RJPT_MANUSCRIPT
DIREVIEW

Acute toxicity Level of Pulutan (*Urena lobata*) Leaf Extract on Zebrafish (*Danio rerio*) and its analysis by *in silico* study

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Abstract

Background: Pulutan (*Urena lobata*) is medicinal plant used to treat some diseases empirically and pre-clinical studies have already established its efficacy. However, its safety has not been evaluated moreover an acute toxicity test has to be performed on different phases of organism life to screen for its safety. The study aims to determine the acute toxicity level of *Urena lobata* (*U. lobata*) leaf extract on embryo phase, juvenile and adult of zebrafish (*Danio rerio*) therefore analyse it by *in silico* methods.

Methods: This is an experimental laboratory study using embryo, juvenile and adult of zebrafish (*Danio rerio*). The leaf of *U. lobata* was extracted by decoction methods and the extract was diluted from 12000 mg/L to 500 mg/L. The animals were exposed to the extracts for 96 hours. Toxicity level of herbs is defined using lethal concentration-50 (LC-50) obtained through linear

regression. *In silico* study was performed using a web-based software application (iLAB ACD) after identification of active compound in *U.lobata* leaf extract.

Results: The LC-50 values of *U. lobata* leaf extract for embryo, juvenile and adult of zebrafish (*Danio rerio*) were 2548 mg/L, 8748 mg/L and 8088 mg/L, respectively. Stigmasterol and β-sitosterol in *U.lobata* showed a high toxicity based on LD-50 value by *in silico* study. Toxicity analysis by the method indicated borderline to moderate reliability level.

Conclusion: Level toxicity of *U. lobata* on zebrafish embryo was moderate (0.5-5 g/L) and it shows teratogenic effect. However, its effects on juvenile and adult fish were considered mild (5-15 g/L). Stigmasterol and β-sitosterol in *U.lobata* are predicted have a toxic effect.

Key words: *acute, , Danio rerio, in silico, toxicity, Urena lobata*

Introduction

Pulutan (*Urena lobata*) is a medicinal plant commonly found in Indonesia. It has been used to cure many diseases, such as cough, malaria, wound, and diabetes empirically [1,2]. Pre-clinical studies of *U. lobata* confirmed that it has anti-diabetic property by inhibiting Dipeptidyl peptidase-4 (DPP-4), broad-spectrum antibacterial and anti-anxiolytic effects.[3,4,5]. Safety of *Urena lobata* must be ensured before using it as a medication. Previous studies showed that administration of *U. lobata* at 3000 mg/kg bw for 28 days did not produce toxicity and death of the rat. However, this herb increased hepatic enzyme and disrupted the structure of hepatocyte and sperm [6,7]. Meanwhile, the long-term exposure of this herb to the rabbits showed that it could destroy of hepatocyte and obstruct the bile duct.[8]

The toxicity of *U.lobata* should be examined and its lethal dose-50 (LD-50) or lethal concentration-50 (LC-50) should be determined as a measure of safety for the herbs [9]. Despite

the data on adult animals, its toxicity should be evaluated in the embryo and juvenile animals to determine the safety *U. lobata* across different life stages. Embryonic evaluation ensures the safety of the herbs on the fetus and pregnancy period while juvenile assessment ensures its safety in the period of rapid growth and development of an organism.[10] Generally, organisms at both stages are more sensitive to xenobiotic agent, including herbs, compared to the adult phase.

The use of zebrafish (*Danio rerio*) as an animal model of toxicity test offers many advantages because they are sensitive to poison, easy to breed and the embryo is transparent therefore, it is easy to observe the internal organs.[11] Almost 70 % of the genes in human is found on *D.rerio,* which implies that human diseases and it can be replicated using this animal.[12] The study aims to evaluate acute toxicity level of *U. lobata* leaf extract on embryo phase, juvenile, and adult zebrafish (*Danio rerio*) and also analyse it by *in silico* study

Methods

2.1 Chemical Sample

Embrionic solution (Magnesium Sulfate, Sodium Chloride, Potasium Chloride, Calcium Chloride.2H_2O), Aquadest, Methanol and Methylen Blue (purchased from Sigma aldrich and E. Merck) Tetramin (Tropical) Artemia (...).

2.2 Sample prepration

U. lobata leaf powder was obtained from Materia Medika, Batu, Malang, Indonesia with certificate number 074/306/101.8/2016. Approximately 50 g of the powdered plant materials were extracted in 250 ml hot water 90ºC for 30 minutes using decoction methods. The extract was diluted for the identification of active compounds and toxicity test.

2.3 Identification of active compounds

Water extract of *U. lobata* was analyzed on a semi-qualitative scale by Liquid Chromatography-Mass Spectra (LC-MS) Accela 1250 pump. Liquid phase contains 0.1 % formic acid in methanol and water. The identification included the 10 active substances from alkaloid (mangiferin), phytosterol (stigmasterol, beta-sitosterol) and flavonoid (luteolin, quercetin, kaempferol, gossypetin, apigenin, chrysoeriol, hypocretin) groups.

2.4 Toxicity Analysis

Active compounds in *U. lobata* leaf extracts were evaluated its toxicity test based on LD-50 value using *in silico* study with a web-based software application (iLAB ACD). It gives a predictive value to determine the dose for *in vivo* study and to confirm its results.

2.5 Acute toxicity test

The assay was based on OECD [13,14] with slight modifications. It was performed in 24 micro well plates for the embryo of *D. rerio*, while the assessment of both juvenile and adult was performed in an aquarium. The treatment was performed in three replicates and each replicate consist of 10 embryos or fish. The *U. lobata* leaf extract was given for 96 hours and the extract was replaced every 24 hours. Death of embryo, juvenile and fish were calculated every 24 hours.[13]

2.6 Statistical Analysis

All data are expressed as the mean ± SD. The LC-50 was determined by linear regression curve fit using SPPS version 16.0.

Results

3.1 Identification of active compounds in *U. lobata* leaf extracts

The active compounds of *U. lobata* leaf extract, can be seen in the figure 1 and table 1. The semi-qualitative analysis by LC-MS showed that the most abundant active compounds in the

extract of *U. lobata* were stigmasterol and gossypetin. Active compounds presented in low concentrations, such as mangiferin, β-sitosterol, and chrysoeriol, were also identified also in the aqueous extracts of *U. lobate*.

3.2 Toxicity analysis of *U. lobata* leaf extracts

The toxicity of the active compounds from *U. lobata* leaf extracts were evaluated based on LD-50 values by *in silico* study and the results are depicted in Table 2. Stigmasterol and β-sitosterol had a low LD-50 values, indicating a high toxicity in rats and mice. Intraperitoneal (i.p) administration of these compounds produced LD-50 lower than per oral (p.o) in the rodents. Toxicity analysis of active compounds in *U. lobata* indicated borderline to moderate reliability level.

3.3 Acute toxicity level of *Urena lobata* leaf extract

Toxicity level of *U. lobata* leaf extract on embryo, juvenile and adult of zebra fish were showed in Table 3, 4 and 5. LC-50 values of *U. lobata* leaf extract were the lowest in embryo (2548.79 mg/L), indicating moderate toxic level, whereas on juvenile (8748.45 mg/L) and adult zebra fish (8088.11 mg/L), the toxicity level was mild.

Discussion

4.1 Identification of active compounds in *U. lobata* leaf extracts

Five active compounds were identified in the water extract of *U. lobata* leaf. They are stigmasterol, gossypetin, β-sitosterol, mangiferin, and chrysoberyl. All of them are secondary metabolite and have biological activity with medicinal values.[15,16] However, like any drugs, they induce adverse reaction and toxic effect in high dose and long-term administration. They may also induce drug-drug interaction.[17]

Stigmasterol and β-sitosterol are phytosterols that are insoluble in water but soluble in most organic solvents and contain one alcohol functional group. Stigmasterol is an unsaturated plant sterol occurring in the plant fats or oils of soybean, calabar bean, rapeseed, and in various medicinal herbs. Studies on laboratory animals treated by stigmasterol found that both cholesterol and sitosterol absorption decreased by 23% and 30%, respectively, over a 6-week period. It also possesses a potential antioxidant, hypoglycemic and anti-thyroid properties.[18,19] β-sitosterol are white, waxy powder with the specific characteristic of odor. They are hydrophobic and soluble in ethanol and chloroform but insoluble in water.[20] It can be found in avocados, cucurbita pepo, corn oil, and soybeans. Studies showed that it possessed anti-cholesterol, anti-inflammatory and immunomodulatory effects.[21]

However, other studies showed that oxidized products of stigmasterol and β-sitosterol increased apoptotic nuclei on hepatoma cells culture and inhibit the growth of cells through cytotoxic effect.[22] No evidence of mutagenicity or genotoxicity of phytosterol was observed. However, toxicity studies on them were limited to 90-day subchronic toxicity studies and a 2-generation reproductive toxicity study in rats.[23] In another 90-days study reported by Kim et al., suppression of body weight gain in rodents of both sexes and infiltration of mononuclear cell in the heart in males at a dose level of 9 g phytosterol esters/kg bw/day were observed.[24] The NOAEL derived from the 90-day subchronic toxicity studies in rats and the 2-generation reproductive toxicity study in rats amounted to be 2.5-6.6 g phytosterol ester/kg bw/day, 1.54-4.1 g phytosterol/kg bw/day and 335-900 mg stigmasterol/kg bw/day.[23]

Gossypetin and chrysoeriol are flavonol or flavone isolated originally from the flowers and the calyx of hibiscus species. Gossypetin shows a high antioxidant, anti-microbial, anti-mutagenic and anti-atherosclerotic effects.[25] This compound is very soluble in chloroform and benzene,

and also moderately soluble in ethanol and ether but insoluble in water. Meanwhile, chrysoeriol is a flavon that provides many health-promoting benefits such as anti-inflammation, anti-cancer, and anti-histamine. It is soluble in alkalies solution and sufficiently soluble in water.[26] Flavonoid has potential toxic effects such as pro-oxidant activity, mitochondrial toxicity, and interaction with drug-metabolizing enzymes. Flavonoid can change into radicals after scavenging free radical, thus increasing stress oxidative and disrupting mitochondria.[27] Interaction of flavonoid with other active compounds or drugs can alter metabolizing enzyme expression and modulate their activity.

Mangiferin is a xanthonoid, and a glucoside of norathyriol. It was found in Mangoes, *Iris unguicularis* and *Anemarrhena asphedelous*. Mangiferin is soluble in hot dilutes ethanol and methanol but insoluble in water. The laboratory study has identified a variety of pharmacology effects of mangiferin, including anti-microbial, antioxidant and anti-diabetic activity effect in rodents.[28,29] In acute toxicity study, no effects was observed after dermal exposure to mangiferin 2000 mg/kg. However, transient dyspnea, flank position and piloerection were observed after oral administration of this xanthone. Intraperitoneal administration in mice induced similar toxicity signs with possible mortality in rodents. Rats orally treated with mangiferin (250-1000 mg/kg) for 28 days did not show any abnormal clinical signs or hematology alteration, when compared to control group. Histopathological alterations like vacuolar degeneration, necrosis, and increment of apoptosis of the acinar cells were observed in the exocrine pancreas of rats at 1000 mg/kg. This suggested that exocrine pancreas was the target organ for mangiferin toxicity.[30]

The composition of active compounds in the extract was influenced by polarity and the choice of extract solvent. The polarity of the extract solvent determines the composition of an active

compound by influencing their solubility in the solvent. The alkaloids, terpenoids, and steroids are soluble in the non-polar solvent like acetone, diethyl ether and hexane. Meanwhile, flavonoids, phenols, and glycosides dissolve better in a polar solvent, such as water and methanol.[31,32] Non-nutrition compounds or secondary metabolites like alkaloid, terpenoid, flavonoid, and steroid are in smaller quantity and they have pharmacologic effects given in appropriate doses.[33] Secondary metabolites are derived from the metabolism of the primary metabolites in plants but sometimes they have a toxic effect especially when used in high dose. Most of flavonoid and terpenoid in herbs have potency as antioxidant, antiseptic and anti-inflammatory whereas steroids can act as anti-inflammatory and sex hormone [34]

4.2 Toxicity analysis of *U. lobata* leaf extracts

Stigmasterol, β-sitosterol and mangiferin in *U. lobata* leaf extract were predicted as a toxic substances trhough *in silico* study. Plant sterols, such as stigmasterol and β-sitosterol, have cytotoxic effect [22,23] while mangiferin is toxic to the exocrine pancreas of the rats as explained above.[30] They contribute to the overall the toxic effects of *U. lobata* in animal testings.

