IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 9, Issue 4 Ver. I (Apr. 2016), PP 01-07
www.iosrjournals.org

Inhibition Test of Clove Leaf Oils to *Fusarium oxysporum* f.sp. *cubense*

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Abstract: The aims of the study is to identify the inhibition of clover leaf oils fraction to the grow of *Fusarium oxysporum* f.sp. *cubense* (*Foc*) and determines the Lethal Concentration 50% (LC_{50}) *Foc* of clove leaf oil. Experiment was done through in vitro methods. Research was initiated by purification of cloves leaf oil, fractionation using vacuum distillation and in vitro test. Research shows that in the minimum concentration of 0.025%, each fraction has ability to inhibit *Foc* grows. The exploration of minimum inhibition levels was done at concentration 0.010% (70 µl), 0.075% (52.5 µl), 0.050% (35 µl), and 0.025% (17.5 µl). Result of the concentration exploration confirm that each fraction has ability to inhibits *Foc* in concentration 17.5 µl (0.025%) about 56,7%. This concentration was used as lower concentration in inhibition. The best inhibition found at Fraction F2 in the concentration 0.025% with inhibition 90% and LC_{50} was about 11.17µL.

Keywords : cloves leaf oil, *Foc, in vitro* test and

I. Introduction

Cloves leaf oils has been known as potential organic pesticides. It has been used as insecticides, bactericides and fungicides. As fungicides, cloves leaf oil has been reported able to inhibit pathogens cause soil born desease. It has been also reported that cloves leaf oils able to inhibits *Sclerotium rolfsii, Sclerotium oryzae, Pyricularia oryzae, Rhizoctonia solani, Fusarium oxysporum, Fusarium batatis, Phytopthora palmivora* and *Phytium* sp. [1] [2] [3]. These pathogens contributes significantly to crop productivity decreases trough black-pod disease and basal stem rot disease [1]. Therefore, mitigation and reduction of pathogens attack to crop is important.

There are numerous biological compounds which area able to minimize pathogens in crops. The uses of organic compound widely applied due to its characteristics as eco-friendly pesticides. The important aspect of cloves leaf oils to inhibits *Fusarium oxysporum* in Panama disease has been considered important in integrated pest and diseases management in banana orchards, as reported from Southeast Asia and Australia in 1876, Panama and some countries in North Pacific in 1950. Panama diseases caused by *Fusarium oxysporum* is important agent for decrease of banana production. In 1940-1960, about 30,000 banana plant in Honduras was attacked by *Fusarium oxysporum* , while in Suriname *Fusarium oxysporum* attacks about 4.000 ha of banana orchards. *Fusarium oxysporum* is important banana disease in Taiwan and other banana producing area in the world [5].

In Indonesia, *Fusarium oxysporum* has reported attacks thousands hectares of banana, especially *kepok* and Cavendish cultivars. In 2007, *Fusarium oxysporum* attacks 10,000 hectares of banana orchards in East Kalimantan. Similar incident also found in south Kalimantan. The spread of pathogens has been reported fast and able to spread 100 km per year [6]. *Fusarium oxysporum* f. sp. *cubense* (*Foc*) has been destroy 163 ha of banana orchards in Aceh. In west Sumatra, *Fusarium oxysporum* f. sp. *cubense* (*Foc*) has reported destroy 1 million banana clump in 2010 and 5 million banana clump, especially *kepok* cultivar [7]. This led to the economic damage about 10 billion rupiah [8]. In Lampung, this pathogens becomes the important diseases to 2000 ha banana Cavendish plantation. In Bali, banana production was decrease from 134,000 tons in 1997 to 58,000 tons in 2002 [9]. Scholars point out that *Fusarium oxysporum* f. sp. *cubense* (*Foc*) was the very serious plant disease. As far, there are no fungicides to inhibit *Fusarium oxysporum* f. sp. *cubense* (*Foc*) [10].

The sustainable agriculture and farming recently accept the ideas to apply bio-pesticides or agent of bio-controls. Numerous plants are sources of the potential compound for bio-pesticides. Clove oil is one of the potential agents for bio-pesticides development. Research about the application of cloves leaf oil to mitigate pathogens has been done in some place in the words [11] [12]. Scholars point out that clove is potential agents for bio-pesticides development to enhance sustainable agriculture practices. In Indonesia, the exploration of potential cloves leaf oils as agent for bio-pesticides is important.

