TOXIC ACTIVITIES OF HEXANE EXTRACT AND COLUMN CHROMATOGRAPHY FRACTIONS OF RODENT TUBER PLANT (*Typhonium flagelliforme* Lodd.) ON *Artemia salina*

*Aktivitas Toksik Ekstrak Heksana dan Fraksi Kromatografi Kolom dari Tanaman Keladi Tikus (*Typhonium flagelliforme *Lodd.) pada* Artemia salina

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ABSTRACT

Rodent tuber (*Typhonium flagelliforme* Lodd.) is a medicinal plant particularly found in Java. The plant is used as an ingredient for conventional cancer treatment. The aim of this study was to determine the toxic activities of crude extracts and column chromatography fractions of rodent tuber on *Artemia salina* larvae. Rodent tuber plant was obtained from the Indonesian Spice and Medicinal Crops Research Institute in Bogor, West Java. The experiment was conducted in the Biology Laboratory of Universitas Pelita Harapan, Tangerang, Banten. Leaves and petioles of the plant were macerated with acetone and the filtrates were evaporated (40°C) to obtain crude extracts. The crude extracts were partitioned with ethyl acetate, followed with hexane, chloroform and butanol. Toxicity test of the extracts was performed using the Brine Shrimp Lethality Test (BSLT) method on *A. salina* larvae. Extract showing the most toxic was fractioned using column chromatography and then tested on the larvae. The experiment was designed in a completely randomized factorial, four replicates for crude extracts and two replicates for the fractions. Treatments were different types of extracts (hexane, chloroform and butanol) at various concentrations (500, 1,000 and 1,500 µg ml^{-1} of 5% Tween solution). Fractions of the column chromatography used were taken from the column number 1, 3 and 10, and tested their toxicities at 200, 400, 600, 800 and 1,000 µg ml^{-1} of 5% Tween solution. Parameters observed were the death of *A. salina* expressed as LC_{50}. The study showed that hexane extract of the petioles had the most toxic to *A. salina* (LC_{50} = 762.08 µg ml^{-1}). Fraction number 10 showed the highest toxic (LC_{50} = 381.07 µg ml^{-1}), whereas the lowest was fraction number 3 (LC_{50} = 653.13 µg ml^{-1}). The study indicates that rodent tuber plant from Bogor is toxic to *A. salina* and further test for its cytotoxic activity is justified.

[***Keywords***: *Typhonium flagelliforme*, hexane extract, column chromatography fraction, toxicity, *Artemia salina*]

ABSTRAK

*Keladi tikus (*Typhonium flagelliforme *Lodd.) merupakan tanaman obat yang khusus ditemukan di Pulau Jawa. Tanaman ini digunakan sebagai bahan untuk pengobatan kanker. Penelitian ini bertujuan untuk mengetahui aktivitas sitotoksik dari ekstrak kasar dan fraksi kromatografi kolom tanaman keladi tikus pada larva* Artemia salina*. Tanaman keladi tikus diperoleh dari Balai Penelitian Tanaman Rempah dan Obat (Balittro), Bogor, Jawa Barat. Penelitian dilakukan di Laboratorium Biologi Universitas Pelita Harapan, Tangerang, Banten. Daun dan tangkai daun tanaman dimaserasi dengan aseton dan filtratnya diuapkan (40°C) untuk memperoleh ekstrak kasar. Ekstrak kasar dipartisi dengan etil asetat, kemudian dilanjutkan dengan heksana, kloroform, dan butanol. Pengujian toksisitas ekstrak dilakukan menggunakan metode* Brine Shrimp Lethality Test *(BSLT) pada larva* A. salina*. Ekstrak yang menunjukkan paling toksik difraksinasi dengan menggunakan kromatografi kolom dan kemudian diuji pada larva. Penelitian dirancang dengan rancangan acak lengkap berfaktor, empat ulangan untuk ekstrak kasar dan dua ulangan untuk fraksi. Perlakuan meliputi jenis ekstrak (heksana, kloroform, dan butanol) dengan beberapa konsentrasi (500, 1.000, dan 1.500 µg ml^{-1} dengan larutan Tween 5%). Fraksi kromatografi kolom yang digunakan adalah dari nomor kolom 1, 3, dan 10; dan diujikan toksisitasnya pada 200, 400, 600, 800, dan 1.000 µg ml^{-1} dengan larutan Tween 5%. Parameter yang diamati adalah kematian* A. salina *yang diekspresikan pada LC_{50}. Penelitian menunjukkan bahwa ekstrak heksana dari tangkai daun mempunyai aktivitas toksik paling tinggi terhadap* A. salina *(LC_{50} = 762.08 µg ml^{-1}). Fraksi nomor 10 menunjukkan aktivitas toksik yang paling tinggi (LC_{50} = 381.07 µg ml^{-1}), dan aktivitas toksik paling rendah adalah fraksi nomor 3 (LC_{50} 653.13 µg ml^{-1}). Penelitian ini menunjukkan tanaman keladi tikus asal Bogor bersifat toksik terhadap* A. salina *dan uji lanjut terhadap aktivitas sitotoksik telah dilakukan.*

