The Poriferasta Compound -5,22E,25-trien-3-ol,22-dehydrocholesterol from *Clerodendrum paniculatum* leaf as inducer Agent of Systemic Resistance on Red Chilli plant *Capsicum annuum L* from *Cucumber Mosaic Virus* (CMV).

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ABSTRACT

The active compound from *Clerodendrum paniculatum* leaf was obtained with induction activity to systemic resistance of cucumber mosaic virus (CMV) to red chilli.

Methanol extract of *Clerodendrum paniculatum* leaf was concentrated and extracted with ethyl acetate. Ethyl acetate extract shows induction activity with inhibition percentage above of 55%. Ethyl acetate extract was separated with column chromatography using kieselgel 60 provide one of compound as white needle crystal with 146-148^{o}C melting point. Based on phytochemistry test and spectroscopy data including : IR, ^{1}H-NMR, ^{13}C-NMR and 2D NMR as well as comparation with literature data was concluded isolated compound was steroid group. The inhibition compound showed 87% inhibition activity to red chilli plants from (CMV) at **300 ppm of concentration.**

Key word: Poriferasta-5,22E,25-trien-3-ol,22-dehydrocholesterol, *Clerodendrum paniculatum,* induction of systemic resistance, CMV

1. Introduction

Clerodenrum is one of the genus from Verbenacea family known as producer of diterpen, steroid, sterol glycoside compound and diterpenoid on some spesies of *C. colebrookianum, C. trichotonum,* dan *C. Inerme.* (Goswami *et al*., 1995; Kawai *et al*., 1999; Yang *et al*., 2000; Kang *et al*., 2003; Pandey *et al*., 2003 dan 2004).

Currently, the use of chemical substances that is successfully isolated has not been found on the research involving *Clerodenrum* genus.

Most of the researches reported that *Clerodenrum* genus is the inducer agent for systemic resistance to pathogenic harm as has been reported by Prasad (Prasad *et al*., 1995; Olivieri *et al*., 1996; Verma *et al*., 1996; Praveen *et al*., 2001). It reported that compound of inducer agent is protein, but for the secondary metabolite compound on this genus is not an inducer agent for systemic resistance of the plant.

One of the genus that is potential in inducing the resistance to red chilli encounter the harm of Cucumber Mosaic Virus (CMV) in the green house is the *Clerodenrum paniculatum* (Hersanti, 2004). This research focus only on the leaf extract used as the inducer agent, therefore, this research would try to discover the active compound.

2. Experiment

2.1.Materials and Methods

Instruments needed for this experiment are glass and other supporting instruments that are usually available in Natural Organic Chemical Lab. The melting point is determined by micro melting point apparatus. Each UV Spectrum dan IR is measured by spectrophotometer Beckman DU-700 dan Shimadzu FTIR 8400. Spectrum ^{1}H and ^{13}C NMR are determined by spectrophotometer JEOL JNM ECA-500 operated at 500 MHz (^{1}H) and 125 MHz (^{13}C) using TMS as standard. Chromatography vacuum liquid (KVC) using Si gel 60 (230-400 Mesh), chromatography column gravity using Si gel 60 (70-230 Mesh), KLT analysis using KLT plate Kieselgel 60 GF_{254} 0,25 mm. All Solvent used in this research is technically qualified and distilled.

Bio assay using red chilli (*Capsicum annuum L.*), carborundum, tobacco leaf that have infected by CMV2-RIV and buffer phosphate solution.

2.2. Material

Using *Clerodenrum paniculatum* as the material.

2.3. Extraction and Isolation.

5 kg of *Clerodenrum paniculatum* leaf is macerated using methanol solvent, then is partitioned by *n*-hexane and ethyl acetate. Each extract is dried at low pressure resulting 705,77 g concentrate extract methanol, 251,75 *n*-hexane 23,5 g and 111,80 g extract of ethyl acetate consentrate .