The activity of the entire active compound in cloves leaf oils has been known widely. As far, research has been paid a lot of attention to the ability of crude oils as component for bio-pesticides, but there is few research to identify the ability of oils fractions, especially from Indonesian clove specimen. The aims of the research were to identify compound of cloves leaf oils which are able to inhibit *Fusarium oxysporum* f.sp. *cubense* isolates (*Foc*).

II. Materials and Methods

Materials

Cloves leaf oils were collected from simple extraction process from Dampit area in Malang, East Java. The *Fusarium oxysporum* f.sp. *cubense* isolates (*Foc*) was collected from banana tissues with fusarium wilt diseases. Potato Dextrose Agar Streptomycin was prepared in laboratory using standard methods. Another material for experiment was encompasses alcohol, Tween 20, eugenol binding materials (NaOH, HCl and Hexane), gas chromatography (GC), active silt and aquadest.

Methods

Preparation of PDAS

Potato-dextrose-agar was made by cutting 200 g of potatoes into small cubes and cooking them in 1,000 ml of sterile aquadest. After potatoes becomes cool, 1.5 gr of dextrose and 1.5 gr agar was added. These materials were mix and transfer to Erlenmeyer. The Erlenmeyer with PDA was sterilized in autoclave at 4 hours. Potato-dextrose-agar streptomycin was created by adding 200 ppm streptomycine antibiotics into PDA media. All of the equipemnt in this study was sterelized in autoclave in 30 minutes at temperature 121^{O}C and presure 1 atm. It was done to minimize microorganism contamination [13].

Isolation and identification of *Fusarium oxysporum f. sp. cubense (Foc)* isolates

Fusarium oxysporum f. sp. *cubense* (*Foc*) sample was isolated from banana with Fusarium wilt diseases from Gondang-Tegal Gondo Village, Karang Ploso sub-district in Malang Regency, East Java. Organs of banana which is suspected infected by *Foc* was cut about 1x1 cm, and sample piece was grown in Petri dish with PDA media. Sample was incubated at room temperature one week for fungal grows. The *Fusarium oxysporum* f. sp. *cubense* (*Foc*) which are grows in PDA media was isolated and purified to provides *Foc* isolates for further experimental steps and analysis. The isolation of *Foc* was done following standard methods [14].

Fractionation distillation of cloves oil

Cloves leaf oil was purified using active loam through dilution techniques by flowing circular pipe into in which there are active loam in the basal of instrument. Slowly, clove oil fall down and collected for further analysis and experiment. Cloves oils were characterized by yellow color and hard aromatic flavor.

Eugenol was produced following standard methods. Crude eugenol analysis was encompasses purity and physical characteristics of eugenol. It is including specific gravity and bias indices. Crude eugenol purification was done through vacuum fractionation distillation [14]. Vacuum fractionation distillation was set up and done following standard methods.

Chromatography (GC) and mass spectrometry (MS) assessment

Cloves leaf oils resulted from fractionation was performed on gas chromatograph instrument interfaced to a mass spectrometer (GC-MS). It was done to identify the compound and its number of cloves leaf oils sample.

Anti fungi bioassay

Fusarium oxysporum f.sp. *cubense* isolates (*Foc*) was gown in PDA within one weeks before used in experiment. Technically, bioassays was done following standart methods by Hadacek & Greger [15]. The *in vitro* anti fungi test was done by adding PDAS with oils fractions with concentrations 0.025%; 0.050%; 0.075% and 0,01%. The highest inhibition value in the lowest concentration was used as highest concentration in the bioassay test. A piece of mycelium of fungal isolates was embedded in 6 mm holes in PDAS medium. Sampel was incubated at temperature room. Observation of myccelium grows and inhibition was made 24 hour after planting. Observation was stoped when michelium grows was maximum.