Intraperitoneal administration of active compounds in *U. lobata* resulted in LD-50 value lower than oral administration. The oral administration will subject the substances to biotransformation process at the liver, such as oxidation, reduction, hydrolysis and conjugation, which may reduce its toxicity.[17] Detoxification of active substances occurs at metabolism phase by microsomal hepatic.[35] The first-pass metabolism occurs more extensively for substances administered orally than intraperitoneally. Besides, bioavailability of the substances is higher with intraperitoneal administration than oral route because they do not enter gastrointestinal organ. Therefore, the damage by digestive and biotransformation enzymes is avoided. The bioactivity of

herbs in the organism is retained while the accumulation and toxicity are increased.[36] Biotransformation is needed to decrease the toxicity of xenobiotics such as drugs, herbs, and chemical substances. However, the biotransformation process produces a more active metabolite or the same activity with parent drug, therefore it modulate its activity.[17]

The LD-50 of *U. lobata* is higher in mice than in the rats~~,~~ It is postulated that the organ capacity to eliminate xenobiotic is limited in mice compared to the rats; thus, the accumulation of active metabolite and toxicity risk are increased.[5,7] Elimination processes, including metabolism and excretion, aims to decrease the bioactivity of active substances.[17] The liver is a major organ of metabolisms, while, kidney, gastrointestinal lumen, blood, and lung eliminate the substances.[35]

4.3 Acute toxicity level of *Urena lobata*

The toxicity level of *U. lobata* leaf extract on embryo is higher compared to juvenile and adult of zebra fish. The embryo is more sensitive to the active compounds of *U. lobata* leaf extract due to the lack of metabolism enzyme and the immaturity of metabolism/excretion organ system; thus increasing the toxicity risk.[17] There is relatively lower level of plasma protein in the embryo, and this caused a higher level of the free drugs circulating in the blood, leading to the increased toxicity risk. Detoxifying enzymes, like glucuronidase, is also limited in an embryo; thus, the active compounds are less inactivated by the metabolic processes.[35] This results in an increased bioactivity and toxicity risk. With regards to the excretion process, glomerulus filtration rate is lower in embryo compared to the adult organism due to the under-developed excretion organs. Hence, the performance of the organ systems to eliminate toxic metabolites is reduced.[17,36] ~~Whereas for the adult organisms, both metabolism and excretion organs have grown better compared to juvenile and embryo. This is proven from the level toxicity of *U. lobata*, which is LC-50 value on embryo lower than juvenile and adults~~. Therefore, the use of *U.*

lobata in pregnancy and infant should be considered carefully based on these safety issues. Dose adjustment of this herb should be considered to adjust for its bioactivity, adverse reaction, and toxicity among the vulnerable groups.

According to *in silico* study, alkaloids like mangiferin and phytosterols like stigmasterol and β-sitosterol in *U. lobata* leaf extract are predicted to be toxic. These substances have the potential to interact with each other, thereby increasing the toxicity of the extract. The interaction between the active compounds in this herb could also modulate their biology activity.[17,34] The compounded effects of the cytotoxic, pro-oxidant and damage to the pancreatic from the active compounds in *U. lobata* should be considered together in explaining its toxicity.[22,24,30]

Conclusion

Stigmasterol and beta sitosterol are predicted to be toxic in *U. lobata* leaf extract. The embryo is more sensitive to xenobiotic agents due to the lack of metabolic enzymes and immaturation of the metabolic/excretion organs. Therefore, *U. lobata* leaf extract could have teratogenic effect. Application of this extract among pregnant women and children should be cautioned.

What is already know on this topic

- The most abundant active compounds in the extract of *U. lobata* were stigmasterol and gossypetin.
- Stigmasterol and β-sitosterol had a low LD-50 value, indicating a high toxicity in both on rat and mouse.
- LC-50 value of *U. lobata* leaf extract was the lowest in embryo with moderate toxicity level while in juvenile and adult of zebra fish was mild.

What this study adds

- Stigmasterol and β-sitosterol were predicted as toxic substances in *U. lobata* leaf extract.
- The toxicity level of *U. lobata* leaf extract on embryo is higher compared to juvenile and adult of zebra fish

Competing interests

The authors declare no competing interest.

Authors' contributions

Yudi Purnomo as designed of the research, Noer Aini wrote the manuscript and data analysis, and Eko Noerhayati revised the manuscript and data analysis

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Tables and figures

Table 1. Active compounds in *U. lobata* leaf extracts

No	Active compounds	Molecule weight	Result
1	Stigmasterol	413	(+++)
2	B-Sitosterol	415	(+)
3	Mangiferin	423	(+)
4	Quercetine	303	(-)
5	Kaempferol	286	(-)
6	Hypolaetin	302	(-)
7	Gossypetin	318	(++)

8	Luteolin	286	(-)
9	Apigenin	270	(-)
10	Chrysoeriol	300	(+)
*(-): negative, (+): weak, (++): moderate, (+++): strong			

333

334 **Table 2.** Analysis of toxicity active compound in *U. lobata* leaf extracts

No	**Active compounds**	**LD-50 (mg/kg) mouse (i.p)**	**LD-50 (mg/kg) mouse (p.o)**	**LD-50 (mg/kg) rat (i.p)**	**LD-50 (mg/kg) rat (p.o)**
1	Stigmasterol	160**	530*	170**	1400**
2	β-Sitosterol	110**	570*	140*	740*
3	Mangiferin	460*	1500**	160**	1900
4	Gossypetin	490*	550**	710	600*
5	Chrysoeriol	290	1100*	700**	1300**
**( ): not reliable, (*): borderline, (**): moderate					

335

336

Table 3. Acute toxicity level of *U. lobata* leaf extract on embryo *D. rerio*

No	Concentration (mg/L)	n	Number of death (%)
1	1000	3	0.00 ± 0.00
2	1500	3	3.33 ± 0.58
3	2500	3	50.00 ± 0.58
4	3000	3	70.00 ± 1.15
5	4000 LC-50 Level Toxicity	3 = =	100.00 ± 0.58 2548.79 mg/L Moderate

Table 4. Acute toxicity level of *U. lobata* leaf extract on juvenile *D. rerio*

No	Concentration (mg/L)	n	Number of death (%)
1	2000	3	0.00 ± 0.00
2	6000	3	10.00 ± 0.00
3	8000	3	30.00 ± 0.00
4	10000	3	70.00 ± 0.00
5	12000 LC-50 Level Toxicity	3 = =	83.33 ± 5.77 8748.45 mg/L Mild

Table 5. Acute toxicity level of *U. lobata* leaf extract on adult *D. rerio*

No	Concentration (mg/L)	n	Number of death (%)
1	500	3	0.00 ± 0.00
2	2000	3	0.00 ± 0.00
3	6000	3	40.00 ± 0.00
4	8000	3	43.33 ± 0.58
5	12000 LC-50 Level Toxicity	3 = =	80.00 ± 0.00 8088.11 mg/L Mild

342

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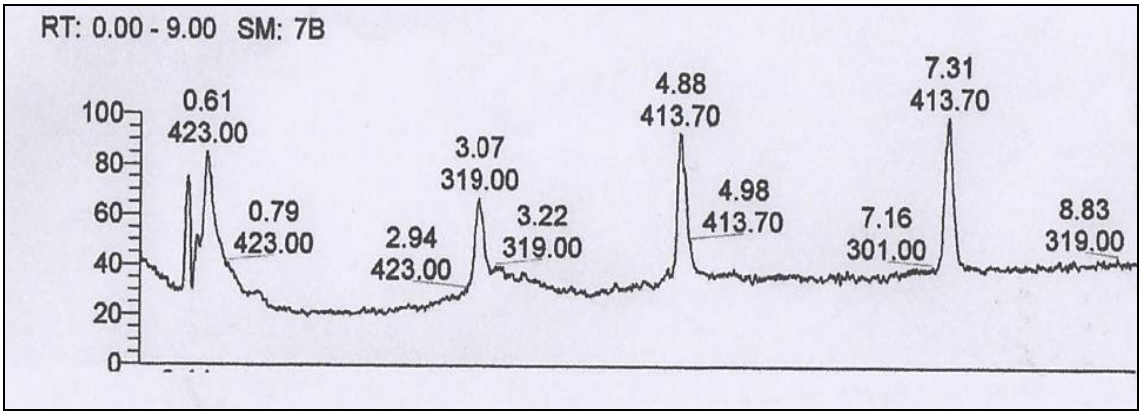

344

345 **Figure 1.** Chromatogram of active compounds in *U. lobata* leaf extracts

346

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Original Article

Acute Toxicity Level of Pulutan (*Urena lobata*) Leaf Extract on Zebrafish (*Danio rerio*) and its Analysis by *In Silico* Study

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ABSTRACT:

Pulutan (*Urena lobata*) is one of medicinal plant used to treat some diseases traditionally and pre-clinical studies have showed its efficacy. However, the study about its safety has not been evaluated completely. An acute toxicity test has to be performed in order to screen for its safety. The objective of study to determine the acute toxicity level of *Urena lobata* (*U. lobata*) leaf extract on embryo phase, juvenile and adult of zebrafish (*Danio rerio*) and the role of its active constituents through *in silico* methods. This was an experimental laboratory study using embryo, juvenile and adult of zebrafish (*Danio rerio*). The leaf of *U. lobata* was extracted by decoction methods and the extract was diluted from 12000 mg/L to 500 mg/L. The animals were exposed to the extracts for 96 hours. Toxicity level of herbs was defined using lethal concentration-50 (LC-50) obtained through linear regression. *In silico* study was performed using a web-based software application (iLAB ACD). The LC-50 values of *U. lobata* leaf extract for embryo, juvenile and adult of zebrafish (*Danio rerio*) were 2548 mg/L, 8748 mg/L and 8088 mg/L, respectively. Acute toxicity level of *U. lobata* on embryo is higher compared to juvenile and adult of zebrafish. After identification of active compound and *in silico* study was performed, Stigmasterol and β-sitosterol in *U. lobata* showed high toxicity level based on LD-50 value. Level toxicity of *U. lobata* on zebrafish embryo was moderate (0.5-5.0 g/L) and it shows teratogenic effect, meanwhile, its effects on juvenile and adult fish were considered mild (5.0-15.0 g/L).

