CHEMICAL COMPOTITION OF *TINOSPORA CRISPA* L. TOWARDS LARVAE OF *AEDES ALBOPICTUS* AS EFFORTS ON HEALTH SUSTAINABILITY IN INDONESIA

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ABSTRAK

Pestisida kimia diperkenalkan oleh manusia pada zaman keemasan peradaban dunia sebelumnya. Setelah Perang Dunia II, telah dikomersialkan oleh ilmuwan untuk pengendalian hama. Selain pestisida kimia ini, para ilmuwan mulai menemukan pestisida dari bahan alam yang biasa dikenal dengan pestisida hayati atau biopestisida. Saat ini, banyak produk alam yang dikomersialkan untuk biopestisida. 'A-Mark Procon' adalah salah satu perusahaan riset yang memproduksi dan memasok biopestisida untuk pertanian, hortikultura dan kehutanan. *Tinospora crispa* merupakan salah satu biopestisida yang potensial. Hal ini umumnya dikenal sebagai Patawali atau Seruntum. *T.crispa* merupakan herba yang tersebar luas di Malaysia, Indonesia, Thailand, Filipina dan Vietnam. Di Indonesia, rebusan batang *T.crispa* digunakan untuk mengobati sakit perut, demam, gonore dan cacingan. Ekstrak batang dan akar digunakan untuk mengobati malaria, abses bakteri dan tekanan darah tinggi. Analisis kromatografi gas – spektrometri massa (GC-MS) pada ekstrak kasar *T.crispa* menunjukkan 16 senyawa telah terdeteksi. Trichloromethane dan hexachloroethane tidak dianggap sebagai senyawa dalam T.crispa karena merupakan bagian dari pelarut kloroform. Ini digunakan sebagai pelarut dan ekstraktan dalam berbagai prosedur manufaktur dan laboratorium, di mana dibuat sebagai produk sampingan senyawa yang mengandung klorin. Senyawa lainnya adalah golongan terpen, alkaloid dan flavonoid. Diisooctyl phthalate merupakan salah satu senyawa aktif yang memiliki toksisitas tinggi, dimana di-n-octyl phthalate (DNOP) memiliki toksisitas yang tinggi dibandingkan dengan di(2-ethylhexyl) phthalate (DEHP).
Kata kunci *: T.crispa*, ketahanan kesehatan, toksikologi

ABSTRACT

Chemical pesticide was introduced by humans on the previous golden age of world civilization. After the World War II, it has been commercialized by scientist for pest-control management. Besides of this chemical pesticide, scientist start to find out pesticide from natural substance, commonly known as biological pesticide or biopesticide. Nowadays, many natural products were commercialized to biopesticides. 'A-Mark Procon' is one of the researches driven company that manufacture and supply biopesticides for agriculture, horticulture and forestry. Tinospora crispa is one of the potential biopesticide. It is commonly known as Patawali or Seruntum. T.crispa is herb widely spread in Malaysia, Indonesia, Thailand, Philippines and Vietnam. In Indonesia, a decoction of the stem of T.crispa is taken to treat stomach-ache, fever, gonorrhea and worms. Stem and root extracts are used to treat malaria, bacterial abscesses and high blood pressure. Gas chromatography – mass spectrometry (GC-MS) analysis on T.crispa crude extract show 16 compounds have been detected. Trichloromethane and hexachloroethane are not considered as compound in T.crispa as they are part of chloroform solvent. It is used as a solvent and extractant in various manufacturing and laboratory procedures, where created as a by-product chlorine-containing compounds. Other compounds are groups of terpenes, alkaloids and flavonoids. Diisooctyl phthalate is one of the active compounds that have high toxicity,which di-n-*octyl phthalate (DNOP) have high toxicity compared to di(2-ethylhexyl) phthalate (DEHP).*
Keywords *: T.crispa*, health sustainability. toxicology

PENDAHULUAN

Chemical pesticide was introduced by humans on the previous golden age of world civilization. After the World War II , it has been commercialized by scientist for pest-control management. Besides of this chemical pesticide, scientist start to find out pesticide from natural substance, commonly known as biological pesticide or biopesticide.

