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Ligan Activity of *Antiaris toxicaria* Lesch. and the Role of Toxicarioside in Crude Extract*: In Silico, In Vitro,* and *In Vivo* Approaches

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Abstract: *Antiaris toxicaria Lesch is one of the endemic plant to Kalimantan Island and some tropical countries in South East Asia region. Traditionally, the sap of plant was used as poison and attached in traditional weapon. The crude extract of the sap plant contain toxic material called Toxicarioside. The aims of the research are to identify the toxicity of the crude extract which are derived from heating and methanol extraction process to Rattus norvegicus mortality and examine ligan Toxicarioside activity using in silico method. Extract toxicity was analyzed using percentages of probit analysis of the R. Norvegicus and a toxicity indicators. In silico analysis on the value of energy released and inhibition* constanta *of some molecule resulting the first confirmation of 2ZKD receptors has higher value -9.12 kcal/mol. Compared to the other nine confirmation, the value of inhibition constanta was the lowest 0.21 µM. Compared to the other nine conformation, receptor 3F8J, the minimum free energy which are able to release was about -6.26 kcal/mol and it has lowest inhibition constanta (25.89 µM). Crude extract of A. toxicaria from heating process has probit value =0.754x - 0.354 and LD50 = 1.017 or 10,339 mg while the extract derived from Methanol extraction has probit value =0.799x - 0.642 and LD50= 1.267 or 18.493 mg. These extract is the very toxic materials and has potentiality as bio-rodenticides.*

Keywords - *Antiaris toxicaria, bio-r*odenticides, *Toxicarioside*

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I. Introduction

Antiaris toxicaria Lesch. (Moraceae) locally known as *upas tree*, distributes at tropical countries and known to be endemic to Kalimantan and some area in tropical countries. The bark has latex, which are used widely as traditional medicine by Dayaks, the local people in Kalimantan. The latex also extracted and used as poison material in traditional weapon, including arrow and blow pipe (*sumpit*).

The phyto-chemical and chemical structure analysis shows that latex of *Antiaris toxicaria* contains Antiarisin (cardenolide)/toxicarioside A and B, and other 17 active compound [1] [2]. The cardenolide can be extracted from the bark of *A. toxicaria* using ethyl-acetate solution [1] [3]. The ethanol extraction of *A. toxicaria* shows the bark has some active compound with cardiotonic effect. It is shown trough the *in vitro* test to the marmot atrium [1] [3] [4] [5].

Some chemical compound found and reported in Toxicarioside I by researchers, including strophanthidin, periplogenin, (3β,5α,14β,17α)-3,14-dihydroxypregnan-20-one, α-tocopherol and 3,4,5-trimethoxy-phenol. These compound has been reported as toxic compound. Previous chemical investigation reported that Toxicarioside *(*previously known as cardenolides) can be isolated from latex of *Antiaris toxicaria* [1] [3] [6], seeds [7] [8], and steam [5], bark [4] [9]. These compound has reported has strong citotoxicity activity [10]. There are, however, potential value of the active compound of *A. toxicaria* as anticancer drug and cardiovascular [11].

Biological mechanism of Toxicarioside effect has been done using animal test and shows that the Toxicarioside active compound was work on cell plasm and in ion regulation Na^+/K^+-ATPase and ATP, Na^+/K^+-ATPase enzyme [12] [13]. Cardenolide glycoside as an active compound of Toxicarioside in cell membrane work to disturb Na^+/K^+ balance in cell membrane. Toxicarioside is a group of active compound called metabolic inhibitors in insect, mammals and humans, or plays as toxic poisons agent [12] [14] [15].