KEYWORDS: acute, *Danio rerio*, *in silico*, toxicity, *Urena lobata*

INTRODUCTION:

Pulutan (*Urena lobata*) is a medicinal plant commonly found in Indonesia. It has been used to treat many diseases, such as cough, malaria, wound, and diabetes empirically[1]. Pre-clinical studies of *U. lobata* confirmed that it has anti-diabetic properties by inhibiting alpha-amylase and alpha-glucosidase, broad-spectrum antibacterial and anti-anxiolytic effects[2,3,4]. The safety of *Urena lobata* must be ensured before using it as an alternative medicine. Previous studies showed that administration of *U. lobata* at 3000 mg/kg bw for 28 days did not produce toxicity and death in rats. However, this herb increased hepatic enzyme and disrupted the structure of hepatocyte and sperm[5,6]. Meanwhile, the long-term exposure of herbs to rabbits showed that it could destroy of hepatocytes and obstruct the bile duct[7].

The toxicity of *U. lobata* must be examined and the lethal dose-50 (LD-50) or lethal concentration-50 (LC-50) should be determined as a parameter of safety for the herbs[7,8]. Despite the data on adult animals, its toxicity should be evaluated in the embryo and juvenile animals to determine the safety *U. lobata* across different life stages[8]. Embryonic evaluation ensures the safety of the herbs on the fetus and pregnancy period, meanwhile juvenile assessment certain its safety in the period of rapid growth and development of an organism[8,9]. Generally, organisms at these stages are more sensitive to xenobiotic agent, including herbs, compared to the adult phase.

The use of zebrafish (*Danio rerio*) as an animal model of toxicity test offers many advantages because they are sensitive to poison and easy to breed. Since the embryo is transparent, it is easy to observe their internal organs[9]. Almost 70 % of the genes in human are found on *D. rerio*, implying that human diseases can be replicated using this animal[10]. The study aims to evaluate acute toxicity level of *U. lobata* leaf extract on embryo phase, juvenile, and adult of zebrafish (*Danio rerio*) and also analyse it by *in silico* approach.

MATERIAL AND METHODS:

Chemical Sample:

First, Embryonic solution containing magnesium sulfate, sodium chloride, potassium chloride, calcium chloride dihydrate is solved in distilled water. All of the chemicals are purchased from Sigma Aldrich and Merck. Others materials include Methylene Blue (Sakkai Pro), Tetramin (Tropical) Artemia (Golden west).

Sample Preparation:

U. lobata leaf powder was obtained from Materia Medika, Batu, Malang, Indonesia with certificate number 074/306/101.8/2016. The powdered plant materials were extracted using decoction methods with ratio herbs and solvent 1:5. The extract was diluted into several concentrations for toxicity test and for the identification of active compounds.

Identification of Active Constituen:

Water extract of *U. lobata* was analysed on a semi-qualitative scale using Liquid Chromatography-Mass Spectra (LC-MS/MS) Accela 1250 pump. The liquid phase contains 0.1 % formic acid in solvent combination (methanol and water). The identification included 10 active substances target from alkaloid (mangiferin), phytosterol (stigmasterol, beta-sitosterol) and flavonoid (luteolin, quercetin, kaempferol, gossypetin, apigenin, chrysoeriol, hypocretin) groups.

Toxicity Analysis:

Active compounds in *U. lobata* leaf extracts were evaluated its toxicity test based on LD-50 value using *in silico* study with a web-based software application (iLAB ACD). It gives a predictive value to determine the dose for *in vivo* study and to confirm its results.

Acute Toxicity Test:

The assay was based on OECD[11,12] with slight modifications. It was performed in 24 microwell plates for the embryo of *D. rerio*, while the assessment of both juvenile and adult was performed in an aquarium. The treatment was performed in three replicates and each replicate consist of 10 embryos or fish. The *U. lobata* leaf extract was given for 96 hours and the extract was replaced every 24 hours. Death of embryo, juvenile and fish was calculated every 24 hours.

Analytical Studies:

For acute toxicity test, the percentage of death are expressed as the mean ± SD and the LC-50 value was calculated using a linear regression curve using SPPS version 16.0.

RESULTS AND DISCUSSION:

Identification of Active Constituent in *U. lobata* Leaf Extracts:

The active compounds of *U. lobata* leaf extract, can be seen in (Fig. 1) and (Table 1). Analysis using LC-MS/MS was obtained that the most abundant active constituent in *U. lobata* extract were stigmasterol and gossypetin. Meanwhile, active constituent presented in low concentrations, like mangiferin, β-sitosterol, and chrysoeriol, were also identified in the aqueous extracts of *U. lobata.*

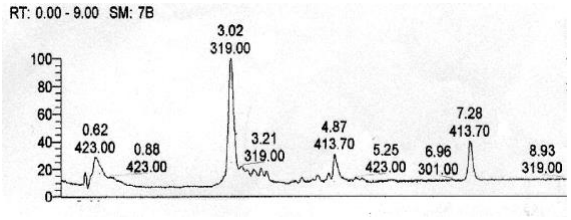

Fig.1: Chromatogram of active constituent in *U. lobata* leaf extracts.

Table 1: Active constituent in *U. lobata* leaf extracts

No	Active compounds	Molecule weight	Result
1	Stigmasterol	413	(++)
2	β-Sitosterol	415	(+)
3	Mangiferin	423	(+)
4	Quercetine	303	(-)
5	Kaempferol	286	(-)
6	Hypolaetin	302	(-)
7	Gossypetin	318	(+++)
8	Luteolin	286	(-)
9	Apigenin	270	(-)
10	Chrysoeriol	300	(+)

Note ; (-): negative, (+): weak, (++): moderate, (+++): strong

Five active constituent were found in the water extract of *U. lobata* leaf. They are gossypetin, stigmasterol, mangiferin, and chrysoeriol. All of them are non-nutrition substances and have pharmacology effect[13,14]. However, like any drugs, they induced adverse reaction and toxic effect in high dose and long-term administration. They may also induce drug-drug interaction[15].

Stigmasterol and β-sitosterol are phytosterols that are soluble in most organic solvents and contain one alcohol functional group. Pre-clinical studies on animals treated by stigmasterol showed that both of cholesterol and sitosterol absorption decreased by 23% and 30%, respectively, over 6 weeks. It also possesses a potential hypoglycemic, antioxidant and anti-thyroid properties[16,17]. β-sitosterol are hydrophobic and soluble in ethanol and chloroform but insoluble in water[18]. Studies showed that it possessed anti-cholesterol, anti-inflammatory and immunomodulatory effects[19].

However, other studies showed that oxidized products of stigmasterol and β-sitosterol increased apoptotic nuclei on hepatoma cells culture and inhibited the growth of cells through cytotoxic effects[20]. No evidence of genotoxicity and mutagenicity of phytosterol was observed. However, toxicity studies on them were limited to 90-day sub chronic toxicity studies and a 2-generation reproductive toxicity study in rats[21]. In another 90-days study reported by *Kim et al., 2002*[22], suppression of body weight gain in rodents of both sexes and infiltration of mononuclear cell in the heart in males at a dose level of 9 g phytosterol esters/kg bw/day were observed. The NOAEL derived from the 90-day sub-chronic toxicity studies in rats and the 2-generation reproductive toxicity study in rats amounted to be 2.5-6.6 g phytosterol ester/kg bw/day, 1.54-4.1 g phytosterol/kg bw/day and 335-900 mg stigmasterol/kg bw/day[21].

Gossypetin and chrysoeriol are flavonol or flavone obtained from the flowers and the calyx of hibiscus species. Gossypetin shows a high antioxidant, anti-mutagenic, anti-microbial and anti-atherosclerotic effects[23]. This

compound is very soluble in benzene and chloroform and also moderately soluble in ether and ethanol but insoluble in water. Meanwhile, chrysoeriol is a flavone that provides many health-promoting benefits such as anti-inflammation, anti-cancer, and anti-histamine. It is soluble in alkalies solution and sufficiently soluble in water[24]. On the other hand, flavonoid has potentially toxic effects, such as pro-oxidant activity, mitochondrial toxicity, and interaction with drug-metabolizing enzymes. Flavonoid can change into radicals compound after scavenging free radical, thus increasing stress oxidative and disrupting mitochondria[25]. Interaction of flavonoid with other active compounds or drugs can alter metabolism enzyme expression and modulate their activity.

Mangiferin is a xanthonoid and a glucoside of norathyriol. Mangiferin is soluble in hot dilutes methanol and ethanol but insoluble in water. The laboratory study has identified a variety of pharmacology effects of mangiferin, including antioxidant, anti-microbial and hypoglycemic effect in rodents[26,27]. In acute toxicity study, no effects were found after dermal exposure to mangiferin 2000 mg/kg. However, flank position, transient dyspnea and piloerection were found after oral administration of this xanthone. Intraperitoneal administration in mice induced similar toxicity signs with possible mortality in rodents. Orally treated on rat with mangiferin (250-1000 mg/kg) for 28 days did not obtain any abnormal clinical signs or hematology alteration, when compared to control group[28]. Histopathological alterations like necrosis, vacuolar degeneration, and increment of apoptosis of the acinar cells were found in the exocrine pancreas of rats at 1000 mg/kg. This suggested that exocrine pancreas was the target organ for mangiferin toxicity[28].

The composition of active constituent the extract was depended on polarity solute and the choice of extract solvent. The polarity of the extracting solvent determines the composition of an active compound by influencing their solubility in the solvent. The alkaloids, terpenoids, and steroids are soluble in the non-polar solvent like acetone, diethyl ether and hexane. Meanwhile, flavonoids, phenols, and glycosides dissolve better in a polar solvent, such as water and methanol[29,30]. Non-nutrition compounds or secondary metabolites like alkaloid, terpenoid, flavonoid, and steroid are in smaller quantity and they have pharmacologic effects given in appropriate doses[30,31]. Secondary metabolites are derived from the metabolism of the primary metabolites in plants, however, sometimes they have a toxic effect, especially when used in high dose. Most of flavonoid and terpenoid in herbs have potency as antioxidant, antiseptic and anti-inflammatory whereas steroids can act as anti-inflammatory and sex hormone[31].

Toxicity Analysis of *U. lobata* Leaf Extracts:

The toxicity of the active constituent from *U. lobata* leaf extracts was evaluated based on LD-50 values by *in silico* study and the results are depicted in Table 2. Stigmasterol and β-sitosterol had a low LD-50 value, indicating high toxicity in rats and mice. Intraperitoneal (i.p) administration of these compounds produced LD-50 lower than per oral (p.o) in the rodents. Toxicity analysis of active compounds in *U. lobata* indicated borderline to moderate reliability level.

Stigmasterol, β-sitosterol and mangiferin in *U. lobata* leaf extract were predicted as a toxic substance through *in silico* study. Plant sterols, such as stigmasterol and β-sitosterol, have cytotoxic effect[20,21], anti-diabetic[2,32] . Meanwhile, mangiferin is toxic to the exocrine pancreas of the rats as explained above[28]. They contribute to the overall the toxic effects of *U. lobata* in animal testings.

