potato Dextrose Broth (PDB) media. PDB was mixed with 200 µL of conidial suspension (2×10^6 conidia/mL) and crude extract of betel leaf, on a petri dish. Extract of betel leaves as treatments with concentrations of 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%, 1 mL each was added to the 29 mL PDB media. The final concentrations of the media-extract mixtures were were 0.003%, 0.006%, 0.010%, 0.013%, and 0.017%.

Healthy stems of bell peppers were washed with distilled water and disinfected in 70% alcohol for 2 min. After that, the bell pepper stems were washed using sterile water three times and cut into cross-sections at a length of ± 1 cm. The chopped stems were put in a petri dish, 10 pieces each. As comparisons, a negative control (PDB media, without fungal suspension and extract) were used along with positive controls (PDB media, with fungal suspension only).

Separation of Methanol Phase and n-Hexane Phase of Betel Leaf Extract. The separation of the extract from the n-hexane and methanol phases was carried out by dissolving 4 mL of methanol extract of betel leaf into a mixture of 200 mL of methanol and 200 mL of n-hexane. It was shaken in a separate funnel until it was evenly mixed. The mixture then incubated for 10 min until it showed separation between methanol and n-hexane phases. After the two phases were separated, the solvent was evaporated in a vacuum rotary evaporator. Both phases were tested for inhibition ability against *F. oxysporum* f.sp. *capsici* on PDA media (Darmadi, 2015).

Active Component Fractionation (Column Chromatography and Thin Layer Chromatography). Fractionation of the active component of betel leaf extract was obtained by column chromatography. The column was passed through eluents with different levels of polarity (n-hexane, dichloromethane, ethyl acetate, acetone, and methanol). The stationary phase used in thin layer chromatography was silica gel (wako gel, particle size 75–150 µm). Thin Layer Chromatography (TLC) was performed by dropping each fraction on a 10 × 10 cm TLC plate (Keisal Gel 60 F254) using a capillary tube to form spots.

The TLC plate was inserted into the TLC chamber equipped with eluent. The eluent used in this study was n-hexane: ethyl acetate (20: 3). The process was ended after the top side of the eluent reached ¾ of the plate height. The TLC plate was taken out and dried, then the spots weres identified by placing the plate into a chamber containing iodine crystals. The iodine vapor caused the spots in TLC plate to change color to slightly brownish so that the TLC results could be observed.

To compare one spot with another from the separation by Thin Layer Chromatography, the retention factor (Rf) unit value was used. The value of Rf is the ratio of the distance from the starting point of the spot to the extent that the spot compared to the distance from the solvent to the highest point calculated from the starting point.

RESULTS AND DISCUSSION

The Inhibition Ability of Betel Leaf Extract Against *F. oxysporum* f.sp. *capsici*. Minimum Inhibitory Concentration (MIC) of betel leaf extract against *F. oxysporum* f.sp. *capsici* was at 0.1% (Table 1). MIC is defined as the lowest concentration of antimicrobials compounds that can inhibit microorganisms after a certain incubation period (Andrews, 2001). Suprapta & Khalimi (2012) reported that betel leaf extract had the same antifungal activity against *F. oxysporum* f.sp. *capsici* with an MIC of 0.3 mg/mL. The variability of antifungal activity was related to the secondary metabolites produced by the plants, whereas the production of secondary metabolites in plants was influenced by the stress level of the plant (Pagare *et al*., 2015). Environmental factors such as water stress could increase secondary metabolites in plants. The response of plants to water stress in addition to reducing growth and productivity could also increase levels of K, proline amino acids, and the content of secondary metabolites (Trisilawati & Pitono, 2012). Abdelmajeed *et al*. (2013) reported that water deficiency treatment increased total phenolic content and antioxidant activity in *Cuminum cyminum* L. seeds. The effect of water deficiency in increasing secondary metabolic activity would improve the quality and efficacy of Simplicia, a medicinal plant. Rahardjo *et al*. (1999) reported that water stress on *Sonchus arvensis* caused an increase of leaf flavonoid levels by 2.11%.