Experiment was set up following completely randomized design with 5 replication and controls. The observed parameters was inhibition levels and LC_{50} of three fraction of cloves leaf oils. The concentration of solutions of treatment was calculated using formula:

$V1.M1 = V2.M2,$

In which:

V1 : initial volume
M1 : initial Molarity
V2 : final volume
M2 : final Molarity

Inhibition ability was calculated using formula:

Inhibition level (P) = D1 – D2/ D1 x 100%, in which

P : inhibition percentage
D1 : fungi colony control diameters
D2 : fungi colony treatments diameters

LC_{50} was calculated and generated from Microsoft excel 2010 programs calculations.

III. Result and Discussion

Compound fractionation of cloves leaf oil

Cloves leaf rich in term of oils which are produced from the distillation of dried fallen and fresh leaf. Scholars confirms confirm that crude oil which is distillated from fresh leaf produce oil with phenol and has sweet aroma [16] [17]. In this study, the vacuum fractionation distillation in Eugenol purification produce three fraction (F), namely fraction with boiling point less than Eugenol (F1), fraction Eugenol (F2) and fraction with boiling point above Eugenol or residue (Table 1).

Six compound which area classified into fraction F1 was called as active compound of cloves leaf oil, encompasses yaitu*:* eugenol, eugenol acetat, trans-Caryophyllene, alpha-Humulene, 4-cycloprophylnorarane, Naphthalene and Furan. Four active compound which are classified as fraction F2 are encompasses Eugenol, Eugenol acetate, Naphthalene, and Buthane. Ten active compound namely Cyclohexasiloxane, Eugenol, alpha-Copaene, trans-Caryophyllene, alpha-Humulene, Tetradecamethylcycloheptasiloxane, Eugenol acetate, delta-cadinene, Caryophyllene oxide and Tetracosamethylcyclododecasiloxane were derived from fraction F3. This fractionation confirms that cloves leaf oils rich in term of potential compound.

This research result is relevant with the previous leaf compound exploration by scholars. Scholars point out that there is two important compounds of cloves leaf oils. First is phenolic with eugenol as a dominant component, and secondly is non-phenolic, including ß-Caryophyllene,α-kububen, α-kopaen, humulen, δ-kadien, and kadina 1,3,5-trien. Leaf of cloves contains Eugenol as active compound about 70-85% [18]. Eugenol content in clove flower was about 90-95%. In branches, it was recorded about 83-95%. Leaf contains about 82-87% of Eugenol [19]. Eugenol is the second largest compound in cloves leaf which are chemically soluble in alcohol. Eugenol ($C_{10}H_{12}O_2$) has been known as a compound with antimicrobial activity that cause morphological malformation in fungal cell and able to destroy cell wall, conidia and fungal hyphae. Antimicrobial activity of Eugenol was caused by secondary alkyl and OH from phenols groups which are very reactive to build hydrogen bound with enzyme [20] [21].

Chromatography (GC) and mass spectrometry examination of cloves leaf oils shows the compound and active materials with its concentration from each active materials of oils. The dominant concentration in leaf oils was the active materials which is contributes to the significant role in fungal species inhibitions.

The profiles of Fraction F1 active compound was given in Fig.1. From six compound of fraction F1, Eugenol was the dominant compound found in cloves leaf (82.82%). This content seems to be related to the affectivity and ability of cloves leaf oils to inhibit *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Scholars state that Eugenol has antimicrobial activity which are able to changes fungi morphology and initiates cell wall degradation [22]. Biochemically, anti-microbial activity of Eugenol to fungal cell was affected by secondary alkyl chain OH of phenol which is very reactive to build hidrogen bond with enzymes. Eugenol able to dissolve fat of fungal cell wall and therefore disturbs cell permeability. One of the serious consequences from such process is related to the loss of cell permeability [23] [24].