Table 2: Analysis of toxicity active compound in *U. lobata* leaf extracts [33]

No	Active compounds	LD-50 (mg/kg) mouse (i.p)	LD-50 (mg/kg) mouse (p.o)	LD-50 (mg/kg) rat (i.p)	LD-50 (mg/kg) rat (p.o)
1	Stigmasterol	160**	530*	170**	1400**
2	β-Sitosterol	110**	570*	140*	740*
3	Mangiferin	460*	1500**	160**	1900
4	Gossypetin	490*	550**	710	600*
5	Chrysoeriol	290	1100*	700**	1300**

**( ): not reliable, (*): borderline, (**): moderate

Intraperitoneal administration of active compounds in *U. lobata* resulted in LD-50 value lower than oral administration. The oral administration will subject the substances to biotransformation process at the liver, such as oxidation, reduction, hydrolysis and conjugation, which may reduce its toxicity[15]. Detoxification of active substances occur at metabolism phase by microsomal hepatic[34]. The first-pass metabolism occurs more extensively for substances administered orally than intraperitoneally. Besides, the bioavailability of the substances is higher with intraperitoneal administration than oral route because they do not enter the gastrointestinal organs. Therefore, the

damage by digestive and biotransformation enzymes is avoided[34]. The bioactivity of herbs in the organism is prolonged, meanwhile, the accumulation and toxicity are increased[35]. Biotransformation is needed to decrease the toxicity of xenobiotics such as drugs, herbs, and chemical substances[34]. However, the biotransformation process may produce a more active metabolite or the same activity with the parent drug. Moreover, it can modulate the activity of the compounds as well as the toxicity[15,36].

The LD-50 of *U. lobata* is higher in mice than in the rats. It is postulated that the organ capacity to eliminate xenobiotic is limited in mice compared to the rat, thus, the accumulation of active metabolite and toxicity risk are increased[37]. Elimination processes, including metabolism and excretion, aims to decrease the bioactivity of active substances[15]. The liver is a major organ of metabolisms, while kidney, gastrointestinal lumen, blood, and lung eliminate the substances[34,37].

Acute Toxicity Level of *Urena lobata* Extract:

Toxicity level of *U. lobata* leaf extract on embryo, juvenile and adult of zebrafish were shown in Table 3, 4 and 5. LC-50 values of *U. lobata* leaf extract were the lowest in embryo (2548.79 mg/L), indicating moderate toxic level, whereas on juvenile (8748.45 mg/L) and adult zebrafish (8088.11 mg/L), the toxicity level was mild.

Table 3: Acute toxicity level of *U. lobata* leaf extract on embryo *D. rerio*

No	Concentration (mg/L)	n	Number of death (%)
1	1000	3	0.00 ± 0.00
2	1500	3	3.33 ± 0.58
3	2500	3	50.00 ± 0.58
4	3000	3	70.00 ± 1.15
5	4000	3	100.00 ± 0.58
	LC-50 Level Toxicity	= =	2548.79 mg/L Moderate

Table 4: Acute toxicity level of *U. lobata* leaf extract on juvenile *D. rerio*

No	Concentration (mg/L)	n	Number of death (%)
1	2000	3	0.00 ± 0.00
2	6000	3	10.00 ± 0.00
3	8000	3	30.00 ± 0.00
4	10000	3	70.00 ± 0.00
5	12000	3	83.33 ± 5.77
	LC-50 Level Toxicity	= =	8748.45 mg/L Mild

Table 5: Acute toxicity level of *U. lobata* leaf extract on adult *D. rerio*

No	Concentration (mg/L)	n	Number of death (%)
1	500	3	0.00 ± 0.00
2	2000	3	0.00 ± 0.00
3	6000	3	40.00 ± 0.00
4	8000	3	43.33 ± 0.58
5	12000	3	80.00 ± 0.00
	LC-50 Level Toxicity	= =	8088.11 mg/L Mild

The toxicity level of *U. lobata* leaf extract on embryo is higher compared to juvenile and adult of zebrafish. The embryo is more sensitive to the active constituent of *U. lobata* leaf extract due to the lack of metabolism enzyme and

the immaturity of metabolism or excretion organ system thus increasing the toxicity risk[15,38]. There is relatively lower level of plasma protein in the embryo, and this caused a higher level of the free drugs circulating in the blood, leading to the increased toxicity risk. Detoxifying enzymes, like glucuronidase, is also limited in an embryo; thus, the active compounds are less inactivated by the metabolic processes[34,39]. This results in increased bioactivity and toxicity risk also. With regards to the excretion process, glomerular filtration rate is lower in embryo compared to the adult organism due to the under-developed excretion organs[40]. Hence, the performance of the organ systems to eliminate toxic metabolites is reduced[15]. Whereas for the adult organisms, both their metabolism and excretion organs have grown better compared to juvenile and embryo. This explains our observation that toxicity level of *U. lobata*, defined by LC-50 value on embryo, is lower in juvenile than adults. Therefore, the use of *U. lobata* in pregnancy and infant should be considered carefully based on these safety issues[41,42]. Dose adjustment of this herb should be considered to adjust for its bioactivity, adverse reaction, and toxicity among the vulnerable groups.

According to *in silico* study, alkaloids like mangiferin and phytosterols like stigmasterol and β-sitosterol in *U. lobata* leaf extract are predicted to be toxic. These substances have the potential to interact with each other, thereby increasing the toxicity of the extract[38]. The interaction between the active compounds in this herb could also modulate their biological activity[15,31]. The compounded effects of the cytotoxic, pro-oxidant and damage to the pancreatic from the active compounds in *U. lobata* should be considered together in explaining its toxicity[28,43].

CONCLUSION:

Stigmasterol and β-sitosterol are predicted to be toxic in *U. lobata* leaf extract. The embryo is more sensitive to xenobiotic agents due to the lack of metabolic enzymes and immaturation of the metabolic and excretion organs. Therefore, *U. lobata* leaf extract could have teratogenic effects. Application of this extract among pregnant women and children should be cautioned.

ACKNOWLEDGEMENT:

This work was funded by Research Grant of Directorate General of Higher Education Indonesia.

CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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RJPT_MANUSCRIPT
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Original Article

Acute Toxicity Level of Pulutan (*Urena lobata*) Leaf Extract on Zebrafish (*Danio rerio*) and its Analysis by *In Silico* Study

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ABSTRACT:

Pulutan (*Urena lobata*) is one of medicinal plant used to treat some diseases traditionally and pre-clinical studies have showed its efficacy. However, the study about its safety has not been evaluated completely. An acute toxicity test has to be performed in order to screen for its safety. The objective of study to determine the acute toxicity level of *Urena lobata* (*U. lobata*) leaf extract on embryo phase, juvenile and adult of zebrafish (*Danio rerio*) and the role of its active constituents through *in silico* methods. This was an experimental laboratory study using embryo, juvenile and adult of zebrafish (*Danio rerio*). The leaf of *U. lobata* was extracted by decoction methods and the extract was diluted from 12000 mg/L to 500 mg/L. The animals were exposed to the extracts for 96 hours. Toxicity level of herbs was defined using lethal concentration-50 (LC-50) obtained through linear regression. *In silico* study was performed using a web-based software application (iLAB ACD). The LC-50 values of *U. lobata* leaf extract for embryo, juvenile and adult of zebrafish (*Danio rerio*) were 2548 mg/L, 8748 mg/L and 8088 mg/L, respectively. Acute toxicity level of *U. lobata* on embryo is higher compared to juvenile and adult of zebrafish. After identification of active compound and *in silico* study was performed, Stigmasterol and β-sitosterol in *U. lobata* showed high toxicity level based on LD-50 value. Level toxicity of *U. lobata* on zebrafish embryo was moderate (0.5-5.0 g/L) and it shows teratogenic effect, meanwhile, its effects on juvenile and adult fish were considered mild (5.0-15.0 g/L).

KEYWORDS: acute, *Danio rerio*, *in silico*, toxicity, *Urena lobata*

INTRODUCTION:

Pulutan (*Urena lobata*) is a medicinal plant commonly found in Indonesia. It has been used to treat many diseases, such as cough, malaria, wound, and diabetes empirically[1]. Pre-clinical studies of *U. lobata* confirmed that it has anti-diabetic properties by inhibiting alpha-amylase and alpha-glucosidase, broad-spectrum antibacterial and anti-anxiolytic effects[2,3,4]. The safety of *Urena lobata* must be ensured before using it as an alternative medicine. Previous studies showed that administration of *U. lobata* at 3000 mg/kg bw for 28 days did not produce toxicity and death in rats. However, this herb increased hepatic enzyme and disrupted the structure of hepatocyte and sperm[5,6]. Meanwhile, the long-term exposure of herbs to rabbits showed that it could destroy of hepatocytes and obstruct the bile duct[7].

The toxicity of *U. lobata* must be examined and the lethal dose-50 (LD-50) or lethal concentration-50 (LC-50) should be determined as a parameter of safety for the herbs[7,8]. Despite the data on adult animals, its toxicity should be evaluated in the embryo and juvenile animals to determine the safety *U. lobata* across different life stages[8]. Embryonic evaluation ensures the safety of the herbs on the fetus and pregnancy period, meanwhile juvenile assessment certain its safety in the period of rapid growth and development of an organism[8,9]. Generally, organisms at these stages are more sensitive to xenobiotic agent, including herbs, compared to the adult phase.

The use of zebrafish (*Danio rerio*) as an animal model of toxicity test offers many advantages because they are sensitive to poison and easy to breed. Since the embryo is transparent, it is easy to observe their internal organs[9]. Almost 70 % of the genes in human are found on *D. rerio*, implying that human diseases can be replicated using this animal[10]. The study aims to evaluate acute toxicity level of *U. lobata* leaf extract on embryo phase, juvenile, and adult of zebrafish (*Danio rerio*) and also analyse it by *in silico* approach.

MATERIAL AND METHODS:

Chemical Sample:

First, Embryonic solution containing magnesium sulfate, sodium chloride, potassium chloride, calcium chloride dihydrate is solved in distilled water. All of the chemicals are purchased from Sigma Aldrich and Merck. Others materials include Methylene Blue (Sakkai Pro), Tetramin (Tropical) Artemia (Golden west).

Sample Preparation:

U. lobata leaf powder was obtained from Materia Medika, Batu, Malang, Indonesia with certificate number 074/306/101.8/2016. The powdered plant materials were extracted using decoction methods with ratio herbs and solvent 1:5. The extract was diluted into several concentrations for toxicity test and for the identification of active compounds.

Identification of Active Constituen:

Water extract of *U. lobata* was analysed on a semi-qualitative scale using Liquid Chromatography-Mass Spectra (LC-MS/MS) Accela 1250 pump. The liquid phase contains 0.1 % formic acid in solvent combination (methanol and water). The identification included 10 active substances target from alkaloid (mangiferin), phytosterol (stigmasterol, beta-sitosterol) and flavonoid (luteolin, quercetin, kaempferol, gossypetin, apigenin, chrysoeriol, hypocretin) groups.

Toxicity Analysis:

Active compounds in *U. lobata* leaf extracts were evaluated its toxicity test based on LD-50 value using *in silico* study with a web-based software application (iLAB ACD). It gives a predictive value to determine the dose for *in vivo* study and to confirm its results.

Acute Toxicity Test:

The assay was based on OECD[11,12] with slight modifications. It was performed in 24 microwell plates for the embryo of *D. rerio*, while the assessment of both juvenile and adult was performed in an aquarium. The treatment was performed in three replicates and each replicate consist of 10 embryos or fish. The *U. lobata* leaf extract was given for 96 hours and the extract was replaced every 24 hours. Death of embryo, juvenile and fish was calculated every 24 hours.

Analytical Studies:

For acute toxicity test, the percentage of death are expressed as the mean ± SD and the LC-50 value was calculated using a linear regression curve using SPPS version 16.0.