In this study, the leaf extract of betel leaf with a concentration of 0.02% inhibits the growth of colonies of *F. oxysporum* f.sp. *capsici* at 95.54%. This showed that the leaf extract of betel leaf has the ability to inhibit the growth of *F. oxysporum* f.sp. *capsici* mycelia. The 100% inhibition was shown at the concentration of 0.03% (Table 1). This result is in line to Sanit (2016) which demonstrated that 0.10% betel leaf extract was able to inhibit the growth of mycelia *Fusarium* sp. amounted to 58.00%, while the 100% inhibition was shown in the extract treatment of 0.25%. Other study also reported that the 1.00% concentration of betel leaf extract had the ability to suppress 100% of the growth of *F. oxysporum* mycelia (Singburaudom, 2015).

The growth of colony *F. oxysporum* f.sp. *capsici* can already be seen after 2 d of incubation on PDA media with no treatment and it become fully grown after 11 d of incubation (Figure 1). Colony growth of *F. oxysporum* f.sp. *capsici* on media containing of betel leaf began extract to appear on the 4th day (0.01% extract treatment) and 10th day (0.02% extract treatment) after inoculation. While the treatments of betel leaf extract with a concentration of 0.03%; 0.04%; and 0.05% did not show any growth of *F. oxysporum* f.sp. *capsici*. Arsih *et al.* (2015) reported that betel leaf extract at concentration of 0.25% also reported to be able to suppress the growth of *F. oxysporum* f.sp *lycopersici* causing Fusarium wilt disease in tomatoes by 68.89%.

The results growth of *F. oxysporum* f.sp. *capsici* on bell pepper stems in vitro in PDB media showed that in the positive control, 0.003%, 0.006%, and 0.010% the disease symptoms appeared on the 3rd day of incubation (Table 2). Whereas the treatment with 0.013% extract, the symptoms was appeared after 4th day.

Table 1. Inhibition ability of betel leaf extract against *F. oxysporum* f.sp. *capsici*

Concentration (%)	Average colony diameter (mm)*	Inhibition rate (%)*
0	89.67	0
0.01	29.00	67.66
0.02	4.00	95.54
0.03	-	100
0.04	-	100
0.05	-	100

*= 11 days of incubation

Different results were shown by 0.017% extract treatment and negative control, in which fungal growth did not start to appear until 4th days after incubation (Figure 2). In vitro test using bell pepper stems showed that the fungus grew first on the stems, then spread on the media.

Inhibition Ability of Methanol and n-Hexane Phase of Betel Leaf Extract Against *F. oxysporum* f.sp. *capsici*. Separation of betel leaf extract into methanol and n-hexane phases resulted in different inhibition of *F. oxysporum* f.sp. *capsici*. The methanol phase showed a larger diameter of inhibition zone than the n-hexane phase. Methanol phase and n-hexane phase showed a diameter of inhibition of 16.33 mm and 6.67 mm, respectively. This showed that the betel leaf extract in the methanol phase has greater inhibition ability against *F. oxysporum* f.sp. *capsici*.

Betel leaf extract macerated with a 1% concentration of chloroform solvent was reported to be effective in reducing the population of *F. oxysporum* f.sp. *lycopersici* in the rhizosphere of tomato was grown compared to treatment with the synthetic fungicide carbendazim (Singha *et al*., 2011). Besides chloroform solvent, ethanol and acetone extract of betel leaves with a concentration of 20% (Neela *et al*., 2014), diethyl ether and ethyl acetate extract of betel leaves (Murugesan *et al*., 2011) were also effective in inhibiting the growth of *F. oxysporum*. A study in Fusarium wilt in banana demonstrated that betel leaf extracts from maceration processes using ethyl acetate, methanol, n-hexane also showed antifungal properties against