Since the discovery of the insecticide dichloro-diphenyl-trichloroethane (DDT) before the Second World War, the widespread use of synthetic insecticides for the control of pests as well as human disease vectors has led to concerns about their toxicity and environmental impact (Mulla and Su 1999; Vatandoost and Vaziri, 2004). Next the focus shifted to resistance, novel biochemical targets, and new chemical approaches for pest control. The current Golden Age of Genetic Engineering has curtailed, but is unlikely to eliminate, chemical use on major crops. Insecticide research, having passed through several Golden Ages, is now in a renaissance of integrating chemicals and biological for sustainable pest control with human safety (Casida and Quistad,1998).

Aedes albopictus is a commonly known in Malaysia beside *Aedes aegypti* as a cause of dengue disease. *A.albopictus* belongs to the family Diptera. These species are effective vectors of dengue because of their ability to breed in artificial containers in and around the house, close to human being (Cheong, 1967). To overcome the population of the dengue's vector, Malaysian Ministry of Health always use thermal fogging method. Misni *et al*(2011) state that pyrethroids in aerosol canister are widely used for controlling adult mosquitoes in household and also by thermal fogging and ULV (Ultra-Low-Volume) spraying at community level. However, there are possibilities that mosquitoes may develop resistance to pyrethroids due to selection pressure (Somboon *et al*., 2003).

Nowadays, many natural products were commercialized to biopesticides. 'A-Mark Procon' is one of the researches driven company that manufacture and supply biopesticides for agriculture, horticulture and forestry. *Tinospora crispa* is one of the potential biopesticide. It is commonly known as Patawali or Seruntum. *T.crispa* is herb widely spread in Malaysia, Indonesia, Thailand, Philippines and Vietnam. In Malaysia, a decoction of the stem of *T.crispa* is taken to treat stomach-ache, fever, gonorrhea and worms. Stem and root extracts are used to treat malaria, bacterial abscesses and high blood pressure (Joseph *et al*, 2009).

The antioxidant and anti-proliferative activity of the aqueous crude extract of Tinospora crispa stem was investigated (Zulkhairi *et al*, 2008). The antihyperglycaemic effect of *T. crispa is used to treat diabetes type II* (Hamdan, 1997). Thus, it is of interest to investigate the toxicity effect of *T.crispa* since a lot of previous studies related to medical branch.

General on Mosquitoes

Mosquitoes are class of insect, belongs to family Diptera. The family includes 3,523 species classified in two subfamilies and 113 genera. The subfamily Anophelinae has three genera and Culicinae has 110 genera segregated into 11 tribes (Harbach, 2011).

From Mosby's Medical Dictionary (8th edition), *Aedes* is a genus of mosquito prevalent in tropical and subtropical regions. Several species are capable of transmitting pathogenic organisms to humans, including dengue fever, equine encephalitis, St. Louis encephalitis, tularemia, and yellow fever. Mosquito larvae make up substantial biomass in aquatic ecosystems globally (Fang, 2010). In water, larvae eat algae and small organisms. They obtained oxygen from air through breathing tube called siphon. But, *Anopheles* are unlike *Culex* and *Aedes* larvae since they do not have a breathing tube, they must lie parallel to the water surface in order to get a supply of oxygen through a breathing opening (Alamada Country Mosquito Abatement Disctrict).

Female mosquito feeds on blood male mosquito feeds on plant juices (WHO, (1972); Burgess and Cowan, (1993). Eggs is laid in a batch singly or individually by the female (Kettle, (1984); Rhodain and Rosen, (1997)) above the water surface on the damp areas. It can remain dry for months but still viable and the egg hatches when they become flooded with water (Goma, 1966; Harwood and James, 1979; Service, 1996).

Life cycle

Life cycle of mosquitoes can be divided into four different stages, which is egg, larvae, pupa and adult. Each stage can be differentiating by their appearance. USA Centre for Disease Control and Prevention (CDC) stated that female mosquitoes lay their eggs on the inner, wet walls of containers with water. Mosquito eggs were sticks together to form a raft about 200-300 eggs. In suitable conditions, mosquito larvae will emerge from eggs. The larvae live in water for a few days before becomes a pupa. Larvae have divided into four instars. When the fourth instars larvae shed its skin, it becomes a pupa. The pupa the undergo metamorphosis to be an adult.

Aedes albopictus

General

Aedes albopictus (Skuse), has been reported in more than 25 countries on the five continents outside its natural region since the end of the 1970s, and is an important vector of several arboviruses, including dengue, yellow fever and diverse types of encephalitis (Mitchell, 1995; Gratz, 2004). *Aedes albopictus*, commonly known as the Asian tiger mosquito, is currently the most invasive mosquito in the world. It is of medical importance due to its aggressive daytime human-biting behavior and ability to vector many viruses, including dengue, La Crosse, and West Nile (Mark *et al*, 2007).