RESULTS AND DISCUSSION:

Identification of Active Constituent in *U. lobata* Leaf Extracts:

The active compounds of *U. lobata* leaf extract, can be seen in (Fig. 1) and (Table 1). Analysis using LC-MS/MS was obtained that the most abundant active constituent in *U. lobata* extract were stigmasterol and gossypetin. Meanwhile, active constituent presented in low concentrations, like mangiferin, β-sitosterol, and chrysoeriol, were also identified in the aqueous extracts of *U. lobata.*

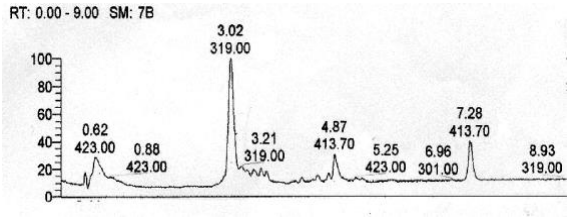

Fig.1: Chromatogram of active constituent in *U. lobata* leaf extracts.

Table 1: Active constituent in *U. lobata* leaf extracts

No	Active compounds	Molecule weight	Result
1	Stigmasterol	413	(++)
2	β-Sitosterol	415	(+)
3	Mangiferin	423	(+)
4	Quercetine	303	(-)
5	Kaempferol	286	(-)
6	Hypolaetin	302	(-)
7	Gossypetin	318	(+++)
8	Luteolin	286	(-)
9	Apigenin	270	(-)
10	Chrysoeriol	300	(+)

Note ; (-): negative, (+): weak, (++): moderate, (+++): strong

Five active constituent were found in the water extract of *U. lobata* leaf. They are gossypetin, stigmasterol, mangiferin, and chrysoeriol. All of them are non-nutrition substances and have pharmacology effect[13,14]. However, like any drugs, they induced adverse reaction and toxic effect in high dose and long-term administration. They may also induce drug-drug interaction[15].

Stigmasterol and β-sitosterol are phytosterols that are soluble in most organic solvents and contain one alcohol functional group. Pre-clinical studies on animals treated by stigmasterol showed that both of cholesterol and sitosterol absorption decreased by 23% and 30%, respectively, over 6 weeks. It also possesses a potential hypoglycemic, antioxidant and anti-thyroid properties[16,17]. β-sitosterol are hydrophobic and soluble in ethanol and chloroform but insoluble in water[18]. Studies showed that it possessed anti-cholesterol, anti-inflammatory and immunomodulatory effects[19].

However, other studies showed that oxidized products of stigmasterol and β-sitosterol increased apoptotic nuclei on hepatoma cells culture and inhibited the growth of cells through cytotoxic effects[20]. No evidence of genotoxicity and mutagenicity of phytosterol was observed. However, toxicity studies on them were limited to 90-day sub chronic toxicity studies and a 2-generation reproductive toxicity study in rats[21]. In another 90-days study reported by *Kim et al., 2002*[22], suppression of body weight gain in rodents of both sexes and infiltration of mononuclear cell in the heart in males at a dose level of 9 g phytosterol esters/kg bw/day were observed. The NOAEL derived from the 90-day sub-chronic toxicity studies in rats and the 2-generation reproductive toxicity study in rats amounted to be 2.5-6.6 g phytosterol ester/kg bw/day, 1.54-4.1 g phytosterol/kg bw/day and 335-900 mg stigmasterol/kg bw/day[21].

Gossypetin and chrysoeriol are flavonol or flavone obtained from the flowers and the calyx of hibiscus species. Gossypetin shows a high antioxidant, anti-mutagenic, anti-microbial and anti-atherosclerotic effects[23]. This compound is very soluble in benzene and chloroform and also moderately soluble in ether and ethanol but insoluble

in water. Meanwhile, chrysoeriol is a flavone that provides many health-promoting benefits such as anti-inflammation, anti-cancer, and anti-histamine. It is soluble in alkalies solution and sufficiently soluble in water[24]. On the other hand, flavonoid has potentially toxic effects, such as pro-oxidant activity, mitochondrial toxicity, and interaction with drug-metabolizing enzymes. Flavonoid can change into radicals compound after scavenging free radical, thus increasing stress oxidative and disrupting mitochondria[25]. Interaction of flavonoid with other active compounds or drugs can alter metabolism enzyme expression and modulate their activity.

Mangiferin is a xanthonoid and a glucoside of norathyriol. Mangiferin is soluble in hot dilutes methanol and ethanol but insoluble in water. The laboratory study has identified a variety of pharmacology effects of mangiferin, including antioxidant, anti-microbial and hypoglycemic effect in rodents[26,27]. In acute toxicity study, no effects were found after dermal exposure to mangiferin 2000 mg/kg. However, flank position, transient dyspnea and piloerection were found after oral administration of this xanthone. Intraperitoneal administration in mice induced similar toxicity signs with possible mortality in rodents. Orally treated on rat with mangiferin (250-1000 mg/kg) for 28 days did not obtain any abnormal clinical signs or hematology alteration, when compared to control group[28]. Histopathological alterations like necrosis, vacuolar degeneration, and increment of apoptosis of the acinar cells were found in the exocrine pancreas of rats at 1000 mg/kg. This suggested that exocrine pancreas was the target organ for mangiferin toxicity[28].

The composition of active constituent the extract was depended on polarity solute and the choice of extract solvent. The polarity of the extracting solvent determines the composition of an active compound by influencing their solubility in the solvent. The alkaloids, terpenoids, and steroids are soluble in the non-polar solvent like acetone, diethyl ether and hexane. Meanwhile, flavonoids, phenols, and glycosides dissolve better in a polar solvent, such as water and methanol[29,30]. Non-nutrition compounds or secondary metabolites like alkaloid, terpenoid, flavonoid, and steroid are in smaller quantity and they have pharmacologic effects given in appropriate doses[30,31]. Secondary metabolites are derived from the metabolism of the primary metabolites in plants, however, sometimes they have a toxic effect, especially when used in high dose. Most of flavonoid and terpenoid in herbs have potency as antioxidant, antiseptic and anti-inflammatory whereas steroids can act as anti-inflammatory and sex hormone[31].

Toxicity Analysis of *U. lobata* Leaf Extracts:

The toxicity of the active constituent from *U. lobata* leaf extracts was evaluated based on LD-50 values by *in silico* study and the results are depicted in Table 2. Stigmasterol and β-sitosterol had a low LD-50 value, indicating high toxicity in rats and mice. Intraperitoneal (i.p) administration of these compounds produced LD-50 lower than per oral (p.o) in the rodents. Toxicity analysis of active compounds in *U. lobata* indicated borderline to moderate reliability level.

Stigmasterol, β-sitosterol and mangiferin in *U. lobata* leaf extract were predicted as a toxic substance through *in silico* study. Plant sterols, such as stigmasterol and β-sitosterol, have cytotoxic effect[20,21], anti-diabetic[2,32] . Meanwhile, mangiferin is toxic to the exocrine pancreas of the rats as explained above[28]. They contribute to the overall the toxic effects of *U. lobata* in animal testings.

Table 2: Analysis of toxicity active compound in *U. lobata* leaf extracts [33]

Active compounds	LD-50 (mg/kg)			
	mouse (i.p)	mouse (p.o)	rat (i.p)	rat (p.o)
Stigmasterol	160**	530*	170**	1400**
β-Sitosterol	110**	570*	140*	740*
Mangiferin	460*	1500**	160**	1900
Gossypetin	490*	550**	710	600*
Chrysoeriol	290	1100*	700**	1300**

**( ): not reliable, (*): borderline, (**): moderate

Intraperitoneal administration of active compounds in *U. lobata* resulted in LD-50 value lower than oral administration. The oral administration will subject the substances to biotransformation process at the liver, such as oxidation, reduction, hydrolysis and conjugation, which may reduce its toxicity[15]. Detoxification of active substances occur at metabolism phase by microsomal hepatic[34]. The first-pass metabolism occurs more extensively for substances administered orally than intraperitoneally. Besides, the bioavailability of the substances is higher with intraperitoneal administration than oral route because they do not enter the gastrointestinal organs. Therefore, the

damage by digestive and biotransformation enzymes is avoided[34]. The bioactivity of herbs in the organism is prolonged, meanwhile, the accumulation and toxicity are increased[35]. Biotransformation is needed to decrease the toxicity of xenobiotics such as drugs, herbs, and chemical substances[34]. However, the biotransformation process may produce a more active metabolite or the same activity with the parent drug. Moreover, it can modulate the activity of the compounds as well as the toxicity[15,36].

The LD-50 of *U. lobata* is higher in mice than in the rats. It is postulated that the organ capacity to eliminate xenobiotic is limited in mice compared to the rat, thus, the accumulation of active metabolite and toxicity risk are increased[37]. Elimination processes, including metabolism and excretion, aims to decrease the bioactivity of active substances[15]. The liver is a major organ of metabolisms, while kidney, gastrointestinal lumen, blood, and lung eliminate the substances[34,37].

Acute Toxicity Level of *Urena lobata* Extract:

Toxicity level of *U. lobata* leaf extract on embryo, juvenile and adult of zebrafish were shown in Table 3, 4 and 5. LC-50 values of *U. lobata* leaf extract were the lowest in embryo (2548.79 mg/L), indicating moderate toxic level, whereas on juvenile (8748.45 mg/L) and adult zebrafish (8088.11 mg/L), the toxicity level was mild.

Table 3: Acute toxicity level of *U. lobata* leaf extract on embryo *D. rerio*

Concentration (mg/L)	n	Number of death (%)
1000	3	0.00 ± 0.00
1500	3	3.33 ± 0.58
2500	3	50.00 ± 0.58
3000	3	70.00 ± 1.15
4000	3	100.00 ± 0.58
		LC-50 = 2548.79 mg/L
		Level Toxicity = Moderate

Table 4: Acute toxicity level of *U. lobata* leaf extract on juvenile *D. rerio*

Concentration (mg/L)	n	Number of death (%)
2000	3	0.00 ± 0.00
6000	3	10.00 ± 0.00
8000	3	30.00 ± 0.00
10000	3	70.00 ± 0.00
12000	3	83.33 ± 5.77
		LC-50 = 8748.45 mg/L
		Level Toxicity = Mild

Table 5: Acute toxicity level of *U. lobata* leaf extract on adult *D. rerio*

Concentration (mg/L)	n	Number of death (%)
500	3	0.00 ± 0.00
2000	3	0.00 ± 0.00
6000	3	40.00 ± 0.00
8000	3	43.33 ± 0.58
12000	3	80.00 ± 0.00
		LC-50 = 8088.11 mg/L
		Level Toxicity = Mild

The toxicity level of *U. lobata* leaf extract on embryo is higher compared to juvenile and adult of zebrafish. The embryo is more sensitive to the active constituent of *U. lobata* leaf extract due to the lack of metabolism enzyme and

the immaturity of metabolism or excretion organ system thus increasing the toxicity risk[15,38]. There is relatively lower level of plasma protein in the embryo, and this caused a higher level of the free drugs circulating in the blood, leading to the increased toxicity risk. Detoxifying enzymes, like glucuronidase, is also limited in an embryo; thus, the active compounds are less inactivated by the metabolic processes[34,39]. This results in increased bioactivity and toxicity risk also. With regards to the excretion process, glomerular filtration rate is lower in embryo compared to the adult organism due to the under-developed excretion organs[40]. Hence, the performance of the organ systems to eliminate toxic metabolites is reduced[15]. Whereas for the adult organisms, both their metabolism and excretion organs have grown better compared to juvenile and embryo. This explains our observation that toxicity level of *U. lobata*, defined by LC-50 value on embryo, is lower in juvenile than adults. Therefore, the use of *U. lobata* in pregnancy and infant should be considered carefully based on these safety issues[41,42]. Dose adjustment of this herb should be considered to adjust for its bioactivity, adverse reaction, and toxicity among the vulnerable groups.