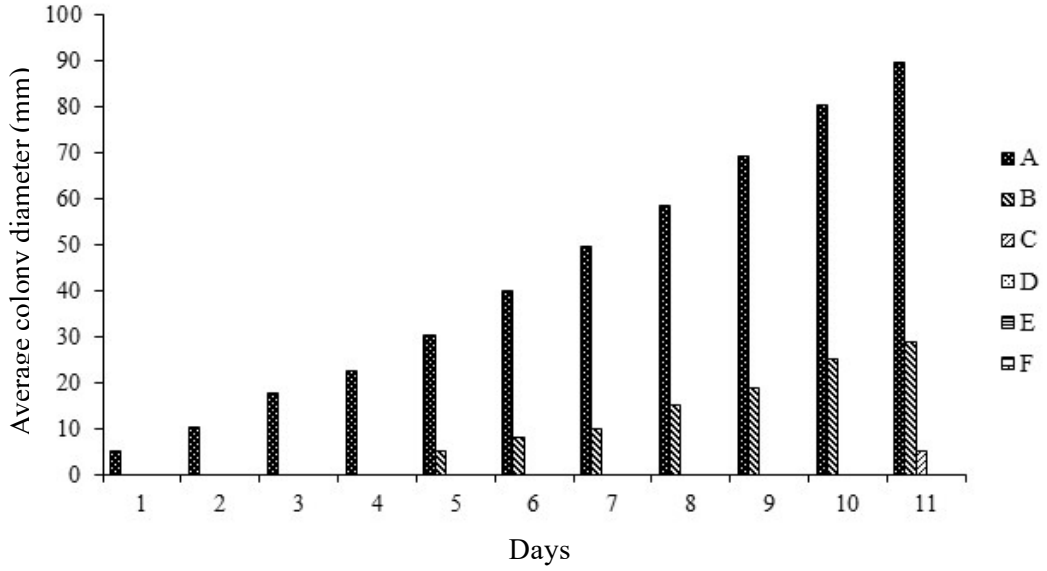

Figure 1. Growth of *F. oxysporum* f.sp. *capsici* on PDA media with the concentration of betel leaf extract. (A) 0%; (B) 0.01%; (C) 0.02%; (D) 0.03%; (E) 0.04%; (F) 0.05%.

Table 2. Growth of *F. oxysporum* f.sp. *capsici* on PDB media with various concentrations of betel leaf extract

Days	Extract concentration (%)						
	0 Negative control	0 Positive control	0.003	0.006	0.010	0.013	0.017
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	-	++	+	+	+	-	-
4	-	++++	+++	++	+	+	-

(-) = The fungi did not grow neither in the bell pepper stems nor in the PDB
(+) = The fungi grew in the surface of bell pepper stems
(++) = The fungi grew in the whole of bell pepper stems
(+++) = The fungi grew in the media
(++++) = The fungi filled the media

F. oxysporum f.sp. *cubense* with methanol extraction result exhibiting the highest inhibitory (Gnanasekaran *et al*., 2015).

That study was in line with Aoki *et al*. (2019), in which methanol extract of betel leaf showed stronger antifungal activity than betel leaf hot water extract with a concentration of 10% against *Plasmopara viticola* causing downy mildew in grapes.

Patil *et al*. (2015) stated that betel leaf extract contained various antimicrobial active ingredients as well. The water, ethanol, methanol, butanol, and acetone extracts of betel leaves each had antimicrobial activity against several fungi and bacteria, however the methanol and butanol extracts showed stronger antimicrobial activity. This shows that the antifungal active compounds in betel extract consist of several active compounds that have different polarity, but the antifungal activity is stronger in the methanol phase extract which has polar properties.