When feeding at the air-water interface, *Aedes* larvae rotated about the axis formed by the respiratory siphon in alternating clockwise and counterclockwise directions, possibly to rest the muscles used to twist the larval body into the U-shaped posture characterizing this feeding behavior (Walker and Merritt, 1991).

Disease vector

In Gabon, the tiger mosquito *Aedes albopictus* was shown to be the main CHIKV vector during the 2007 outbreak (Paupy *et al*, 2012). About two-fifths of the world's populations are at risk of catching dengue according to the World Health Organization (WHO 2003). *A.albopictus* is a competent vector of many viruses including dengue fever (CDC 2001; Rios and Maruniak 2004) and Eastern equine encephalitis virus (Mitchell *et al*. 1992; Rios and Maruniak 2004).

Tinospora crispa

General Information

Tinospora crispa is a climbing plant, grows widely scattered in the primary rainforests, up to an elevation of 1,000 m. The older stem becomes woody and knobbly with very prominent tubercles (Joseph *et al*, 2009). The bitter tasted stem provided effective remedy for various ailments such as tooth and stomach aches, coughs, asthma, pleurisy, fever, and other viral and bacterial infections (Perry, 1980; Muhammad and Mustafa, 1994). The flowers become red and white when matured.

METHODS

Materials and Extraction of Samples

Tinospora crispa plant's stem was collected at field then the stem is chopped down into small pieces. After the cutting, the stem was dried for three days in shade. Markert (1995) discussed the purpose of drying the sample material is to protect it against microbial decomposition during subsequent storage and to acquire a constant reference value by determining the dry weight as opposed to the fresh weight, which is difficult to quantify. 200 g of *T.crispa* dried stem was soaked in 2000 ml chloroform in big conical flask, totally covered by aluminium foil to prevent the chloroform from evaporate. This sample is kept in dark cupboard for 24 hours.

Collection of *Aedes albopictus* larvae

Mosquito larvae were collected at Institute of Biological Sciences (ISB) garden, UM.

Bioassays with *T.crispa* crude extract

Bioassay is an assay designed to analyze any compound by use a suitable biological system like animals, tissues and etc. Firstly, preparations of *T.crispa* crude extract concentration into part-per-million (ppm), 1×10^{-6}. To prepare concentration of 1000 ppm, 1 ml of *T.crispa* crude extract was added into 1000 ml distilled water. The mixture was shaken to get well separately crude. Four milliliter of the mixture was drowning out into a 30 ml glass vial bottle. Then, ten *A.albopictus* larvae were added into the vial containing 1000 ppm of the crude. The time of adding larvae were recorded for analyze the time of death. Data was taken for every hour after the time start until 72^{nd} hour. Other concentrations prepared are 250 ppm, 500 ppm, 3000 ppm and 5000 ppm. Control treatment was done; each vial is added with 4 ml of distilled water. Each ppm concentration prepared is replicate ten times, so each ppm concentration needs one hundred mosquito larvae. Hence, 600 mosquito larvae have been used to undergo this bioassay

Flash Column Chromatography

Flash column chromatography is a technique used to separate complex mixtures of compounds. It namely flash column because we use compressed air to push the solvent through the column. So, it is a quick process.

Before prepare the column chromatography, appropriate column is selected. Small pieces of glass wool have been used to support the selected column. Silica gel powder is used as an adsorbing material into the column. The silica gel is then packed into the column. Column chromatography was washed two times with 10 ml slurry of 100% hexane. Pouring of this slurry is continuously added to avoid formation of sorbent. Next, the column was loaded with one milliliter of concentrated *T.crispa* crude extract. At the same time, the eluent is starting to collect. After that, 10 ml of solvent 100% hexane is carefully added to wash the sample into the column material.

Next step is eluting the column. Through this flash column chromatography, an adsorption column was developed by gradient, where there is gradual change in solvent composition. According to Boyer (2009), the composition of eluting solvent can be changed by the continuous mixing of two difference solvents to gradually change the ratio of the two solvents. Solvents used are 100% Hexane and Diethyl ether. Total volume for each solvent ratio is 10 ml. Table below show the ratio of the solvent used to elute the column.