Table 1. Fractions of clove leaf oil based on boiling point

Fraction compound	Formula	Compound name	Percentage (%)
F1	$C_{10}H_{12}O_2$	Eugenol	82.82
	$C_{12}H_{12}O_3$	Eugenol acetat	*
	$C_{15}H_{24}$	trans-Caryophyllene alpha-Humulene	16.06
	$C_{10}H_{16}$	4-Cycloprophylnorarane	0.47
	$C_{20}H_{32}$	Naphthalene	0.66
	C_6H_{10}	Furan	*
F2	$C_{10}H_{12}O_2$	Eugenol	99.48
	$C_{12}H_{14}O_3$	Eugenol acetate	*
	$C_{20}H_{32}$	Naphthalene	0.52
	C_5H_9N	Butane	

F3	$C_{12}H_{36}O_6$	Cyclohexasiloxane	1,59
	$C_{10}H_{12}O_2$	Eugenol	54.29
	$C_{15}H_{24}$	alpha-Copaene	1.29
	$C_{15}H_{24}$	trans-Caryophyllene	32.20
	$C_{15}H_{24}$	alpha-Humulene	5.88
	$C_{14}H_{42}O_7$	Tetradecamethylcycloheptasiloxane	1.99
	$C_{12}H_{14}O_3$	Eugenol acetate	0.76
	$C_{15}H_{24}$	delta-Cadinene	0.61
	$C_{15}H_{24}O$	Caryophyllene oxide	1.05
	$C_{16}H_{48}O_8$	Tetracosamethylcyclododecasiloxane	0.33

*Not fond or found in limited number, but read during GCMS reading

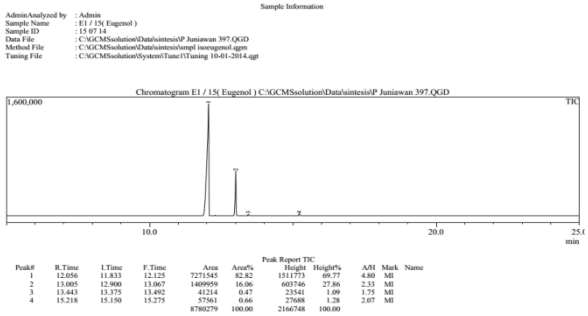

Fig. 1. Profiles of the active compound of Fraction F1.

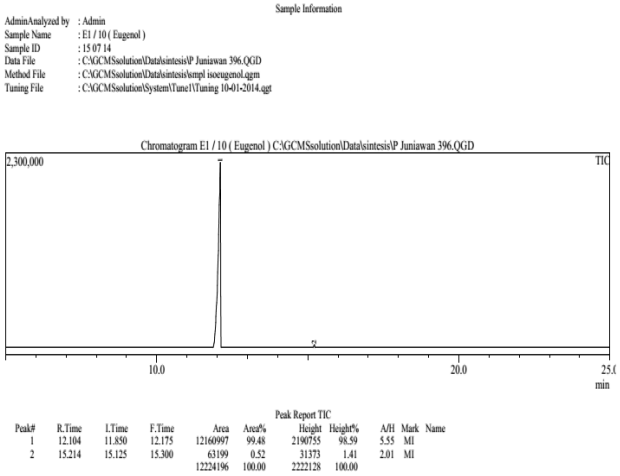

Fig. 2. Profiles of the active compound of Fraction F2.

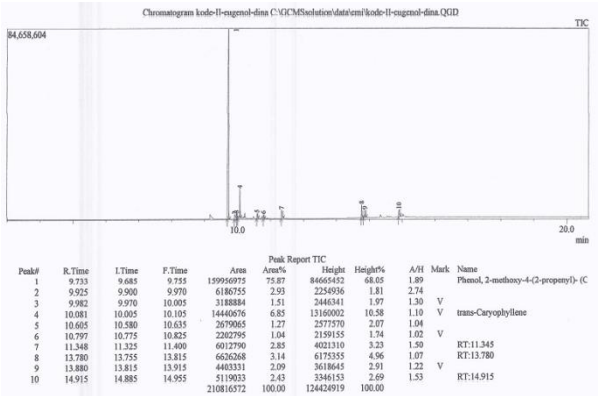

Fig. 3. Profiles of the active compound of Fraction F3.

Eugenol was also dominant in fraction F2 and F3 (Fg.2 and 3). There are also high concentration of Eugenol in fraction F2 (99.48%), compared to the fraction F1 and F3. Eugenol in each fraction seems to be the important component fungal grows inhibition. The high concentration has high inhibition to pathogens. Scholars

point out that Eugenol causes fungi-statics activity. There are positive correlation between antifungal activity and phenol content. Strong antimicrobial activity has correlation with Eugenol compound which are found in cloves leaf oils [21] [25].