The latex of *A. toxicaria* has brown color, while the crude extract by n-hexane, Ethyl acetate and methanol solvent has red color. As far, the application of crude extract of *A. toxicaria* are rarely studied and

implemented in crop pest and disease management. It is especially important o replace the intensive use of chemical pesticide in reducing crop pest and disease. *In silico* and *in vitro* test to active compound of *A. toxicaria* to white mouse is important to implemented as a crucial step in the development of biological agent to control mouse pest in agriculture sector. The aims of the research is to identify the toxicity of crude extract which are extracted through methanol extraction and heating techniques to *R.norvegicus* and *in silico* activity of ligans of Toxicarioside.

II. Methods

Plant sap simplicia of *Antiaris toxicaria* Lesch was collected from wild population in Barong Tongkok Sub-Regency, East Kalimantan, Indonesia. In order to verify the name of plant material, a identification of plant by botanical expert was done in Mulawarman University. The collected latex was filtered and packaged into anti-toxic bottle (1 Liter) and soaked into n-hexane (1 L×2) solution in room temperature.

The extracted material from n-hexane was separated into crude extract and residue. The residue (called n-hexane fraction) was evaporated using vacuum rotating evaporator in under 50ºC. The crude extract which are derived from n-hexane extraction consist of two material; residue (called ethyl acetate fraction) and crude extract. The ethyl acetate fraction was evaporated using vacuum rotating evaporator in under 50ºC.

Residue derived from ethyl acetate extraction was extracted using methanol to produce methanol fraction. From initial analysis, the number of n-hexane fraction after evaporation produce 3 ml, and it is nor enough to the further n-hexane fraction test. Therefore, this material was not used in this study for further active compound test.

There are distillation difficulties to separate latex of *A. toxicaria* from its essential oil and therefore heating techniques was implemented. In this method, latex was heated in temperature 200^{o}C and stirred continuously. The active compound of the collected material was tested using TLC.

The Toxicarioside content was confirmed using LC-MS/MS, and the determination of relative amount percentage of Toxicarioside was based on the percentage of peak area [13]. Result of the LC-MS in steroid (anticarioside) group found 14 peaks and 13 peak methanol fraction. Toxicarioside was detected with retention time 557.1109, 405.254-408.2664 m/z. In the methanol extract methods, there are fragmentation in the sugar molecule with retention time 324.3192, 409.1581, 410,10.1827, 534.1992, 751.5303 m/z in various spectrum (Li et al. 2010) (Fig.1).