Thin Layer Chromatography (TLC) Analysis

Thin layer chromatography (TLC) was done to determine the most suitable solvent system for isolation of the chemical compounds from *Tinospora crispa* and also to monitor the elution of the chemical components from flash column chromatography. In this study, silica gel 60 F254 TLC plates from MERCK were used for analysis. Samples use in TLC analysis are taken from column chromatography collection,where they are eluent collection from solvent 70% Hexane:30% Diethyl ether, 60% Hexane:40% Diethyl ether, 40% Hexane:60% Diethyl ether and Ethyl acetate.

In the first step, two droplets of sample were spotted on about 1.0 cm from the bottom of the TLC plate by using a fine capillary tube. The spotted sample was left to dry in room temperature and then developed in a TLC developing tank which had been conditioned with premixed solvent system. Once it was developed, the TLC plate was removed from tank and subsequently dried. Next, each TLC plate was viewed under short (254 nm) ultra violet light. Spot seen was marked for bioassay treatment.

RESULTS

Table 1. Bioassay result of mosquito larvae mortality treated with crude extract at different ppm concentration.

Treatment	12 hour	24 hour	36 hour	48 hour	60 hour	72 hour
Control	0.1	1.3	2	2.3	2.7	2.8
250 ppm	0.1	0.5	0.6	1.1	4.8	9.3
500 ppm	0.7	0.7	0.8	1.1	2	3
1000 ppm	3.2	3.4	3.5	3.6	3.8	6.1
3000 ppm	10	10	10	10	10	10
5000 ppm	10	10	10	10	10	10

Flash column chromatography produce twenty collection of eluents. This collection was collected in small vials. The fractions are then selected to perform bioassay. Following table is the result of mosquito larvae mortality treated with it.

Table 2. Bioassay result of mosquito larvae mortality treated with Flash column chromatography fraction collection.

Treatment	12 hour	24 hour	36 hour	48 hour	60 hour	72 hour
100% Hexane	1.0	2.4	4.1	5.0	6.3	7.6
70% Hexane:30% Diethyl ether	0.7	1.8	4.1	5.6	7.1	8.7
60% Hexane:40% Diethyl ether	0.4	0.9	1.5	3.4	5.4	8.0
40% Hexane:60% Diethyl ether	0	0.4	1.9	3.1	4.1	5.4
Ethyl acetate	0.4	1.5	2.2	3.3	4.3	6.1

Table 3. Bioassay result of mosquito larvae mortality treated with TLC spots.

Treatment	12 hour	24 hour	36 hour	48 hour	60 hour	72 hour
70% Hexane:30% Diethyl ether	0.5	2.9	4.8	8.4	8.7	9.9
60% Hexane:40% Diethyl ether	0.4	0.8	1.5	3.4	5.6	7.7
40% Hexane:60% Diethyl ether	0.4	1.4	5.8	8.1	9.0	9.7
Ethyl acetate	0	1.0	1.7	5.1	6.6	9.1