The concentration of inhibition

Inhibition test aims to identify the impact of concentration to the inhibition of cloves leaf oils to *Foc*. The technique was similar with bioassay methods with wide rage dosage and it was assumed able to inhibits fungal grows. In this study, the inhibition concentration of fraction F1 was started from 0.01% (70 µl); 0.075% (52.5 µl); 0.05% (35 µl) and 0.025% (17.5 µl). The highest inhibition value in the lowest concentration was used as highest concentration in next test steps. Experiment shows numerous impacts to inhibition (Table 2).

Table 2. Inhibition levels of cloves leaf oils fraction

Concentration (µL)	Fungi colony treatments diameters (cm)	Fungi colony control diameters (cm)	Inhibition levels (%)
Inhibition levels of cloves leaf oils Fraction 1 (F1) in fungi			
70.0	0.00	9.00	100.00
52.5	0.00	9.00	100.00
35.0	0.80	9.00	91.11
17.5	1.00	9.00	88.88
Inhibition levels of cloves leaf oils Fraction 2 (F2) in fungi			
70.0	0.00	9.00	100.00
52.5	0.00	9.00	100.00
35.0	0.70	9.00	92.22
17.5	1.40	9.00	84.44
Inhibition levels of cloves leaf oils Fraction 3 (F3) in fungi			
70.0	0.00	9.00	100.00
52.5	0.00	9.00	100.00
35.0	0.70	9.00	92.22
17.5	1.40	9.00	84.44

The evidence of inhibition of numerous cloves leaf concentration to *Foc* was given in Fig. 4. Inhibition rate of cloves leaf oils above 0.025% was high, even seems some fungal mycelia able to grow at concentration 0.05% .

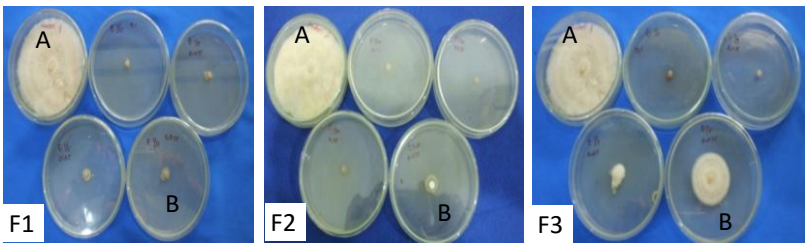

Fig.4 Inhibition levels of cloves oils n minimum dosages. F1= under boiling point, F2=in boiling point F3=residues. Notes: A=control, B=treatment.

Inhibition ability of Fraction 1 (F1) of cloves leaf oils to Foc.

In the seconds steps, test was done to identify inhibition ability of fraction F1 to inhibits *Foc* using four treatment and five replication. The tested concentration treatments was 0.025% (17,5 µL); 0.0125 (8,75 µL); 0,00625% (4,4 µL) and 0.003125% (2.2 µL). Result of the observation of the ability of Fraction F1 to inhibits fungal *Foc* was shown in Fig. 5

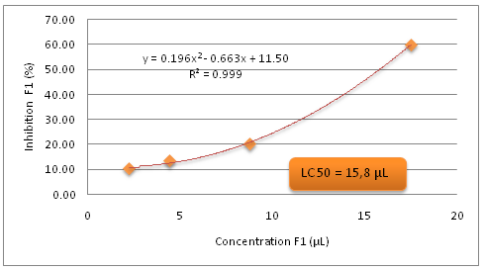

Fig. 5. Percentage of fungal *Foc* inhibition at fraction F1 in some concentration

The inhibition ability of cloves leaf oil in Fraction F1 shows that in the concentration 17.5 µL (0.025%) has highest inhibition level (60%). This is similar with the inhibition ability in exploration stages. In the concentration 8.75 µL the inhibition ability was decrease three times, but it is not relevant with the inhibition decrease in the next concentration. Therefore, it is important to determine the proper LC_{50} to generates the value references for pathogens mitigations. The highest value of LC_{50} was found in fraction F1.