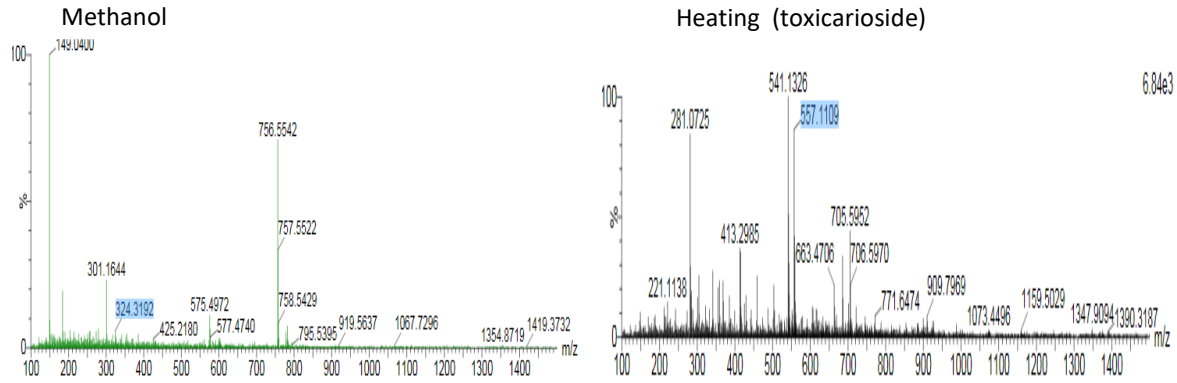


Fig. 1. TIC of Methanol fraction and latex which are heating using LC-MS/MS

Probit analysis

The healthy *Rattus norvegicus* with normal growth by weight 130-150 gr. was used as an experimental animals. Before experimental using animals was done, an ethical clearance aspects regarding the usage of animals was practiced. The animals ethical clearance was obtained from Komisi Etik Penelitian Kesehatan Fakultas Kedokteran Universitas Mulawarman (no. 13-KEPK-FK/III/2017). *R. norvegicus* was conditioned in laboratory within one week before experiment was done. *R. norvegicus* was kept in standard environmental condition (29 ± 2◦C). *R. norvegicus* fed by standard pellet diet and mineral water through *ad libitum* methods. Before treatment, *R. norvegicus* was maintain without fed one night, but animal still permitted to access water mineral.

Statistical analysis.

The toxicity test of active compound of *A. toxicaria* which area extracted through Methanol extraction techniques is part of the screening methods to identify active compound on particular plant. Chemical compound of pant was referred as toxic material when LD50 ≤ 200 ppm [12]. Result of the toxicity test was given in Table 1.

In vivo inhibition activity of Toxicarioside was defined as the mean ± SEM and analyzed using ANOVA and followed by DMRT test. ANOVA (Sig. P<0,05). was done using SPSS software version 15.0. LD50 value was determined from inhibition percentage plot versus concentration and calculated using probit regression analysis. LD50 was defined as an extract concentration that contain Toxicarioside which are able to kill experimental animals, the *R.norvegicus* in this study. This is also Toxicarioside activity in cell membrane of *R.norvegicus*. Probit analysis shows that the latex of *A. Toxicaria* which are extracted from heating process has probit value =0.754x - 0.354 and LD50= 1.017 or 10.339 mg while the methanol extract has probit value =0.799x - 0.642and LD50=1.267 or 18.493 mg. These compound are identified as very toxic materials to kill *R. norvegicus* (Fig. 1.).

In vivo research

Meyer, et. al. (1982) method was implemented to study the sample toxicity of active compound in plant material [16]. In each toxicity test, one treatment will have one repetition. In each treatment, about 15 *Rattus norvegicus* were used. Sample was solubilized with water and each sample was tested following the concentration 0 mg, 5 mg, 20 mg, 40 mg, and 100 mg. Material from original sap, anticariocide and control was done through oral fed in three times of treatment repetition. The mouse cage was set up under lamp light and observed for 24 hours. The calculation of living and dead mouse was done to determine the toxicity level (Lethal Dosage 50/LD50) of given materials. Data was corrected to the mortality control using Abbot formula, while probit analysis was generated from LD50, LD75 and LD90 test results [17].The toxicity level status was given in Table 1.

Table 1. Relationship between LC50 and Toxicity Category

Categories	LC50 values
Super toxic	≤ 5 mg/kg
Very toxic	5-50 mg/kg
Toxic	50-500 mg/kg
Toxic medium	0.5-6 g/kg
Mild toxic	5-15 g/kg
Practically non-toxic	> 15 g/kg

***In Silico* Mechanism.**

Docking process between ligans and protein receptors was done using *Autodock Tools 1.5.6* program. Docking process was done in size and grid box position in default conditions. Docking parameter which were used includes *Genetic Algorithm* and *output docking* definition with parameter *Lamarckian GA(4.2.)*. Docking validity process was done using *Autodock Vina* with similar size and grid box position to that process in docking process using *Autodock Tools 1.5.6*. From ten models which are resulted from ten models which are resulted from docking analysis, one model with stabile conformation with lowest and k_i and ΔG was selected. The value of k_i and ΔG shows that the ability of ligans to interact with active sites of very strong proteins receptors.

III. Result and Discussion

The docking analysis includes occurring integration analysis, docking result confirmation with lowest *ki* value, docking data collection including binding energy, and electrostatic energy. The LD50 of Toxicarioside in killing 50% of mouse was used as reference to estimate the ability Toxicarioside to kill mouse [14]. Result of the analysis shows that LD50 of each fractions was difference. Fraction resulted from probit analysis shows that the sap plant of *A. toxicaria* which are resulted heating process has probit value =0.754x - 0.354 and LD50= 1.017 or 10.339 mg, while the extract that derived from methanol extract has probit value 0.799x - 0.642 and LD50= 1.267 or 18.493 mg.