[***Kata kunci****:* Typhonium flagelliforme, *ekstrak heksana, fraksi kromatografi kolom, toksisitas,* Artemia salina]

INTRODUCTION

Indonesia is a country that rich in its biodiversity, including medicinal plants. Many plants have potential as sources of bioactive compounds for herbal medicines. One of them is rodent tuber plant (*Typhonium flagelliforme*). In Indonesia, the plant is mostly found in Java at the altitude of 1-300 m above the sea level (Essai 1995). The plant has triangular leaves, white and round tubers (Syahid 2008). Rodent tuber plant can grow up to 30 cm height and can only grow in the bushes that are not exposed to direct sunlight (Mohan *et al*. 2008). The plant is also found in India, Australia, eastern Asia and the forests of Malaysia, especially the eastern and northern Malaysia (Sai *et al*. 2000).

Medicinal use of rodent tuber plant as an ingredient for herbal cancer treatment has been suggested by Choo *et al*. (2001a). Rodent tuber has a cytotoxic activity against cancer cell growth (Mohan *et al*. 2008). *In vitro* studies of non-polar extracts of rodent tuber showed a significant cytotoxic activity against murine lymphoid (Neoh 1992), P388 murine leukaemia (Choo *et al*. 2001a), NCI-H23 human lung carcinoma, and T-47D human breast carcinoma cell lines (Chan *et al*. 2005).

Some chemical compounds of *T. flagelliforme* have been identified as anticancer. Hexane extracts containing saturated hydrocarbons and aliphatic acids (Choo *et al*. 2001b) and ethyl-acetate extracts containing flavonoid named isovitexin (Farida *et al*. 2012) are reported as anticancer. Huang *et al*. (2004) found glycosides, sterols and cerebrosides in the rodent tuber roots which have anti-hepatotoxic activity.

The purpose of this study was to determine the toxic activities of crude extracts and column chromatography fractions of rodent tuber plant on *Artemia salina*.

MATERIALS AND METHODS

Extraction

Leaves and petioles of rodent tuber plant were obtained from the Indonesian Spice and Medicinal Crops Research Institute at Bogor, West Java. These plant samples were cut, weighed and put in a separate glass beaker. Both are macerated with acetone and stand for 24 hours. Two replicates were prepared. The samples were then filtered using a filter paper (Whatman paper No. 1) and the filtrates were evaporated with a rotary evaporator at 40°C to obtain crude plant extracts.

Partition

The crude extracts 3,015 ml of leaves and 2,225 ml of petioles were placed in the separatory funnels and added with ethyl-acetate (ethyl-acetate : crude extract = 1:2). The mixtures were shaken repeatedly and allowed to stand until two phases were formed; the bottom part was water and the upper one was ethyl-acetate phase. The ethyl-acetate phase was extracted and evaporated in a rotary evaporator at 40°C to dryness. The ethyl-acetate phase was then partitioned using hexane as non-polar solvent, chloroform as semi-polar solvent, and butanol as polar solvent. The hexane, chloroform and butanol phases were evaporated as mentioned above, transferred into a sample vial and dried in a desicator. Each phase was tested for its toxicity to *A. salina*.

Toxicity Test

Toxicity test was conducted using Brine Shrimp Lethality Test (BSLT) on *A. salina*. As much as 100 mg of *A. salina* eggs were placed in a hatch chamber containing 500 ml of sterile sea water under constant aeration for 24-48 hours until hatched. *A. salina* larvae of second stage were added into test tubes (20 larvae per tube) containing 4 ml of sterile sea water.