80 g of ethyl acetate is separated using chromatography vacuum liquid with adsorbent Silica gel and eluent *n*-hexane- ethyl acetate with gradient (10:0 – 0:10) producing nine fractions FE1-FE9 (each 2 g; 2,75 g; 4,50 g; 5,55 g; 6,35 g; 6,85 g; 7,45 g; 7,95 g; 8,25 g). FE3 fraction 4,50 g is then chromatographed column with *n*-hexane eluent: metal chloride : acetone (8,5 : 1 : 0,5) producing 7 fractions namely FE3.1-FE3.7.

Intensity of CMV disease is Fraction FE3.4 (178 mg) is re-crystallized and resulting pure compound in a form of white needle crystal (35 mg) with melting point of 146-148^{o}C. Every step of isolation in bio assay is used to determine the inducing systemic resistance agent on red chilli previously infected by CMV.

3. Structure of Elucidation

The molecule structure of that compound is elucidated based on data IR, ^{1}H-NMR, ^{13}C-NMR and 2D NMR.

4. Bio Assay

Each fraction used for bio assay is concentrated as follows:

1. Extract with 9000 ppm of concentration
2. Extract with 6000 ppm of concentration
3. Extract with 3000 ppm of concentration
4. *Clerodenrum paniculatum* leaf as comparison
5. Water as control
6. Ethanol as control

Before conducting the bio assay, Extract is previously mixed with carborundum so that the extract can be absorbed into plant cells without causing the plant tissue dead. Every extract is applied to the first and the second leaf above cotyledon of red chilli, then after 30 minutes, rinse with water. After 24 hrs CMV inoculation is done, that is by applying the filtrate of tobacco leaf infected by the CMV2-RIV mixed with buffer phosphate solvent on the third and the fourth leaf (above the two leaves that have been applied by the extract).

5. Observation

The observed parameters are:

1. The observed from the first symptom of CMV appear (7 times observation with three day interval). The intensity of CMV is calculated with formula:

description:

I = insect Intensity
n = number of plant in every attacks category
v = rate scale in each attacks category
V= rate scale from the highest attacks category
N= number of observed plants

Attacks scales based on Dolores (1996) are as follow:

0 = plant do not show virus attack symptoms
1 = plant shows a light mosaic symptom, or the symptom is not systemically distributed
2 = plant shows a moderate mosaic symptom
3 = plant shows a heavy mosaic symptom or heavy stripes without shrinking or malformed leaf

4 = a heavy mosaic symptom or heavy stripes with shrinking or malformed leaf
5 = a very heavy symptom or very heavy stripes with shrinking or severe malformed leaf, dwarf or dead.

2. All of the CMV intensity attack is written into disease development graphic. According to Louws *et al*. (1996) the total *Area Under Diseases Progress Curve*/AUDPC) is calculated by the formulae:

Description:

Yi+1 = observation data -i+1
t i+1 = observation time ke-i+1
Yi = observation data ke-1
ti = observation time ke-1

3. The percentage of CMV inhibition resulted from plant extract application is calculated based on the formulae:

6. Result and Discussion

Spectroscopy analysis of IR, ^{1}H-NMR and ^{13}C-NMR compound isolated and guided by bio assay, produced systemic resistance inducer agent on red chilli plant infected by CMV. Based on the compound data analysis, the compound is steroid group with functions cluster positions obtained from spectroscopy analysis NMR 2D.