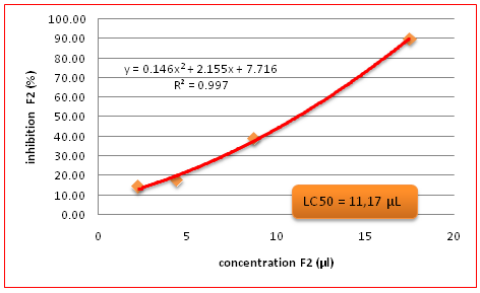

Fig. 6. Percentage of fungal *Foc* inhibition at fraction F2 in some concentration Inhibition ability of Fraction 2 (F2) of cloves leaf oils to *Foc*.

In the similar concentration with Fraction 1 (F1) 17.5 µL (0,025%), Fraction 2 (F2) shows highest inhibition ability 90%. This trend similar with the lowest concentration, the Fraction F2 shows better inhibition impact (Fig.6). The content of Eugenol seems contributes to the inhibition.

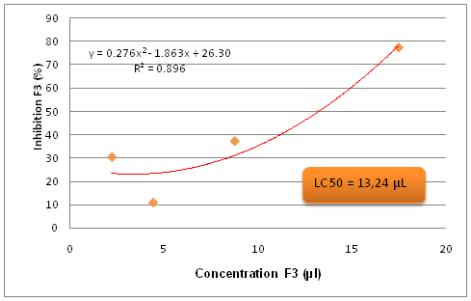

Fig. 7. Percentage of fungal *Foc* inhibition at fraction F3 in some concentration Inhibition ability of Fraction 3 (F3) of cloves leaf oils to *Foc*.

Different result was shown in Fraction 3 (F3). Range of value was higher (84.44%) compared to the Fraction F1 (80.00%) and fraction F2 (90.67%) (Fig.7). The Eugenol concentration was not different compared to the fractions F2 and F1, but the content of active compound was relatively differs. Scholars point out that, number of active compound and the increase of concentration of each fractions has positive correlation with fungi grows inhibition [1] [23] [26].

Three fractions of cloves leaf oils shows the variation in the ability to inhibits fungal grows. The content of Eugenol seems to be important to determine such variations. This data shows that Eugenol has significant role in *Foc* grows. This findings is similar with the previous research [2] [18] [23] [24]. Eugenols is an antimicrobial compound that are able to cause malformation of fungal morphology. This compound also able to destroy cell wall, conidia and hyphae of fungal individual. Eugenol has fungi-statics activity [21]. Chitinase has been reported as one of the enzyme with its ability to destroy cell wall. Chitinase provides different impact to different fungal species [27]. *Fusarium oxysporum* (*Fo*) resistance to chitinase due to its cell wall composition. The outer layer of cell wall consist of 50-60% glicoprotein which area able to protect mycelium surface. Inside cell there are also chitin and glucan.

The activity of Chitinase require other enzymes, especially in its relationship with fungal inhibition activity. Experiment using Foc mycelium confirm that chitinase able to destroy cell wall of *Foc*. Fungal mycelium which is interact with chitinase shows morphological changes. Chitinase able to digest chitin of cell wall. Cell wall degradation lead to the serious problems of physiological process and therefore case fungi degradation. This phenomena called fungi-toxicity.

IV. Conclusion

There are three fraction found from cloves leaf oils distillations and fractionation from Dampit , East Java. Fraction F1 of cloves leaf oil contains eugenol, eugenol asetat, trans-Caryophyllene, alpha-Humulene, 4-cycloprophylnorarane, Naphthalene and Furan. Fraction F2 contains Eugenol, Eugenol asetate, Naphthalene,

and Buthane. Fraction F3 contains Ten active compound namely Cyclohexasiloxane, Eugenol, alpha-Copaene, trans-Caryophyllene, alpha-Humulene, Tetradecamethylcycloheptasiloxane, Eugenol asetate, delta-cadinene, Caryophyllene oxide and Tetracosamethylcyclododecasiloxane. This fractionation confirms that cloves leaf oils rich in term of potential compound. The fraction F2 has highest content of Eugenol (99.48%). This fraction has highest inhibition ability (90%) and lowest LC_{50} t (11.7 µl).

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