GC-MS RESULTS

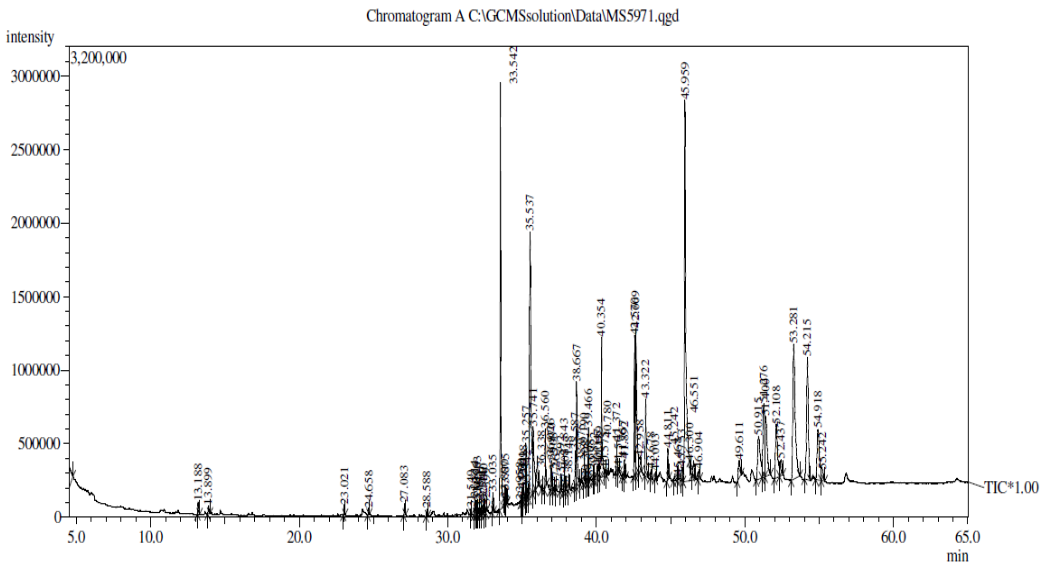


Figure 1. Chromatogram of Crude extract

Gas chromatography-mass spectrometry (GC-MS) for *Tinospora crispa* crude extract was run for 65 minutes. Mass spectrum data of separated components obtained are match with the library.

Table 4. Active compounds of Sample A, *T.crispa* crude extract detected by GC-MS.

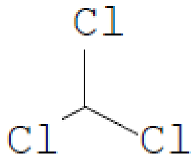
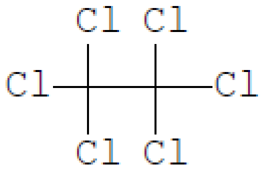
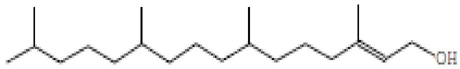
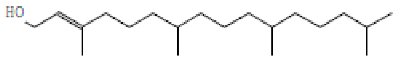
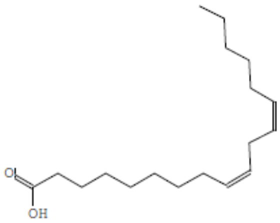
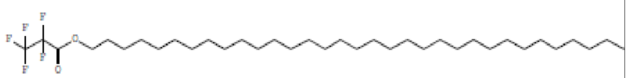
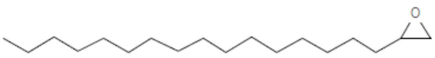
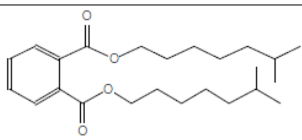
No.	Chemical Compound	Structure
1.	Trichloromethane	
2.	Hexachloroethane	
3.	3,7,11,15-tetramethyl-2-hexadecen-1-ol	
4.	2-Pentadecanone	
5.	n-Hexadecanoic acid / Palmitic acid	
6.	(2*E*,7*R*,11*R*)-3,7,11,15-tetramethyl-2-hexadecen-1-ol / Phytol	
7.	*cis*, *cis*-9,12-Octadecadienoic acid / Linoleic acid	
8.	Octatriacontyl pentafluoropropionate	
9.	Dotriacontane	
10.	2-Hexadecyloxirane	
11.	Pentatriacontane	
12.	Diisooctyl phthalate	
13.	Tetrapentacontane	
14.	Hexatriacontane	
15.	1-Heptacosanol	
16.	Tetracontane	

Table 5. Compounds analyzed by GC-MS on Sample B.

No.	**Chemical Compound**	**Structure**
1.	Eicosane	
2.	Dotriacontane	
3.	Hexatriacontane	
4.	Tetracontane	

Table 6. Compounds analyzed by GC-MS on sample C

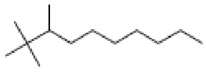
No.	**Chemical Compound**	**Structure**
1.	2,2,3-Trimethyldecane	
2.	Eicosane	
3.	Tetracontane	
4.	Dotriacontane	
5.	Tetrapentacontane	

Table 7. Compounds analyzed by GC-MS on sample D.

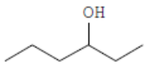
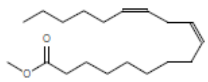
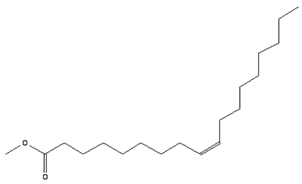
No.	**Chemical Compound**	**Structure**
1.	3-Hexanol	
2.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	
3.	Methyl trans-9-octadecenoate	
4.	Methyl cis-9-octadecenoate	
5.	Eicosane	
6.	Hexatriacontane	
7.	Tetrapentacontane	

Table 8. Compounds analyzed by GC-MS on sample E.