The collected compound of inducer agent is in a form of needle crystal, colourless with melting point at 146-147^{o}C. Infra red Spectrum showed the presence of absorbent ribbons of OH clusters (3429 cm^{-1}), this is due to the fact that there is a special absorbance for the strecth of C-O (1056 cm^{-1}). Double band C=C is seen as the appearance of absorbance area 1377 cm^{-1}, 1643 cm^{-1} extending absorbance C=C from olefin linear and 1458 cm^{-1} extending C=C from olefin cyclic. Detailed analysis on NMR spectra showed that there are 29 carbon signals, those signals consisted of five methyl carbon atoms (CH_3), 9 methylene carbon (CH_2) dan 10 methyne carbon atoms (CH). Methyne carbon comprises seven carbons atom sp^3 and three carbon atom sp^2. Signal for five methyl carbon appear on δc 12,2; 12,3; 19,6; 20,4; and 21,0 ppm, and these data is supported by spectrum ^{1}H NMR at δ_H (ppm) 0,68 (s); 0,83 (t, *J*= 7,4 H_z); 0,99 (s); 0,87 (d, *J* = 6,4 H_z);and 1,64 (S). Signals for 9 carbon methylene atoms appear at δc 37,4; 31,7; 42,4 31,8; 21,2; 25,9; 24,5; 40,4; 21,0 and 109,7 ppm. Signal for methyne comprises seven carbons sp^3 at δc 72,0; 32,0; 50,3; 57,0; 56,0; 39,8; and 52,2 ppm and three carbons sp^2 at δc 121,9; 130,2; and 137,4 ppm. These data is supported by ^{1}H NMR at δ_H (ppm), 3,52 (m); 5,36 (d *J* = 4,8); 1,50 (m); 1,49 (m); 1,43 (m); 1,53 (m); 2,27 (m); and three protons sp2 5,36 (d J = 4,8 H_z , 5,19 (1H, dd, *J* = 15, 1 ; 8,8 H_z) and 5,17 (1H, dd, *J* = 15,1; 8,8 H_z). The presence of disubstitution trans C=C (C22- C23) is shown by signal ^{1}H NMR that abdorbed at δ_H 5,19 (1H, dd, *J* = 15, 1 ; 8,8 H_z) and 5,17 (1H, dd, *J* = 15,1; 8,8 H_z). This facr is also supported by signal C24 whic is more protected by δc 52,2 ppm. The proton ethylenic is bound respectively by carbon absorbing at δc130,2 ppm (C22) and 137,4 ppm (C23).

The shifting of chemical carbon on active compound with literature data is shown on table 3. Spectrum ^{1}H and ^{13}C NMR, DEPT, and HMBC is shown on Table 2.

Table 2. The correlation between proton and carbon compound **2** according to investigation result of spectrum ^{1}H-^{13}C HMQC and ^{1}H-^{13}C HMBC

No. C	δ_C (ppm)	Kind of C	δ_H (ppm) HMQC	δ_C (ppm) HMBC
24	52,2	CH	2,41(1H,m)	130,2 (C-22); 137,4 (C-23); 148,8 (C-25); 25,9 (C-28)
26	109,7	CH_2	6.68 (1H,s); 4,69(1H,s)	20,4 (C-27); 52,2 (C-24)
29	12,3	CH_3	0,83 (3H,s)	25,9 (C-28)

Table 3. The chemical shift of pure (1), Cholesterol (II*) and stigma sterol (III**) from spectrum Resonance Magnet core carbon -13

Carbon	I The shifting of ^{13}C pure isolate $C_{29}H_{46}O$ (ppm)	II shifting of ^{13}C cholesterol $C_{27}H_{46}O$ (ppm)	III shifting of ^{13}C Stigmasterol $C_{29}H_{50}O$ (ppm),
1	37,4	37,5	37,21
2	31,7	31,6	31,69
3	72,0	71,3	71,81
4	42,4	42,4	42,35
5	141,0	141,2	140,80
6	121,9	121,3	121,69
7	31,8	32,0	31,94
8	32,0	32,3	31,94
9	50,3	50,5	50,20
10	36,7	36,5	36,56
11	21,2	21,2	21,11
12	28,9	28,3	39,74
13	42,5	42,4	42,35
14	57,0	56,9	56,91
15	24,5	24,3	24,39
16	40,4	40,0	28,96
17	56,0	56,5	56,06
18	12,2	12,0	12,07
19	19,6	19,4	19,42
20	39,8	35,8	40,54
21	21,0	18,8	21,11
22	130,2	36,4	138,37
23	137,4	24,1	129,32
24	52,2	39,6	51,29
25	148,8	28,0	31,94
26	109,7	22,8	21,26
27	20,4	22,5	19,02
28	25,9		25,44
29	12,3		12,17

7. The result of bio assay

In conducting bio assay, each fraction from maceration, partition and chromatography column 0,045 g are diluted into 5 ml ethanol for 9000 ppm of concentration, next, 1.33 ml is diluted up to 2 ml into 6000 ppm of concentration dan 0,66 ml for 3000 ppm of concentration. The overall result from bio assay, maceration, partition, chromatography column are displayed on table 4, 5, 6, and 7.