Inhibition Ability of Betel Leaf Extract Fraction Against *F. oxysporum* f.sp. *capsici*. Fractionation of the betel leaf extract by column chromatography resulted in 44 fractions. Testing of *F. oxysporum* f.sp. *capsici* was carried out on those 44 fractions. The results showed that, there were seven fractions showing the inhibition capability against *F. oxysporum* f.sp. *capsici* namely F9, F10, F11, F12, F13, F19, and F20 (Figure 3; Table 3).

Thin Layer Chromatography (TLC) was carried out on these seven fractions to determine the best eluent as a separator for the active antifungal compound of *F. oxysporum* f.sp. *capsici*. The TLC results showed that the best eluent was mixture of n-hexane: ethyl acetate (20: 3) (Figure 3).

Based on the Rf value, there were seven different fractions (Table 3). There were fractions F10 (Rf = 0.55) and F20 (Rf = 0.13) that had single Rf value meaning that the fractions probably contained single groups of compounds. Meanwhile, there were fractions that had multiple Rf values one such as F9, F11, F12, F13, and F19 and probably each contained more than one compound. These fractions probably contained similar groups of compounds (F13 and F19 have the same Rf value, namely 0.08 and 0.16). There were also groups of fractions consisting of different types of compounds (F13 contained a group of compounds that were not present in F19 indicated by the Rf value of 0.23, and vice versa F19 contained a group of compounds that were not present in F13 notified by Rf value of 0.29).

The diameters of the inhibition zones produced by the fractions were also different. For example, fraction F19 had a larger diameter of inhibition than fraction F11. This was presumably due to the different types of active compounds in the two fractions as well as differences in the synergy of active compound groups in these fractions. The fractions F9, F11, F12, F13, and F19 had more than one Rf value, indicating that the active ingredients in the fractions probably consisted of two or more groups of active compounds.

Betel leaf extract contained various active ingredients that have antimicrobial properties (Datta *et al*., 2011). The antifungal in methanol extract of betel leaves was produced from a mixture of more than two compounds (Aoki *et al*., 2019). The same phenomenon

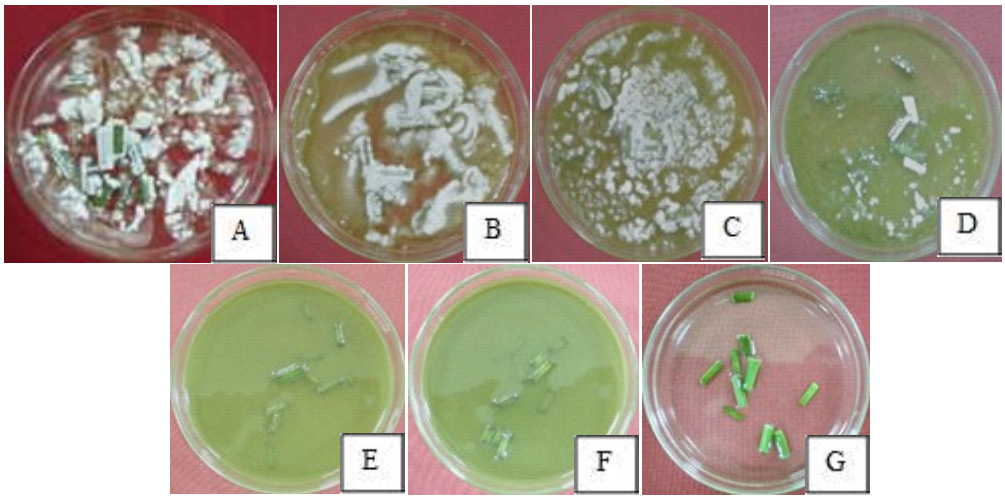

Figure 2. Growth of *F. oxysporum* f.sp. *capsici* on the PDB with various treatments after 3 days of incubation. (A) positive control, (B) 0.003% extract, (C) 0.006%, (D) 0.010%, (E) 0.013%, (F) 0.017%, (G) negative control.

happened to betel leaves extracted with other organic solvents. Chloroform extract of betel leaves contained terpenoids, saponins, phlobatanins, flavonoids, phytosterols, phenols, and tannins (Singha, 2011). Gas chromatography-mass spectroscopic analysis (GC-MS) of betel leaf extract showed the content of alkaloids, fatty acids, phenols, alcohols, flavonoids, terpenes, coumarin, and organic acids (Foo *et al*., 2015).