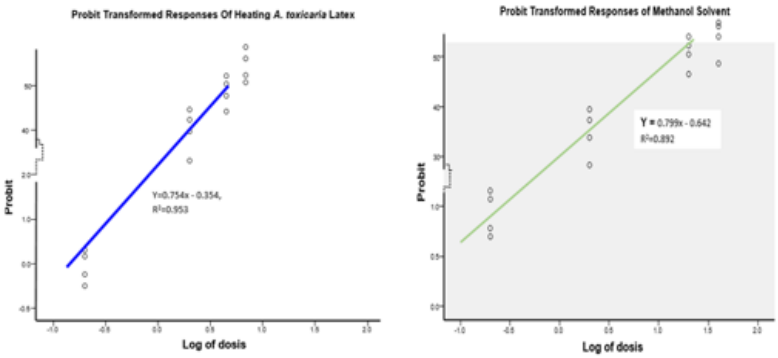
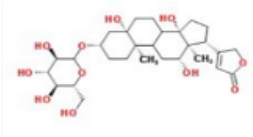


Fig. 2. Graph of the relationship between log and extract concentration

The lowest *Ki* value of Toxicarioside was about 0.21µM with binding energy -9.12 kcal/mol. This means that Toxicarioside very toxic and able to kill *R. norvegicus* in very fast rate, or it can be said receptors acceptor very sensitive to Toxicarioside.

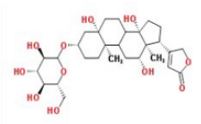
Table 2. Release value and inhibition constanta of some molecules using LC-MS/MS analysis
Compared to the other nine confirmation, the first confirmation at receptor 2ZKD shows that the highest free

Compound	Receptor	AutodockTools1.5.6		Autodock Vina	
		Binding Energy (kcal/mol)	k_i(uM)	ΔG_{aff}(kcal/mol)	*%Error*
Toxicarioside k 	2ZKD	-9.12	0.21	-8.5	7.29
	2ZKO	-7.81	1.88	-6.1	0.28
	2ZKF	-7.17	5.54	-6.9	4.87
	2ZKG	-6.53	16.28	-7.2	0.09
	3F8I	-6.29	24.53	-2.2	1.86
	3F8J	-6.26	25.89	-7.4	0.15

energy was -9.12 kcal/mol, Autodoct vina -8.5kcal/mol, result that collected from docking using software Autodoct Tools and Autodoct Vina shows different ΔG % error < 10%, means that these two docking methods able to use (Table 2).This confirmation has the lowest inhibition constanta (Ki) (0.21 µM),The highest released free energy will have lowest inhibition constanta. The low constanta value shows the stability of R-L complex and the strength of association or molecule binding in the development of stable complex, and Toxicarioside was inhibited at its homolog receptor (2ZKD) [18]. Ligans is the molecules that able to produce signal as a sign that there are interaction with receptors (proteins) target. The relationship between ligan and receptors is the loading function, hydrophobicity and molecule structure [19].

Docking process of ligan and macromolecule resulting Ki value 10 (conformation model), indicates the strong interaction between ligans and macromolecule. Strong interaction influence the instability of cell plasm movement and activities and regulations of Na^+/K^+-ATPase ions and ATP and Na^+/K^+-ATPase enzymes [20]. Ligan-macromolecule weak, and *Ki* high, and therefore it is easy for ligans macromolecules in dissociation status. Ligan consist of substrate, inhibitors and neurotransmitter which are able to bind receptor proteins and able to changes the chemical confirmation by changes of 3D orientation of molecules; while the protein receptors is the functional component. The activity of ligans receptors describes the affinity level which are not only interaction between L-R, but also shows the ability of solution to support chemical bindings in solution. Solvent provides chemical environment that are suitable to L-R adapt [21].The different of solvent treatment that used to extract sap of *A. toxicaria* is important aspect in differentiate LD50 value in the mortality test using *R. norvigicus*. Extraction using methanol and heating influence the material in latex fragmented into other materials and therefore decreasing the ability to inhibit interaction between Ligan-Receptor and the mortality of *R. norvigicus*was highest and fast [15].