No.	**Chemical Compound**	**Structure**
1.	Eicosane	
2.	Tetracontane	
3.	Tetracosane	
4.	Hexatriacontane	
5.	Tetrapentacontane	

Table 9. Compounds analyzed by GC-MS on sample F

No.	Chemical Compound	Structure
1.	Eicosane	
2.	Hexatriacontane	
3.	Tetrapentacontane	

Table 10. Compounds analyzed by GC-MS on sample G

No.	Chemical Compound	Structure
1.	Eicosane	
2.	Hexatriacontane	
3.	Tetrapentacontane	

Table 11. Compounds analyzed by GC-MS on sample H (Column collection eluent 70:30)

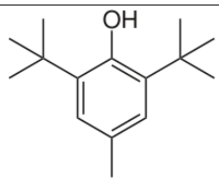
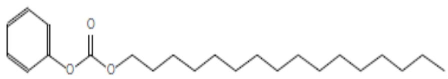
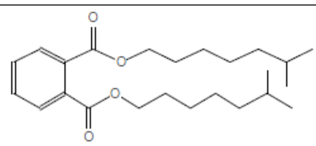
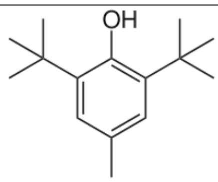
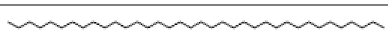
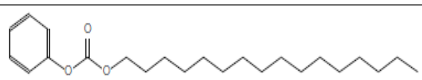
No.	Chemical Compound	Chemical Formula	Structure
1.	Butylated Hydroxytoluene	C15H24O	
2.	Eicosane	C20H42	
3.	Hexatriacontane	C36H74	
4.	Carbonic acid, hexadecyl phenyl ester	C23H38O3	
5.	1,2-Benzenedicarboxylic acid, diisooctyl ester / Diisooctyl phthalate	C24H38O4	
6.	Tetrapentacontane	C54H110	

Table 12. Compounds analyzed by GC-MS on sample I (Column collection eluent 60:40)

No.	Chemical Compound	Chemical Formula	Structure
1.	Butylated Hydroxytoluene	C15H24O	
2.	Eicosane	C20H42	
3.	Hexatriacontane	C36H74	
4.	Carbonic acid, hexadecyl phenyl ester	C23H38O3	

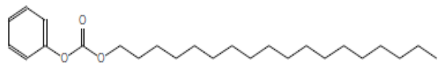
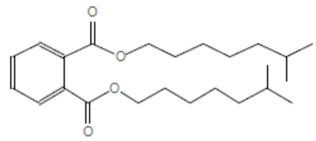
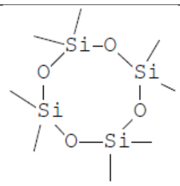
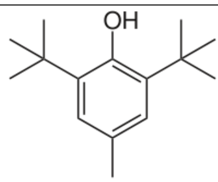
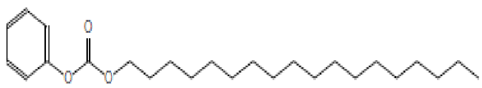
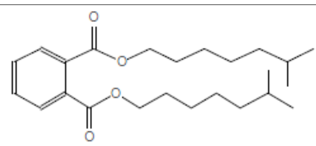
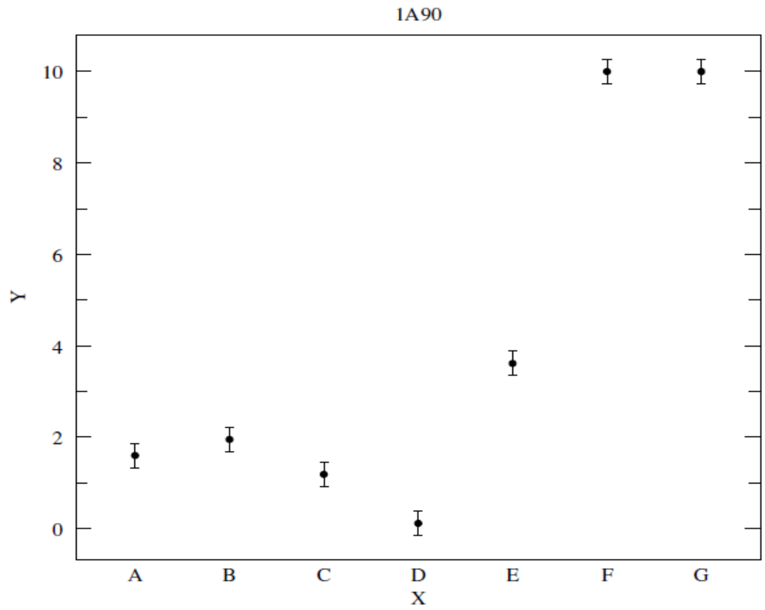
5.	Carbonic acid, octadecyl phenyl ester	C25H42O3	
6.	1,2-Benzenedicarboxylic acid, diisooctyl ester / Diisooctyl phthalate	C24H38O4	
7.	Tetrapentacontane	C54H110	