Rongai *et al*. (2014) reported that the relationship between Total Phenolic Content (TPC) in several extracts was related to the ability to inhibit the growth of *F. oxysporum* mycelia. Plant extracts having strong antifungal activity are generally high in phenols. In line with this, Singburaudom (2015) stated that, betel leaves contained phenols with the active compound of hydroxychavicol having a fungistatic and fungicidal effect on plant pathogenic fungi. Similar results were reported by Ali *et al*. (2010) stating that, hydroxychavicol from betel leaf showed antifungal activity against the 124 tested fungi.

The anti-fungal activity of the fractionated betel leaf extract was higher than the anti-fungal activity of the crude extract. This could be seen from the F19 fraction test that created 39.33 mm of inhibition zone, while the crude extract in the preliminary test only produced 34.5 mm of inhibitin zone. The larger diameter of inhibition zone after fractionation was probably due to the pure active compound resulting from the fractionation, as well as the lack of anti-synergy among the various compounds contained in the crude extract of the betel leaves.

Inhibitory ability of betel leaf extract against *F. oxysporum* f.sp. *capsici* showed that the plant had the potential to be developed as a natural pesticide. Various antifungal active compounds in betel leaf extract gave the advantage in the application of natural pesticides in the field, because various active compounds will reduce the resistance of pathogens. Further research is needed to determine the antifungal compounds in the betel leaf extract, especially against *F. oxysporum* f.sp. *capsici*.

CONCLUSION

Methanol extract of betel leaves effectively inhibited the growth of *F. oxysporum* f.sp. *capsici* with

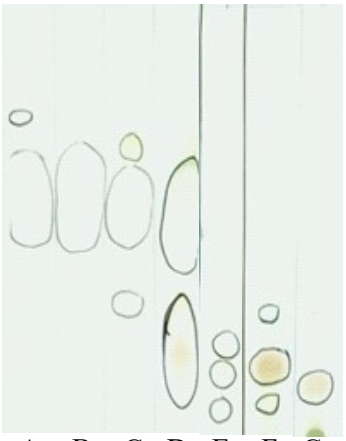

Figure 3. Spots on TLC. (A) F9; (B) F10; (C) F11; (D) F12; (E) F13; (F) F19; (G) F20.

Table 3. The inhibition ability of each fraction of betel leaf extract against *F. oxysporum* f.sp. *capsici*

Fraction number	Diameter of inhibition zone (mm)	Rf
F9	10.67	Rf 1 = 0.55; Rf 2 = 0.74
F10	8	Rf = 0.55
F11	6	Rf 1 = 0.31; Rf 2 = 0.55; Rf 3 = 0.66
F12	14	Rf 1 = 0.20; Rf 2 = 0.51
F13	27.67	Rf 1 = 0.08; Rf 2 = 0.16; Rf 3 = 0.23
F19	39.33	Rf 1 = 0.08; Rf 2 = 0.16; Rf 3 = 0.29
F20	14.67	Rf 1 = 0.13

MIC of 0.01%. Betel leaf extract with a concentration of 0.03% was able to inhibit the growth of *F. oxysporum* f.sp. *capsici* by 100%, and at a concentration of 0.017% was able to inhibit the growth of *F. oxysporum* f.sp. *capsici* on the bell pepper stems by 100%. Fractionation of betel leaf extract using column chromatography resulted in seven fractions that had inhibitory power against *F. oxysporum* f.sp. *capsici*. This showed that the betel leaf extract containeds several compounds with antifungal properties.

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