Samples of the crude plant extracts to be tested were dissolved in 0.5% Tween 80 solution at different concentrations, i.e. 500, 1,000 and 1,500 µg ml^{-1}, whereas the concentrations of the column chromatography fractions were 200, 400, 600, 800 and 1,000 µg ml^{-1}. Each concentration was inserted into a test tube containing active *A. salina* larvae and added with sterile sea water up to 5 ml. Each test was repeated four times, except for column chromatography fraction which were twice. Control treatments were tubes with *A. salina* larvae in sterile saline water alone (no extract or Tween 80) and *A. salina* with 0.5% Tween 80. All the prepared test tubes were incubated for 24 hours at room temperature. Number of dead larvae was counted every day. Larvae were declared dead if no movement of larvae for 10 seconds of observation (Krishnakumar *et al*. 2007) or disoriented motion (irregular motion) but spinning at one point (Nurhayati *et al*. 2006).

Thin Layer Chromatography (TLC)

Extracts of the rodent tuber plant samples showing the highest toxic activity were spotted on TLC plate

and put in the chamber that already saturated with a mixture solvent of ethyl-acetate and hexane at the ratio of 1:1, 1:1.5, 1:2 and 1:3. Solvent ratio with spot that has been well-separated was then analyzed in the column chromatography.

Column Chromatography

Cotton ball and slurry of silica gel 60 (silica gel mixed with hexane solvent and allowed to stand overnight) as much as 40 are inserted into the column, stirred frequently so there was no air cavity trapped in the middle of the column, then saturated for 30 minutes. Hexane extracts of the rodent tuber plant were then inserted in the upper limit of the silica gel and the hexane-ethyl-acetate solvents (100 µl) at ratio of 1:4, 1:3, 1:2 and 1:1 were continuously introduced simultaneously with the opening of the tap column, followed with ethyl-acetate-methanol solvent at the ratio of 8:2. Five ml of each separated fractions were collected in a sample bottle till the whole extracts separated. Fractions were analyzed on the TLC with a solvent ratio that showed good separation as determined before and Rf value of each fraction was recorded. Fractions that have the same spot were mixed and used for toxicity test on *A. salina* as described above.

Statistical Analysis

Data on mortality were analyzed with a probit analysis to calculate lethal concentration dose (LC_{50}). Statistical significance of the data was analyzed using ANOVA and the means were compared using Tukey's pairwise comparisons at P = 0.05.

RESULTS AND DISCUSSION

Amount of crude extracts obtained from the leaves and petioles of the rodent tuber varied depend on different solvents used. The extract contained higher non-polar components because more extracts produced using hexane (Table 1). The non-polar compounds might include fatty acids, lipids, waxes, sterols, some alkaloids and terpenoids as mentioned by Otsuka (2006) and Seidel (2006). However, the plant extract also contained semi-polar and polar compounds as extracted with chloroform and butanol solvents.

The study was in agreement with previous work of Lai *et al.* (2008) which produced higher extract of rodent tuber plant using hexane. High extraction from the study may be attributed to the technical preparation of the plant. In our study, leaves and petioles of the tuber plant samples were only air-dried to avoid decomposition of any thermolabile compounds. In addition, the samples were used as soon as possible to avoid the risk of artifact formation and decomposition or isomerization of extract constituents (Jones and Kinghorn 2006). However, several researchers used dried samples of rodent tuber plant in their experiments. Mohan *et al*. (2011) used dry leaves and tubers of rodent tuber without any defect reported. Nurrochmad *et al*. (2011) used oven dried (60°C) samples and macerated with 96% ethanol for five days. Putra *et al*. (2012) dried the sample at 45°C for 48 hours.

Table 1. The weight of rodent tuber plant extracts from different solvents.