From the four extracts with different concentrate on bio assay, 2 of extracts gave a high response as an anti viral compound on red chilli plant. The inhibition concentration is consistent ranging from the low up to the high concentration.

The bio assay indicated that the methanol and ethyl fraction acetate is active as a fraction that is able to induce systemic resistance to red chilli plant from CMV virus. Then, separation and purification are carried out to ethyl acetate fraction to collect pure active isolate as a compound that induced the systemic resistance on red chilli plant.

Table 4 The Extracts gained from Maceration, and Partition are potential as the systemic resistance inducer agent on red chilli plant from CMV virus.

No	Kinds of extract/ concentration	Incubation period (day)	AUDPC	Penghambatan (%)
1	Extract Methanol 3000 ppm	15	30	94,11
2	Extract Methanol 6000 ppm	15	165	67,65
3	Extract Methanol 9000 ppm	15	172,5	66,18
4	Extract Hexane 3000 ppm	15	194,85	61,79
5	Extract Hexane 6000 ppm	28	19,95	96,09
6	Extract Hexane 9000 ppm	15	149,55	70,68
7	Extract Ethyl acetate 3000 ppm	15	19,8	96,12
8	Extract Ethyl acetate 6000 ppm	15	169,95	66,68
9	Extract Ethyl acetate 9000 ppm	15	190,05	62,74
10	Extract H_2O 3000 ppm	21	75	85,29
11	Extract H_2O 6000 ppm	0	0	100
12	Extract H_2O 9000 ppm	21	45	91,18
13	Ethanol	15	457,5	10,29
14	Control	12	510	0
15	*Clerodendrum paniculatum*	0	0	100

Ethyl acetate fraction 111,80 g is separated with open liquid chromatography column using adsorbent silica gel size 70 -230 mesh, using the mixed hexane solvent: ethyl acetate increase in gradient with the rise of 25 % started from hexane 100 %. The result of separation column with the same stain pattern was able to collect 9 fractions namely E_1, E_2, E_3, E_4, E_5, E_6, E_7, E_8, and E_9, afterwards, the 9 fractions are examined by bio assay.

Two out of nine fractions collected from the separation were tested namely fraction Fr E_4 and fraction Fr E5 where the resistance percentage was below 55%. Both of two fractions from the tested three concentration gave resistance below 55%, while the fraction E_2, E_3, E_6, E_7, E_8, and E_9 gave the varied resistance from the three concentration tested which was above 50% in inducing resistance systemic on red chilli.

Tabel 5 : Incubation period, AUDPC and the percentage of CMV disease resistance on red chilli plant induced by fractions gained from Ethyl acetate

No	Kinds of extract/ concentration	Incubation period (hari)	AUDPC	Penghambatan (%)
1	Fraction E_1 300 ppm	16	252	60,75
2	Fraction E_1 600 ppm	13	96	85,05
3	Fraction E_1 900 ppm	0	0	100
4	Fraction E_2 300 ppm	10	636	0,93
5	Fraction E_2 600 ppm	10	576	10,28
6	Fraction E_2 900 ppm	13	48	92,52
7	Fraction E_3 300 ppm	0	0	100
8	Fraction E_3 600 ppm	10	12	98,13
9	Fraction E_3 900 ppm	10	468	27,10
10	Fraction E_4 300 ppm	7	444	30,84
11	Fraction E_4 600 ppm	13	492	23,36
12	Fraction E_4 900 ppm	10	510	20,56
13	Fraction E_5 300 ppm	7	702	-9,34
14	Fraction E_5 600 ppm	10	552	14,01
15	Fraction E_5 900 ppm	10	468	27,10
16	Fraction E_6 300 ppm	7	732	-14,01