According to *in silico* study, alkaloids like mangiferin and phytosterols like stigmasterol and β-sitosterol in *U. lobata* leaf extract are predicted to be toxic. These substances have the potential to interact with each other, thereby increasing the toxicity of the extract[38]. The interaction between the active compounds in this herb could also modulate their biological activity[15,31]. The compounded effects of the cytotoxic, pro-oxidant and damage to the pancreatic from the active compounds in *U. lobata* should be considered together in explaining its toxicity[28,43].

CONCLUSION:

Stigmasterol and β-sitosterol are predicted to be toxic in *U. lobata* leaf extract. The embryo is more sensitive to xenobiotic agents due to the lack of metabolic enzymes and immaturation of the metabolic and excretion organs. Therefore, *U. lobata* leaf extract could have teratogenic effects. Application of this extract among pregnant women and children should be cautioned.

ACKNOWLEDGEMENT:

This work was funded by Research Grant of Directorate General of Higher Education Indonesia.

CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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ISSN 0974-3618 (Print)
0974-360X (Online)

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RESEARCH ARTICLE

Acute Toxicity Level of Pulutan (*Urena lobata*) Leaf Extract on Zebrafish (*Danio rerio*) and its Analysis by *In Silico* Study

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ABSTRACT:

Pulutan (*Urena lobata*) is one of medicinal plant used to treat some diseases traditionally and pre-clinical studies have showed its efficacy. However, the study about its safety has not been evaluated completely. An acute toxicity test has to be performed in order to screen for its safety. The objective of study to determine the acute toxicity level of *Urena lobata* (*U. lobata*) leaf extract on embryo phase, juvenile and adult of zebrafish (*Danio rerio*) and the role of its active constituents through *in silico* methods. This was an experimental laboratory study using embryo, juvenile and adult of zebrafish (*Danio rerio*). The leaf of *U. lobata* was extracted by decoction methods and the extract was diluted from 12000 mg/L to 500 mg/L. The animals were exposed to the extracts for 96 hours. Toxicity level of herbs was defined using lethal concentration-50 (LC-50) obtained through linear regression. *In silico* study was performed using a web-based software application (iLAB ACD). The LC-50 values of *U. lobata* leaf extract for embryo, juvenile and adult of zebrafish (*Danio rerio*) were 2548 mg/L, 8748 mg/L and 8088 mg/L, respectively. Acute toxicity level of *U. lobata* on embryo is higher compared to juvenile and adult of zebrafish. After identification of active compound and *in silico* study was performed, Stigmasterol and β-sitosterol in *U. lobata* showed high toxicity level based on LD-50 value. Level toxicity of *U. lobata* on zebrafish embryo was moderate (0.5-5.0 g/L) and it shows teratogenic effect, meanwhile, its effects on juvenile and adult fish were considered mild (5.0-15.0 g/L).

KEYWORDS: Acute, *Danio rerio*, *in silico*, toxicity, *Urena lobata*.

INTRODUCTION:

Pulutan (*Urena lobata*) is a medicinal plant commonly found in Indonesia. It has been used to treat many diseases, such as cough, malaria, wound, and diabetes empirically[1]. Pre-clinical studies of *U. lobata* confirmed that it has anti-diabetic properties by inhibiting alpha-amylase and alpha-glucosidase, broad-spectrum antibacterial and anti-anxiolytic effects[2,3,4]. The safety of *Urena lobata* must be ensured before using it as an alternative medicine. Previous studies showed that administration of *U. lobata* at 3000mg/kg bw for 28 days did not produce toxicity and death in rats. However, this herb increased hepatic enzyme and disrupted the structure of hepatocyte and sperm[5,6].

Meanwhile, the long-term exposure of herbs to rabbits showed that it could destroy of hepatocytes and obstruct the bile duct[7].

The toxicity of *U. lobata* must be examined and the lethal dose-50 (LD-50) or lethal concentration-50 (LC-50) should be determined as a parameter of safety for the herbs[7,8]. Despite the data on adult animals, its toxicity should be evaluated in the embryo and juvenile animals to determine the safety *U. lobata* across different life stages[8]. Embryonic evaluation ensures the safety of the herbs on the fetus and pregnancy period, meanwhile juvenile assessment certain its safety in the period of rapid growth and development of an organism[8,9]. Generally, organisms at these stages are more sensitive to xenobiotic agent, including herbs, compared to the adult phase.

The use of zebrafish (*Danio rerio*) as an animal model of toxicity test offers many advantages because they are sensitive to poison and easy to breed. Since the embryo is transparent, it is easy to observe their internal organs[9]. Almost 70 % of the genes in human are found on *D. rerio,* implying that human diseases can be replicated using this animal[10]. The study aims to evaluate acute toxicity level of *U. lobata* leaf extract on embryo phase, juvenile, and adult of zebrafish (*Danio rerio*) and also analyse it by *in silico* approach.

MATERIAL AND METHODS:

Chemical Sample:

First, Embryonic solution containing magnesium sulfate, sodium chloride, potassium chloride, calcium chloride dihydrate is solved in distilled water. All of the chemicals are purchased from Sigma Aldrich and Merck. Others materials include Methylene Blue (Sakkai Pro), Tetramin (Tropical) Artemia (Golden west).

Sample Preparation:

U. lobata leaf powder was obtained from Materia Medika, Batu, Malang, Indonesia with certificate number 074/306/101.8/2016. The powdered plant materials were extracted using decoction methods with ratio herbs and solvent 1:5. The extract was diluted into several concentrations for toxicity test and for the identification of active compounds.

Identification of Active Constituen:

Water extract of *U. lobata* was analysed on a semi-qualitative scale using Liquid Chromatography-Mass Spectra (LC-MS/MS) Accela 1250 pump. The liquid phase contains 0.1 % formic acid in solvent combination (methanol and water). The identification included 10 active substances target from alkaloid (mangiferin), phytosterol (stigmasterol, beta-sitosterol) and flavonoid (luteolin, quercetin, kaempferol, gossypetin, apigenin, chrysoeriol, hypocretin) groups.

Toxicity Analysis:

Active compounds in *U. lobata* leaf extracts were evaluated its toxicity test based on LD-50 value using *in silico* study with a web-based software application (iLAB ACD). It gives a predictive value to determine the dose for *in vivo* study and to confirm its results.

Acute Toxicity Test:

The assay was based on OECD[11,12] with slight modifications. It was performed in 24 microwell plates for the embryo of *D. rerio*, while the assessment of both juvenile and adult was performed in an aquarium. The treatment was performed in three replicates and each replicate consist of 10 embryos or fish. The *U. lobata* leaf extract was given for 96 hours and the extract was replaced every 24 hours. Death of embryo, juvenile and fish was calculated every 24 hours.

Analytical Studies:

For acute toxicity test, the percentage of death are expressed as the mean±SD and the LC-50 value was calculated using a linear regression curve using SPPS version 16.0.

RESULTS AND DISCUSSION:

Identification of Active Constituent in *U. lobata* Leaf Extracts:

The active compounds of *U. lobata* leaf extract, can be seen in (Fig. 1) and (Table 1). Analysis using LC-MS/MS was obtained that the most abundant active constituent in *U. lobata* extract were stigmasterol and gossypetin. Meanwhile, active constituent presented in low concentrations, like mangiferin, β-sitosterol, and chrysoeriol, were also identified in the aqueous extracts of *U. lobata.*

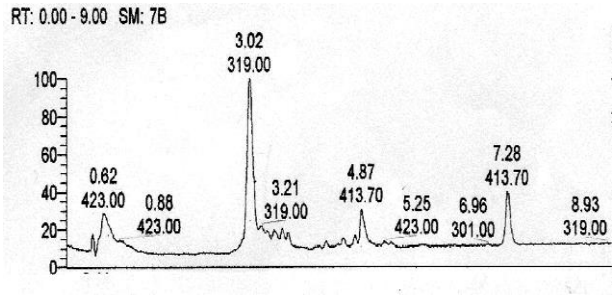

Fig.1: Chromatogram of active constituent in *U. lobata* leaf extracts.

Table 1: Active constituent in *U. lobata* leaf extracts

S. No	Active compounds	Molecule weight	Result
1	Stigmasterol	413	(++)
2	β-Sitosterol	415	(+)
3	Mangiferin	423	(+)
4	Quercetine	303	(-)
5	Kaempferol	286	(-)
6	Hypolaetin	302	(-)
7	Gossypetin	318	(+++)
8	Luteolin	286	(-)
9	Apigenin	270	(-)
10	Chrysoeriol	300	(+)

Note ; (-): negative, (+): weak, (++): moderate, (+++): strong

Five active constituent were found in the water extract of *U. lobata* leaf. They are gossypetin, stigmasterol, mangiferin, and chrysoeriol. All of them are non-nutrition substances and have pharmacology effect[13,14]. However, like any drugs, they induced adverse reaction and toxic effect in high dose and long-term administration. They may also induce drug-drug interaction[15].

Stigmasterol and β-sitosterol are phytosterols that are soluble in most organic solvents and contain one alcohol functional group. Pre-clinical studies on animals treated by stigmasterol showed that both of cholesterol and sitosterol absorption decreased by 23% and 30%, respectively, over 6 weeks. It also possesses a potential hypoglycemic, antioxidant and anti-thyroid properties[16,17]. β-sitosterol are hydrophobic and soluble in ethanol and chloroform but insoluble in water[18]. Studies showed that it possessed anti-cholesterol, anti-inflammatory and immunomodulatory effects[19].

However, other studies showed that oxidized products of stigmasterol and β-sitosterol increased apoptotic nuclei on hepatoma cells culture and inhibited the growth of cells through cytotoxic effects[20]. No evidence of genotoxicity and mutagenicity of phytosterol was observed. However, toxicity studies on them were limited to 90-day sub chronic toxicity studies and a 2-generation reproductive toxicity study in rats[21]. In another 90-days study reported by *Kim et al., 2002*[22], suppression of body weight gain in rodents of both sexes and infiltration of mononuclear cell in the heart in males at a dose level of 9g phytosterol esters/kg bw/day were observed. The NOAEL derived from the 90-day sub-chronic toxicity studies in rats and the 2-generation reproductive toxicity study in rats amounted to be 2.5-6.6g phytosterol ester/kg bw/day, 1.54-4.1g phytosterol/kg bw/day and 335-900mg stigmasterol/kg bw/day[21].

Gossypetin and chrysoeriol are flavonol or flavone obtained from the flowers and the calyx of hibiscus species. Gossypetin shows a high antioxidant, anti-mutagenic, anti-microbial and anti-atherosclerotic effects[23]. This compound is very soluble in benzene and chloroform and also moderately soluble in ether and ethanol but insoluble in water. Meanwhile, chrysoeriol is a flavone that provides many health-promoting benefits such as anti-inflammation, anti-cancer, and anti-histamine. It is soluble in alkalies solution and sufficiently soluble in water[24]. On the other hand, flavonoid has potentially toxic effects, such as pro-oxidant activity, mitochondrial toxicity, and interaction with drug-metabolizing enzymes. Flavonoid can change into radicals compound after scavenging free radical, thus increasing stress oxidative and disrupting mitochondria[25]. Interaction of flavonoid with other active compounds or drugs can alter metabolism enzyme expression and modulate their activity.