Phase	Weight of extracted plant samples (g)	
	Leaf	Petiole
Hexane	7.56	6.25
Chloroform	3.54	2.78
Butanol	2.67	2.43

Crude Extract Toxicity

Percentage of mortality and LC_{50} values of tested rodent tuber extracts were presented on Tables 2-4. Extract having the highest toxic to the *A. salina* larvae was then extracted in hexane. At highest concentration (1,500 µg ml^{-1}) hexane leaf extract caused 87.50% mortality and hexane petiole extract caused 92.50% mortality (Tables 2 and 3). Butanol extract was also toxic to *A. salina,* whereas chloroform extract was not toxic because at the highest concentration (1,500 µg ml^{-1}), mortality percentage did not reach 50%. Meyer (1982) and Anderson *et al.* (1991) stated that an extract showed a cytotoxic activity in BSLT if it can cause the death of 50% of test animals (LC_{50} value) at concentrations of <1,000 mg ml^{-1} (Nurhayati *et al.* 2006). Present study indicated that leaf and petiole of the tuber plant contain toxic substances to *A. salina* and may be used for medical purposes.

When viewed from the LC_{50} value, the extracts which had the highest toxic properties were rodent tuber's petiole extract of hexane phase with LC_{50} value of 762.08 µg ml^{-1}, while the extract which had the lowest toxic properties was rodent tuber's leaves of butanol phase with LC_{50} values of 933.25 µg ml^{-1}

Table 2. Toxicity of leaf crude extracts of rodent tuber on *Artemia salina* larvae.

Plant extract	Mortality (%)				
	CWOT[1)]	CWT[2)]	500	1,000	1,500
			.....(µg ml^{-1}).....		
Hexane phase	0.00a	0.00a	17.50b	52.50c	87.50e
Chloroform phase	0.00a	0.00a	0.00a	6.25ab	22.50c
Butanol phase	0.00a	0.00a	20.00b	46.25d	80.00e

Numbers in the same column followed with the same letter are not significantly different at $\alpha = 0.05$.
[1)]CWOT = control without 0.5% Tween 80
[2)]CWT = control with 0.5% Tween 80

Table 3. Toxicity of petiole crude extracts of rodent tuber on *Artemia salina* larvae.

Plant extract	Mortality (%)				
	CWOT[1)]	CWT[2)]	500	1,000	1,500
			.....(µg ml^{-1}).....		
Hexane phase	0.00a	0.00a	26.25b	56.25c	92.50d
Chloroform phase	0.00a	0.00a	1.25a	7.50b	28.75c
Butanol phase	0.00a	0.00a	26.25b	53.75c	82.50e

Numbers in the same column followed with the same letter are not significantly different at $\alpha = 0.05$.
[1)]CWOT = control without 0.5% Tween 80
[2)]CWT = control with 0.5% Tween 80

Table 4. LC_{50} values of crude extracts of leaves and petioles of rodent tuber on *Artemina saliva* larvae.

Extract sample	LC_{50} value (µg ml^{-1})	
	Leaf	Petiole
Hexane phase	857.04	762.08
Chloroform phase	Non-active	Non-active
Butanol phase	933.25	827.94

(Table 4). The extract is said to be toxic because it had LC_{50} values <1,000 mg/ml. If the LC_{50} value of an extract sample is smaller, it has the higher toxicity. This suggests that the most active compounds based on the BSLT method is the petioles of rodent tuber in hexane phase, which will then be followed by column chromatography.

Column Chromatography

TLC of hexane extract of rodent tuber petiole revealed 10 fractions having different values of Rf (Table 5). However, only fractions 1, 3 and 10 were enough to be tested on *A. salina*, and the remaining fractions were not sufficient. TLC plate material used is silica gel which has a polar property. Sample components

Table 5. Column chromatography fractions and Rf values of the hexane petiole extract of rodent tuber.

Fraction		Spot color		Rf value
		Without UV	UV	
1	A	Yellow	Brown	0.96
	B	White	Yellow	0.86
2		Dark green	Green	0.82
3	A	Dark green	Dark green	0.82
	B	Dark green	Yellow	0.65
4	A	Dark green	Yellow	0.82
	B	Green yellowish	Yellow	0.69
5	A	White	Yellow	0.82
	B	Green brownish	Yellow brownish	0.65
	C	Yellow greenish	Yellow	0.51
6	A	White	Yellow	0.80
	B	Green brownish	Green brownish	0.67
	C	Soft green	Yellow brownish	0.59
	D	Yellow greenish	Yellow	0.49
7	A	White	Yellow	0.80
	B	Soft green	Yellow	0.69
	C	Soft green	Yellow brownish	0.56
	D	Green yellowish	Green	0.23
8		Yellow	Green	0.71
9	A	Soft green	Orange	0.96
	B	Yellow	Yellow brownish	0.88
10		Soft green	Black	0.08

will migrate along with the eluent that migrate through the TLC plate, but at different levels so that there is separation. More polar compounds will interact very strongly on its adsorbent and more likely to remain on its adsorbent, while less polar compounds do not have a strong interaction with its adsorbent. Less polar compounds will quickly migrate to the top of TLC plate compared to the more polar compounds. It causes more polar compounds to have more lower Rf value (Wall 2005).