T**able3**. Ligan-Receptor and Amino acid that resulted from Docking Ligan Toxicarioside molecule to 6 Receptor

No	ligan	Receptors	Amino acids	Notes
1	toksikarioside 	2ZKD	ASN421, ARG438, VAL451, ARG457, ARG457, ALA468, ALA468, ALA468, ALA468, GLY469, ALA468, GLY469, ALA468, GLY469, ALA468, GLY470, ALA468, GLY470, GLY469, GLY470, TYR483, VAL409	ARG= Arginine GLY= Glycine VAL = Valine LYS= Lysine ASN= Asparagine
		2ZKO	SER42, ARG46, ASP39, ARG46, ASP39	SER= Serine
		2ZKF	ARG457, ARG457, GLY488, LYS505, ASN510, SER486, GLY456, SER486, LYS505, GLY456, HIS455	HIS= Histidine ASN=Aspartat TYR = Tyrosine
2		2ZKG	ARG558, TRP594	TRP = Tryptophan
3		3F8i	GLY453, TYR483, ARG496, ALA452, VAL451, TYR471	
4		3F8J	ASP476 Unfavorable donor	

Amino acid residue in the interaction between Toxicarioside and receptor 2ZKD, 2ZKF, 2ZKG, 2ZKO. 3F8I, 3F8J resulting six amino acid, namely aspartate acid, arginine, alanine, glysine, tyrosine and valine. In receptor 2ZKO there are 3 amino acid (ser, arg, asp), receptor 2 ZKF interact with 7 amino acid, receptor 2 ZKG with 2 amino acid, receptor 3F8i interact with 5 amino acid and receptor 3 F8J interact with 1 amino acid (Table 3.).The intensity of interaction shows the inhibition activity was dynamic and specific. The binding of Toxicarioside k and amino acid in cell plasm was shown by violet color in Fig. 3 and represent the condition of Toxicarioside ligans in releasing Hydrogen binding with amino acid that cause protein denaturation. Result of the docking molecule was given in Fig. 3C. Green color represent the acceptors that accept hydrogen ion

interaction in this interaction. The interaction can be occurring in two direction, from ligans to amino acids or amino acids to ligans. Toxicarioside with –OH able to works in wide spectrum, but it is depend on the number of its acceptors. It influence the wide opportunities of interaction level and it is influence the plasm cell. The plasm cell disturbance occurs due to instability of ion Na^+/K^+-ATPase pump and ATP, Na^+/K^+-ATPase enzymes in the high concentration of ligans. The absorption of ligans in blood will lead to the mouse mortality.

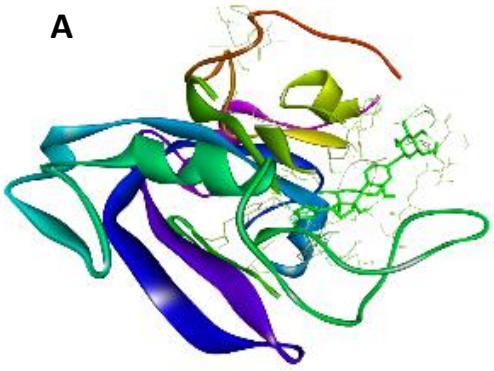
Result of the docking validity using Autodock V shows that reaction from interaction between ligan – Toxicarioside in active sites of receptor protein of *R. norvegicus* can be spontaneously. This is shown by ΔG below 0 and percentage value <10% (Table 2). Result of the docking using Autodock Tools 1.5.6 also shows that the reaction occurs spontaneously. Result of the docking validation shows that the docking methods able to use.