Mangiferin is a xanthonoid and a glucoside of norathyriol. Mangiferin is soluble in hot dilutes methanol and ethanol but insoluble in water. The laboratory study has identified a variety of pharmacology effects of mangiferin, including antioxidant, anti-microbial and hypoglycemic effect in rodents[26,27]. In acute toxicity study, no effects were found after dermal exposure to mangiferin 2000mg/kg. However, flank position, transient dyspnea and piloerection were found after oral administration of this xanthone. Intraperitoneal administration in mice induced similar toxicity signs with possible mortality in rodents. Orally treated on rat with mangiferin (250-1000mg/kg) for 28 days did not obtain any abnormal clinical signs or hematology alteration, when compared to control group[28]. Histopathological alterations like necrosis, vacuolar degeneration, and increment of apoptosis of the acinar cells were found in the exocrine pancreas of rats at 1000 mg/kg. This suggested that exocrine pancreas was the target organ for mangiferin toxicity[28].

The composition of active constituent the extract was depended on polarity solute and the choice of extract solvent. The polarity of the extracting solvent determines the composition of an active compound by influencing their solubility in the solvent. The alkaloids, terpenoids, and steroids are soluble in the non-polar solvent like acetone, diethyl ether and hexane. Meanwhile, flavonoids, phenols, and glycosides dissolve better in a polar solvent, such as water and methanol[29,30]. Non-nutrition compounds or secondary metabolites like alkaloid, terpenoid, flavonoid, and steroid are in smaller quantity and they have pharmacologic effects given in appropriate doses[30,31]. Secondary metabolites are derived from the metabolism of the primary metabolites in plants, however, sometimes they have a toxic effect, especially when used in high dose. Most of flavonoid and terpenoid in herbs have potency as antioxidant, antiseptic and anti-inflammatory whereas steroids can act as anti-inflammatory and sex hormone[31].

Toxicity Analysis of *U. lobata* Leaf Extracts:

The toxicity of the active constituent from *U. lobata* leaf extracts was evaluated based on LD-50 values by *in silico* study and the results are depicted in Table 2. Stigmasterol and β-sitosterol had a low LD-50 value, indicating high toxicity in rats and mice. Intraperitoneal (i.p) administration of these compounds produced LD-50 lower than per oral (p.o) in the rodents. Toxicity analysis of active compounds in *U. lobata* indicated borderline to moderate reliability level.

Stigmasterol, β-sitosterol and mangiferin in *U. lobata* leaf extract were predicted as a toxic substance through *in silico* study. Plant sterols, such as stigmasterol and β-sitosterol, have cytotoxic effect[20,21], anti-diabetic[2,32]. Meanwhile, mangiferin is toxic to the exocrine pancreas of the rats as explained above[28]. They contribute to the overall the toxic effects of *U. lobata* in animal testings.

Table 2: Analysis of toxicity active compound in *U. lobata* leaf extracts [33]

S. No	Active compounds	LD-50 (mg/kg) mouse (i.p)	LD-50 (mg/kg) mouse (p.o)	LD-50 (mg/kg) rat (i.p)	LD-50 (mg/kg) rat (p.o)
1	Stigmasterol	160**	530*	170**	1400**
2	β-Sitosterol	110**	570*	140*	740*
3	Mangiferin	460*	1500**	160**	1900
4	Gossypetin	490*	550**	710	600*
5	Chrysoeriol	290	1100*	700**	1300**
**( ): not reliable, (*): borderline, (**): moderate					

Intraperitoneal administration of active compounds in *U. lobata* resulted in LD-50 value lower than oral administration. The oral administration will subject the substances to biotransformation process at the liver, such as oxidation, reduction, hydrolysis and conjugation, which may reduce its toxicity[15]. Detoxification of active substances occur at metabolism phase by microsomal hepatic[34]. The first-pass metabolism occurs more extensively for substances administered orally than intraperitoneally. Besides, the bioavailability of the substances is higher with intraperitoneal administration than oral route because they do not enter the gastrointestinal organs. Therefore, the damage by digestive and biotransformation enzymes is avoided[34]. The bioactivity of herbs in the organism is prolonged, meanwhile, the accumulation and toxicity are increased[35]. Biotransformation is needed to decrease the toxicity of xenobiotics such as drugs, herbs, and chemical substances[34]. However, the biotransformation process may produce a more active metabolite or the same activity with the parent drug. Moreover, it can modulate the activity of the compounds as well as the toxicity[15,36].

The LD-50 of *U. lobata* is higher in mice than in the rats. It is postulated that the organ capacity to eliminate xenobiotic is limited in mice compared to the rat, thus, the accumulation of active metabolite and toxicity risk are increased[37]. Elimination processes, including metabolism and excretion, aims to decrease the bioactivity of active substances[15]. The liver is a major organ of metabolisms, while kidney, gastrointestinal lumen, blood, and lung eliminate the substances[34,37].

Acute Toxicity Level of *Urena lobata* Extract:

Toxicity level of *U. lobata* leaf extract on embryo, juvenile and adult of zebrafish were shown in Table 3, 4 and 5. LC-50 values of *U. lobata* leaf extract were the lowest in embryo (2548.79mg/L), indicating moderate toxic level, whereas on juvenile (8748.45mg/L) and adult zebrafish (8088.11mg/L), the toxicity level was mild.

Table 3: Acute toxicity level of *U. lobata* leaf extract on embryo *D. rerio*

No	Concentration (mg/L)	n	Number of death (%)
1	1000	3	0.00 ± 0.00
2	1500	3	3.33 ± 0.58
3	2500	3	50.00 ± 0.58
4	3000	3	70.00 ± 1.15
5	4000	3	100.00 ± 0.58
	LC-50	=	2548.79 mg/L
	Level Toxicity	=	Moderate

Table 4: Acute toxicity level of *U. lobata* leaf extract on juvenile *D. rerio*

No	Concentration (mg/L)	n	Number of death (%)
1	2000	3	0.00 ± 0.00
2	6000	3	10.00 ± 0.00
3	8000	3	30.00 ± 0.00
4	10000	3	70.00 ± 0.00
5	12000	3	83.33 ± 5.77
	LC-50	=	8748.45 mg/L
	Level Toxicity	=	Mild

Table 5: Acute toxicity level of *U. lobata* leaf extract on adult *D. rerio*

No	Concentration (mg/L)	n	Number of death (%)
1	500	3	0.00 ± 0.00
2	2000	3	0.00 ± 0.00
3	6000	3	40.00 ± 0.00
4	8000	3	43.33 ± 0.58
5	12000	3	80.00 ± 0.00
	LC-50	=	8088.11 mg/L
	Level Toxicity	=	Mild

The toxicity level of *U. lobata* leaf extract on embryo is higher compared to juvenile and adult of zebrafish. The embryo is more sensitive to the active constituent of *U. lobata* leaf extract due to the lack of metabolism enzyme and the immaturity of metabolism or excretion organ system thus increasing the toxicity risk[15,38]. There is relatively lower level of plasma protein in the embryo, and this caused a higher level of the free drugs circulating in the blood, leading to the increased toxicity risk. Detoxifying enzymes, like glucuronidase, is also limited in an embryo; thus, the active compounds are less inactivated by the metabolic processes[34,39]. This results in increased bioactivity and toxicity risk also. With regards to the excretion process, glomerular filtration rate is lower in embryo compared to the adult organism due to the under-developed excretion organs[40]. Hence, the performance of the organ systems to eliminate toxic metabolites is reduced[15]. Whereas for the adult organisms, both their metabolism and excretion organs have grown better compared to juvenile and

embryo. This explains our observation that toxicity level of *U. lobata*, defined by LC-50 value on embryo, is lower in juvenile than adults. Therefore, the use of *U. lobata* in pregnancy and infant should be considered carefully based on these safety issues[41,42]. Dose adjustment of this herb should be considered to adjust for its bioactivity, adverse reaction, and toxicity among the vulnerable groups.

According to *in silico* study, alkaloids like mangiferin and phytosterols like stigmasterol and β-sitosterol in *U. lobata* leaf extract are predicted to be toxic. These substances have the potential to interact with each other, thereby increasing the toxicity of the extract[38]. The interaction between the active compounds in this herb could also modulate their biological activity[15,31]. The compounded effects of the cytotoxic, pro-oxidant and damage to the pancreatic from the active compounds in *U. lobata* should be considered together in explaining its toxicity[28,43].

CONCLUSION:

Stigmasterol and β-sitosterol are predicted to be toxic in *U. lobata* leaf extract. The embryo is more sensitive to xenobiotic agents due to the lack of metabolic enzymes and immaturation of the metabolic and excretion organs. Therefore, *U. lobata* leaf extract could have teratogenic effects. Application of this extract among pregnant women and children should be cautioned.

ACKNOWLEDGEMENT:

This work was funded by Research Grant of Directorate General of Higher Education Indonesia.

CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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ISSN 0974-3618 (Print)
0974-360X (Online)

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RESEARCH ARTICLE

Acute Toxicity Level of Pulutan (*Urena lobata*) Leaf Extract on Zebrafish (*Danio rerio*) and its Analysis by *In Silico* Study

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ABSTRACT:
Pulutan (*Urena lobata*) is one of medicinal plant used to treat some diseases traditionally and pre-clinical studies have showed its efficacy. However, the study about its safety has not been evaluated completely. An acute toxicity test has to be performed in order to screen for its safety. The objective of study to determine the acute toxicity level of *Urena lobata* (*U. lobata*) leaf extract on embryo phase, juvenile and adult of zebrafish (*Danio rerio*) and the role of its active constituents through *in silico* methods. This was an experimental laboratory study using embryo, juvenile and adult of zebrafish (*Danio rerio*). The leaf of *U. lobata* was extracted by decoction methods and the extract was diluted from 12000 mg/L to 500 mg/L. The animals were exposed to the extracts for 96 hours. Toxicity level of herbs was defined using lethal concentration-50 (LC-50) obtained through linear regression. *In silico* study was performed using a web-based software application (iLAB ACD). The LC-50 values of *U. lobata* leaf extract for embryo, juvenile and adult of zebrafish (*Danio rerio*) were 2548 mg/L, 8748 mg/L and 8088 mg/L, respectively. Acute toxicity level of *U. lobata* on embryo is higher compared to juvenile and adult of zebrafish. After identification of active compound and *in silico* study was performed, Stigmasterol and β-sitosterol in *U. lobata* showed high toxicity level based on LD-50 value. Level toxicity of *U. lobata* on zebrafish embryo was moderate (0.5-5.0 g/L) and it shows teratogenic effect, meanwhile, its effects on juvenile and adult fish were considered mild (5.0-15.0 g/L).

KEYWORDS: Acute, *Danio rerio*, *in silico*, Toxicity, *Urena lobata*.

INTRODUCTION:
Pulutan (*Urena lobata*) is a medicinal plant commonly found in Indonesia. It has been used to treat many diseases, such as cough, malaria, wound, and diabetes empirically[1]. Pre-clinical studies of *U. lobata* confirmed that it has anti-diabetic properties by inhibiting alpha-amylase and alpha-glucosidase, broad-spectrum antibacterial and anti-anxiolytic effects[2,3,4]. The safety of *Urena lobata* must be ensured before using it as an alternative medicine. Previous studies showed that administration of *U. lobata* at 3000mg/kg bw for 28 days did not produce toxicity and death in rats. However, this herb increased hepatic enzyme and disrupted the structure of hepatocyte and sperm[5,6].

Received on 12.07.2020 Modified on 13.07.2021
Accepted on 01.04.2022
Research J. Pharm. and Tech. 2022; 15(6):2477-2482.
DOI: 10.52711/0974-360X.2022.00413

Meanwhile, the long-term exposure of herbs to rabbits showed that it could destroy of hepatocytes and obstruct the bile duct[7].