The use of column chromatography in the present study is beneficial because it is easy and commonly used in most of laboratories. The method was also used by Reid and Sarker (2006) for isolation of natural products. However, Mohan *et al*. (2011) used vacuum liquid chromatography in their study.

Toxicity Test on Fractions of Rodent Tuber

Fraction 1, 3 and 10 from the chromatography column were followed by a toxicity test against *A. salina*. The number of death larvae obtained was from the toxicity test and then calculated the percentage of mortality and its LC_{50} value by probit analysis. The results of toxicity test and LC_{50} value of rodent tuber fractions can be seen in Table 6. Based on Table 6, fraction that

Table 6. Toxicity of hexane petiole rodent tuber fractions on *Artemia salina* larvae.

Sample	Mortality (%)						
	CWOT[1)]	CWT[2)]	200	400	600	800	1,000
			.....(µg ml^{-1}).....				
Fraction 1	0.00a	0.00a	7.50ab	22.50c	40.00d	57.50f	87.50g
Fraction 3	0.00a	0.00a	10.00ab	22.50bc	35.00de	60.00f	77.50h
Fraction 10	0.00a	0.00a	17.50c	40.00d	70.00f	92.50i	97.50i

Numbers in the same column followed with the same letter are not significantly different at $\alpha = 0.05$.
[1)]CWOT = control without 0.5% Tween 80.
[2)]CWT = control with 0.5% Tween 80.

had the most toxic was fraction 10 with the mortality value of 97.50% at concentration of 1,000 µg ml^{-1}.

Toxicity assay showed that fraction 10 was the most toxic on *A. salina* as shown from its LC_{50} value (381.07 µg ml^{1}) followed with fraction 1 (LC_{50} = 599.79 µg ml^{-1}) and fraction 3 (LC_{50} = 653.13 µg ml^{-1}). The extract is said to be toxic because it had LC_{50} value of <1,000 µg ml^{-1}. If the LC_{50} value of an extract sample is smaller, it has the higher toxicity. From the results that LC_{50} value of 381.07 ug ml^{-1} was toxic. The obtained compound from the extracts of rodent tuber has the potential to be developed and studied further.

Based on the chromatography results, active compounds in the crude extracts and fractions of rodent tuber were semi-polar and non-polar compounds such alkaloids, saponins, flavonoids, glycosides and terpenoids (Seidel 2006; Syahid 2008). The presence of these compounds in all plant parts of the rodent tuber used, irrespective of the solvent used, indicates that this plant is a valuable medicinal plant. Previous study showed that rodent tuber plant is potential antibiotic (Nathiya and Dorcus 2012), antioxidant (Akinmoladun *et al.* 2010) and anti-cancer (Lai *et al*. 2008). Recent study by Mohan *et al*. (2011) showed that rodent tuber plant induced apoptosis on human T4 lymphoblastoid (CEMss). Nurrochmad *et al*. (2011) also found anti-cancer activity of the plant on human breast cancer t47d cells. Tuber extract of the plant also inhibited proliferation of MCF-7 human breast cancer cell-line (Putra *et al*. 2012). Therefore, rodent tuber plant is one the most promising medicinal plant to be developed. Mass propagation of the plant is urgent. Use of *in vitro* culture to propagate the plant has been initiated.

CONCLUSION

Rodent tuber plant, especially the hexane phase of the petiole extracts of the plant was highly toxic to *A. salina* (LC_{50} = 762.08 µg ml^{-1}). The fraction 10 of its chromatography was the most toxic (LC_{50} = 381.0 µg ml^{-1}). The study indicates that rodent tuber plant from Bogor is justified for further test for its cytotoxic activity.

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