The toxicity test of active compound of *A. toxicaria* resulted from Methanol extraction and heating process is the screening methods to identify the active compound in plant extract. The chemical component of plant said as toxic when LD50 ≤ 200 ppm [12]. Result of the toxicity test of extract which are obtained from methanol extraction and latex heating was given in Table 1.

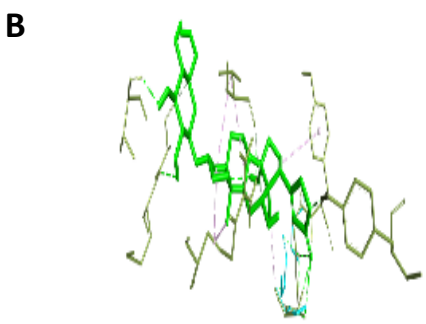
The toxicity extract of plant sap of *A. toxicaria* was done to identify toxicity level of extract to *R. norvegicus*. Result of the experiment shows that there are different concentration of extract between material extracted using methanol and heating process to kill *R. norvegicus* (Table 3 and Fig. 1). Result of the study shows that active compound that area extracted by methanol extraction techniques provides significant mortality impact at different concentration. Extract methanol and heating has mortality level until 100%.

The highest mortality rate of *R. norvegicus* up to 50% caused by latex of *A. toxicaria* which area extracted using methanol and heating methods occurs in the extract concentration 5-40 mg. It can be can occurs after 72 hours of treatments. Extract derived from heating process of *A. toxicaria* latex shows the fast mortality within 72 hours after treatment.

Result of the toxicity analysis shows that increase of extract concentration has positive correlation with the high mortality of *R. norvegicus*. This result shows that crude extract of *A. toxicaria* latex shows that active compound in plant active and has high bioactivity. This means, in the low level of concentration, the material has toxicity impact to *R. norvegicus*.