Table 13. Compounds analyzed by GC-MS on sample J (Column collection eluent 40:60)

No.	**Chemical Compound**	**Chemical formula**	**Structure**
1.	Cyclotetrasiloxane	C8H24O4Si4	
2.	Butylated Hydroxytoluene	C15H24O	
3.	Carbonic acid, octadecyl phenyl ester	C25H42O3	
4.	Carbonic acid, hexadecyl phenyl ester	C23H38O3	
5.	1,2-Benzenedicarboxylic acid, diisooctyl ester / Diisooctyl phthalate	C24H38O4	

Result of bioassay treatment on ppm concentration was then analysed by statistical analysis, Analysis of Variance (ANOVA).



A – Control
B – 250 ppm
C – 500 ppm
E – 1000 ppm
F – 3000 ppm
G – 5000 ppm
X – ppm concentration
Y – Mean

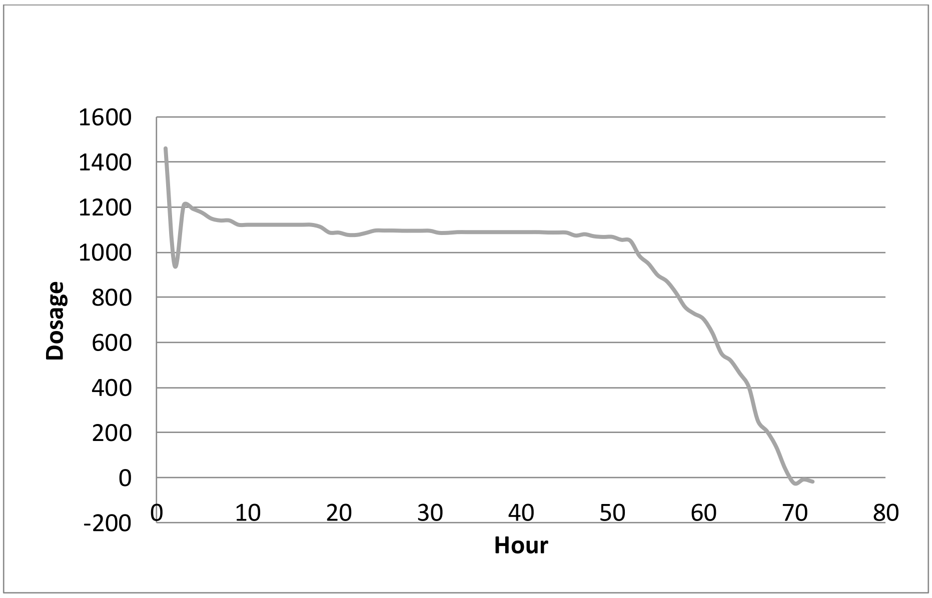


Figure 2. Dosage needed to kill larvae mosquito against time(hour)

CONCLUSION

Active compounds from Tinospora crispa extract have been identified. GC-MS analysis determined 14 compounds which is 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 2-

Pentadecanone, n-Hexadecanoic acid, (2*E*,7*R*,11*R*)-3,7,11,15- tetramethyl-2-hexadecen-1-ol, Linoleic acid, Octatriacontyl pentafluoropropionate, Dotriacontane, 2-Hexadecyloxirane, Pentatriacontane, Diisooctyl phthalate, Tetrapentacontane, Hexatriacontane, Tetracontane and 1-Heptacosanol. From this study, Diisooctyl phthalate was identified as compound that has high toxicity, which kill the *Aedes albopictus* larvae. Minimum dosage of *T.crispa* extract kill the mosquito larvae in longer time compared to higher dosage. Finally, this topic can be further study for maximize the uses of biopesticide for worldwide in term of sustainability of health in Indonesia.

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