No	Kinds of extract/ concentration	Incubation period (hari)	AUDPC	Penghambatan (%)
17	Fraction E_6 600 ppm	0	0	100
18	Fraction E_6 900 ppm	7	738	-14,95
19	Fraction E_7 300 ppm	19	78	87,85
20	Fraction E_7 600 ppm	10	510	20,56
21	Fraction E_7 900 ppm	7	522	18,69
22	Fraction E_8 300 ppm	21	6	99,06
23	Fraction E_8 600 ppm	0	0	100
24	Fraction E_8 900 ppm	10	252	60,75
25	Fraction E_9 300 ppm	10	294	54,21
26	Fraction E_9 600 ppm	10	240	62,62
27	Fraction E_9 900 ppm	0	0	100
28	Ethanol	7	1158	80,37
29	H_20	10	642	0
30	*Clerodendrum paniculatum* leaf	0	0	100

Table 6. Incubation period, AUDPC and the percentage of CMV disease resistance on red chilli plant induced by fractions E_3

No	Kinds of extract/concentration	Incubation period (day)	AUDPC	Penghambatan (%)
1	Fraction $E_{3.1}$ 150 ppm	16	36	92
2	Fraction $E_{3.1}$ 300 ppm	0	0	100
3	Fraction $E_{3.1}$ 450 ppm	16	564	-11,90
4	Fraction $E_{3.2}$ 150 ppm	21	12	97,62
5	Fraction $E_{3.2}$ 300 ppm	13	288	42,86
6	Fraction $E_{3.2}$ 450 ppm	10	102	79,76
7	Fraction $E_{3.3}$ 150 ppm	16	90	82,14
8	Fraction $E_{3.3}$ 300 ppm	10	156	69,04
9	Fraction $E_{3.3}$ 450 ppm	10	60	88
10	Fraction $E_{3.4}$ 150 ppm	21	24	95,23
11	Fraction $E_{3.4}$ 300 ppm	16	54	89,28
12	Fraction $E_{3.4}$ 450 ppm	13	228	54,76
13	Fraction $E_{3..5}$ 150 ppm	13	234	53,57
14	Fraction $E_{3..5}$ 300 ppm	10	336	33,33
15	Fraction $E_{3..5}$ 450 ppm	10	762	-51,19
16	Fraction $E_{3..6}$ 150 ppm	10	240	52,38
17	Fraction $E_{3..6}$ 300 ppm	7	852	-69,05
18	Fraction $E_{3..6}$ 450 ppm	10	282	44,05
19	Fraction $E_{3..7}$ 150 ppm	7	336	33,33
20	Fraction $E_{3.7}$ 300 ppm	7	840	-66,66
21	Fraction $E_{3.7}$ 450 ppm	7	780	-54,76
22	Ethanol	10	498	1,19
23	*Clerodendrum paniculatum* as comparison	10	198	60,71
24	Control	10	504	0

Generally, there were 6 examined fractions which were potential as systemic resistance inducer agent on red chilli. This could be seen obviously from the AUDPC value and the achieved percentage of resistance. However, some concentrate of these 6 fractions functioning as inducer agent of systemic resistance on fraction E_3 (3000 ppm) incubation period 0, AUDPC 0, inhibition percentage 100%. Fraction E_3, the highest inhibition ,100% is continued by separating using chromatography second column. The bio assay result is shown on Table 6.

The result of bio assay on table 6 shows a varied inhibition percentage and fraction $E_{3.7}$ show inhibition percentage below 50 %. The other fractions namely $E_{3.1}$, $E_{3.2}$, $E_{3.3}$, $E_{3.4}$, $E_{3.5}$ and $E_{3.6}$ generally gave a varied inhibition percentage from the three tested fractions. In general, the tested fractions with 300 ppm typically gave a 50% inhibition. Therefore, for the next test, concentration variation are 450 ppm, 300 ppm dan 150 ppm. One of the fractions resulted from the second column gained from second column separation name fraction fr $E_{3.4}$ (78 mg) was re-crystallized with n- hexane and ethyl acetate, since this fraction showed the formation of needle crystal. By re-crystallizing the fraction $E_{3.4}$, pure isolate fraction in forms of needle crystal is resulted and 2 fractions from re-crystallization were named fraction $E_{3.4.1}$ and fraction $E_{3.4.2}$. Those three fraction s is then examined using bio assay. The result of bio assay isolate fraction and re-crystallization can be seen on Table 7

CONCLUSION

The potential inducer agent gained from maceration and partition applied on red chilli is the fraction ethyl acetate where the percentage of inhibition is good and consistent for the three examined sensitive concentrates.