The Toxicarioside k ligans position in cell plasm



The Toxicarioside k ligans position in cell plasm in Receceptor2ZKD

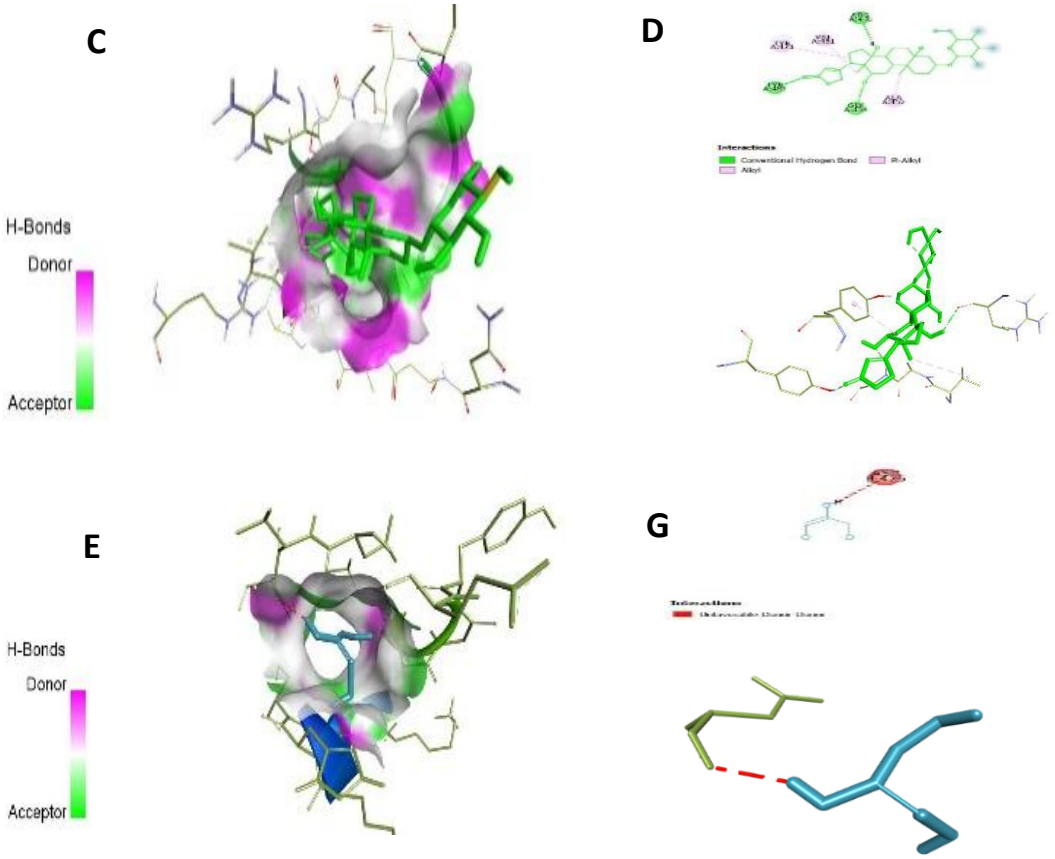


Fig.3. Models of T*oxicarioside* cells plasm and the position of ligans to receptors

Table 4. The mortality rate and toxicity of methanol and heating extract of Latex *A. toxicaria*.

Extraction methods	Concentration (mg)	Mortality ± SD (%)a 72 JSP	Lethal Dosages (Log Dosages)	Toxicity criteria
Heating	0.5	0.6667±8.6744 e	0.25 = 0.685	Very toxic
	5	1.084 ±25.8399 d	0.5 = 1.017	
	20	1.754 ±56.1786 c	0.75 = 1.348	
	40	1.754 ±61.4363 b	0.90 = 1.547	
	100	1.7857± 99.1071a		
Methanol extraction	0.5	0.99178±8.2542 e	0.25 = 0.954	Very toxic
	5	1.084 ±25.8399 d	0.5 = 1.267	
	20	0.9953 ±50.9858 c	0.75 = 1.580	
	40	1.0255 ±74.5145 b	0.90 = 1.768	
	100	1.7857 ±99.1071 a		

Description : a) The average value (corrected) ± SD (standard deviation). Mean followed by the same letter are not significantly different by Duncan's multiple test (α = 0,05). JPS = Hours After Treatment. b) Frank Lu toxicity criteria

Toxicity test to *R. norvegicus* (Tabel 4), shows that extract of plant latex of *A. toxicaria* extracted from heating and methanol extraction process has LD50 value 1.267 or 18.493 mg. This conform that theses material has high toxicity impact or very poisonous material to *R. norvegicus*. Result of this experiment shows that the extract of latex of *A. toxicaria* has potentiality as Bio-rodenticides to control rodent.

IV. Conclusion

Validation docking using Autodock Tools 1.5.6 Autodock Vina shows there are no different of docking result, this mean that both docking methods can used. Compared to the other nine conformation, the first confirmation in receptors 2ZKD shows highest free energy that can be released was -9.12 kcal/mol and lowest inhibition constanta (Ki) 0.21 µM. Amino acid residue in the interaction between Toxicarioside k and receptor 2ZKD, 2ZKF, 2ZKG, 2ZKO. 3F8I, 3F8J resulting six amino acid, namely aspartate acid, arginine, alanine, glysine, tyrosine and valine.

Probit analysis shows that the latex of *A. toxicaria* which are extracted from heating process has probit value =0.754x - 0.354 and LD50= 1.017 or 10,339 mg while the methanol extract has probit value =0.799x -

0.642 and LD50= 1.267 or 18.493 mg. Result of the toxicity analysis shows that increase of extract concentration has positive correlation with the high mortality of *R. norvegicus*. These extract was toxic influence and therefore has potentiality to use as bio-rodenticides.

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