Tabel 7 Incubation period, AUDPC and the percentage of CMV disease resistance on red chilli plant induced by fraction $E_{3.4}$ gained from re-crystallization

No	Kinds of extract/concentration	Incubation period (day)	AUDPC	Penghambatan (%)
1	Isolate + H_2O 150 ppm	16	76	79
2	Isolate + H_2O 300 ppm	19	46	87
3	Isolate + H_2O 450 ppm	19	98	73
4	Isolate + C_2H_5OH 150 ppm	16	114	69
5	Isolate + C_2H_5OH 300 ppm	19	64	82
6	Isolate + C_2H_5OH 450 ppm	10	296	20
7	Fraction $E_{3.4.1}$ 150 ppm	10	228	38
8	Fraction $E_{3.4.1}$ 300 ppm	16	112	69
9	Fraction $E_{3.4.1}$ 450 ppm	13	119	68
10	Fraction $E_{3.4.2}$ 150 ppm	19	54	85
11	Fraction $E_{3.4.2}$ 300 ppm	10	168	54
12	Fraction $E_{3.4.2}$ 450 ppm	7	332	10
13	Ethanol	7	486	-30
14	Control	7	372	0
15	*Clerodendrum paniculatum* as comparison	19	146	60

Component from fraction ethyl acetate (111,80 g) chromatographed by open column, resulted 9 fractions. The nine fractions are applied to red chilli plant to find out which fraction is potential as the anti viral compound. All of the fractions showed different inhibition percentage with the control and gained 6 out of nine tested fractions that generally showed 55% inhibition. However, there are some tested fractions and concentrate showed inhibition below 55%.

Fractions showing 55% percentage : Fraction E_1, (300 ppm) 60,75%, E_1 (600 ppm) 85,05%, E_1 (900 ppm) 100%. Fraction E_2 (900 ppm) 92,52%. Fraction E_3 (300 ppm) 100%, E_3 (600 ppm) 98,13%. Fraction E_8 (300 ppm) 88,79%, dan E_8 (600 ppm). Fraction E_9 (600 ppm) 57,09%. Those fractions above show a percentage above 55%, fraction E_3 (430 mg) with inhibition 100% (300 ppm) continued by an

open liquid column chromatography separation resulting 7 fractions. Fractions with the same stain pattern from the thin layered chromatography is combined. Each fraction is named fraction $E_{3.1}$, $E_{3.2,}$ $E_{3.3,}$ $E_{3.4,}$ $E_{3.5,}$ $E_{3.6}$ dan $E_{3.7}$.Those 7 fractions examined by bio assay with the 3 concentrates resulted a various inhibition percentage. Commonly, with 300 ppm of concentration gave an inhibition above 55% in average, unless for fraction $E_{3.7}$ inhibition percentage was 33,33% and fraction $E_{3.6}$ 52,38%.

One of the fractions; fr $E_{3.4}$ (178 mg) is re-crystallized by n-hexane and ethyl acetate and resulted a white needle crystal. By re-crystallizing fraction $E_{3.4}$ giving fraction pure isolate in a form of white needle crystal and 2 fractions from re-crystallization named fraction $E_{3.4.1\ and}$ fraction $E_{3.4.2}$ the 3 fractions is the examined by bio assay. Fraction with pure isolate was diluted by ethanol and water.

The bio assay shows that fraction on 300 ppm of concentration indicate the inhibition above 50% for the overall fractions. The pure Isolate compound give a high activity compared to unpurified fraction. The pure Isolate compound activity is diluted with either ethanol or water at 300 ppm of concentration showed a 100 % inhibition, this fact proved that the purer the tested material the more the activity will be. The presence of other materials in tested material will decrease the activity, therefore it is assumed that other compounds in the tested material contain antagonise.

Based on the data analysis of spectroscopy UV, IR and NMR; it is concluded that the active compound is Poriferasta-5,22E,25-trien-3-ol 22-dehydrochlosterol with molecular formulae $C_{29}H_{46}O$.

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