The toxicity of *U. lobata* must be examined and the lethal dose-50 (LD-50) or lethal concentration-50 (LC-50) should be determined as a parameter of safety for the herbs[7,8]. Despite the data on adult animals, its toxicity should be evaluated in the embryo and juvenile animals to determine the safety *U. lobata* across different life stages[8]. Embryonic evaluation ensures the safety of the herbs on the fetus and pregnancy period, meanwhile juvenile assessment certain its safety in the period of rapid growth and development of an organism[8,9]. Generally, organisms at these stages are more sensitive to xenobiotic agent, including herbs, compared to the adult phase.

The use of zebrafish (*Danio rerio*) as an animal model of toxicity test offers many advantages because they are sensitive to poison and easy to breed. Since the embryo is transparent, it is easy to observe their internal organs[9]. Almost 70 % of the genes in human are found on *D. rerio*, implying that human diseases can be replicated using this animal[10]. The study aims to evaluate acute toxicity level of *U. lobata* leaf extract on embryo phase, juvenile, and adult of zebrafish (*Danio rerio*) and also analyse it by *in silico* approach.

MATERIAL AND METHODS:

Chemical Sample:

First, Embryonic solution containing magnesium sulfate, sodium chloride, potassium chloride, calcium chloride dihydrate is solved in distilled water. All of the chemicals are purchased from Sigma Aldrich and Merck. Others materials include Methylene Blue (Sakkai Pro), Tetramin (Tropical) Artemia (Golden west).

Sample Preparation:

U. lobata leaf powder was obtained from Materia Medika, Batu, Malang, Indonesia with certificate number 074/306/101.8/2016. The powdered plant materials were extracted using decoction methods with ratio herbs and solvent 1:5. The extract was diluted into several concentrations for toxicity test and for the identification of active compounds.

Identification of Active Constituen:

Water extract of *U. lobata* was analysed on a semi-qualitative scale using Liquid Chromatography-Mass Spectra (LC-MS/MS) Accela 1250 pump. The liquid phase contains 0.1 % formic acid in solvent combination (methanol and water). The identification included 10 active substances target from alkaloid (mangiferin), phytosterol (stigmasterol, beta-sitosterol) and flavonoid (luteolin, quercetin, kaempferol, gossypetin, apigenin, chrysoeriol, hypocretin) groups.

Toxicity Analysis:

Active compounds in *U. lobata* leaf extracts were evaluated its toxicity test based on LD-50 value using *in silico* study with a web-based software application (iLAB ACD). It gives a predictive value to determine the dose for *in vivo* study and to confirm its results.

Acute Toxicity Test:

The assay was based on OECD[11,12] with slight modifications. It was performed in 24 microwell plates for the embryo of *D. rerio*, while the assessment of both juvenile and adult was performed in an aquarium. The treatment was performed in three replicates and each replicate consist of 10 embryos or fish. The *U. lobata* leaf extract was given for 96 hours and the extract was replaced every 24 hours. Death of embryo, juvenile and fish was calculated every 24 hours.

Analytical Studies:

For acute toxicity test, the percentage of death are expressed as the mean±SD and the LC-50 value was calculated using a linear regression curve using SPPS version 16.0.

RESULTS AND DISCUSSION:

Identification of Active Constituent in *U. lobata* Leaf Extracts:

The active compounds of *U. lobata* leaf extract, can be seen in (Fig. 1) and (Table 1). Analysis using LC-MS/MS was obtained that the most abundant active constituent in *U. lobata* extract were stigmasterol and gossypetin. Meanwhile, active constituent presented in low concentrations, like mangiferin, β-sitosterol, and chrysoeriol, were also identified in the aqueous extracts of *U. lobata*.

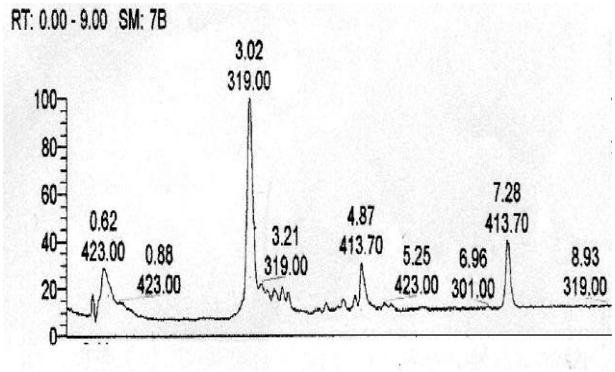

Fig.1: Chromatogram of active constituent in *U. lobata* leaf extracts.

Table 1: Active constituent in *U. lobata* leaf extracts

No	Active compounds	Molecule weight	Result
1	Stigmasterol	413	(++)
2	β-Sitosterol	415	(+)
3	Mangiferin	423	(+)
4	Quercetine	303	(-)
5	Kaempferol	286	(-)
6	Hypolaetin	302	(-)
7	Gossypetin	318	(+++)
8	Luteolin	286	(-)
9	Apigenin	270	(-)
10	Chrysoeriol	300	(+)

Note ; (-): negative, (+): weak, (++): moderate, (+++): strong

Five active constituent were found in the water extract of *U. lobata* leaf. They are gossypetin, stigmasterol, mangiferin, and chrysoeriol. All of them are non-nutrition substances and have pharmacology effect[13,14]. However, like any drugs, they induced adverse reaction and toxic effect in high dose and long-term administration. They may also induce drug-drug interaction[15].

Stigmasterol and β-sitosterol are phytosterols that are soluble in most organic solvents and contain one alcohol

functional group. Pre-clinical studies on animals treated by stigmasterol showed that both of cholesterol and sitosterol absorption decreased by 23% and 30%, respectively, over 6 weeks. It also possesses a potential hypoglycemic, antioxidant and anti-thyroid properties[16,17]. β-sitosterol are hydrophobic and soluble in ethanol and chloroform but insoluble in water[18]. Studies showed that it possessed anti-cholesterol, anti-inflammatory and immunomodulatory effects[19].

However, other studies showed that oxidized products of stigmasterol and β-sitosterol increased apoptotic nuclei on hepatoma cells culture and inhibited the growth of cells through cytotoxic effects[20]. No evidence of genotoxicity and mutagenicity of phytosterol was observed. However, toxicity studies on them were limited to 90-day sub chronic toxicity studies and a 2-generation reproductive toxicity study in rats[21]. In another 90-days study reported by *Kim et al., 2002*[22], suppression of body weight gain in rodents of both sexes and infiltration of mononuclear cell in the heart in males at a dose level of 9g phytosterol esters/kg bw/day were observed. The NOAEL derived from the 90-day sub-chronic toxicity studies in rats and the 2-generation reproductive toxicity study in rats amounted to be 2.5-6.6g phytosterol ester/kg bw/day, 1.54-4.1g phytosterol/kg bw/day and 335-900mg stigmasterol/kg bw/day[21].

Gossypetin and chrysoeriol are flavonol or flavone obtained from the flowers and the calyx of hibiscus species. Gossypetin shows a high antioxidant, anti-mutagenic, anti-microbial and anti-atherosclerotic effects[23]. This compound is very soluble in benzene and chloroform and also moderately soluble in ether and ethanol but insoluble in water. Meanwhile, chrysoeriol is a flavone that provides many health-promoting benefits such as anti-inflammation, anti-cancer, and anti-histamine. It is soluble in alkalies solution and sufficiently soluble in water[24]. On the other hand, flavonoid has potentially toxic effects, such as pro-oxidant activity, mitochondrial toxicity, and interaction with drug-metabolizing enzymes. Flavonoid can change into radicals compound after scavenging free radical, thus increasing stress oxidative and disrupting mitochondria[25]. Interaction of flavonoid with other active compounds or drugs can alter metabolism enzyme expression and modulate their activity.

Mangiferin is a xanthonoid and a glucoside of norathyriol. Mangiferin is soluble in hot dilutes methanol and ethanol but insoluble in water. The laboratory study has identified a variety of pharmacology effects of mangiferin, including antioxidant, anti-microbial and hypoglycemic effect in rodents[26,27]. In acute toxicity study, no effects were found after dermal exposure to mangiferin 2000mg/kg. However, flank position, transient dyspnea and piloerection were found after oral administration of this xanthone. Intraperitoneal administration in mice induced similar toxicity signs with possible mortality in rodents. Orally treated on rat with mangiferin (250-1000mg/kg) for 28 days did not obtain any abnormal clinical signs or hematology alteration, when compared to control group[28]. Histopathological alterations like necrosis, vacuolar degeneration, and increment of apoptosis of the acinar cells were found in the exocrine pancreas of rats at 1000 mg/kg. This suggested that exocrine pancreas was the target organ for mangiferin toxicity[28].

The composition of active constituent the extract was depended on polarity solute and the choice of extract solvent. The polarity of the extracting solvent determines the composition of an active compound by influencing their solubility in the solvent. The alkaloids, terpenoids, and steroids are soluble in the non-polar solvent like acetone, diethyl ether and hexane. Meanwhile, flavonoids, phenols, and glycosides dissolve better in a polar solvent, such as water and methanol[29,30]. Non-nutrition compounds or secondary metabolites like alkaloid, terpenoid, flavonoid, and steroid are in smaller quantity and they have pharmacologic effects given in appropriate doses[30,31]. Secondary metabolites are derived from the metabolism of the primary metabolites in plants, however, sometimes they have a toxic effect, especially when used in high dose. Most of flavonoid and terpenoid in herbs have potency as antioxidant, antiseptic and anti-inflammatory whereas steroids can act as anti-inflammatory and sex hormone[31].

Toxicity Analysis of *U. lobata* Leaf Extracts:

The toxicity of the active constituent from *U. lobata* leaf extracts was evaluated based on LD-50 values by *in silico* study and the results are depicted in Table 2. Stigmasterol and β-sitosterol had a low LD-50 value, indicating high toxicity in rats and mice. Intraperitoneal (i.p) administration of these compounds produced LD-50 lower than per oral (p.o) in the rodents. Toxicity analysis of active compounds in *U. lobata* indicated borderline to moderate reliability level.

Stigmasterol, β-sitosterol and mangiferin in *U. lobata* leaf extract were predicted as a toxic substance through *in silico* study. Plant sterols, such as stigmasterol and β-sitosterol, have cytotoxic effect[20,21], anti-diabetic[2,32]. Meanwhile, mangiferin is toxic to the exocrine pancreas of the rats as explained above[28]. They contribute to the overall the toxic effects of *U. lobata* in animal testings.

Table 2: Analysis of toxicity active compound in *U. lobata* leaf extracts [33]

Active compounds	LD-50 (mg/kg)			
	mouse (i.p)	mouse (p.o)	rat (i.p)	rat (p.o)
Stigmasterol	160**	530*	170**	1400**
β-Sitosterol	110**	570*	140*	740*
Mangiferin	460*	1500**	160**	1900
Gossypetin	490*	550**	710	600*
Chrysoeriol	290	1100*	700**	1300**

**( ): not reliable, (*): borderline, (**): moderate

Intraperitoneal administration of active compounds in *U. lobata* resulted in LD-50 value lower than oral administration. The oral administration will subject the substances to biotransformation process at the liver, such as oxidation, reduction, hydrolysis and conjugation, which may reduce its toxicity[15]. Detoxification of active substances occur at metabolism phase by microsomal hepatic[34]. The first-pass metabolism occurs more extensively for substances administered orally than intraperitoneally. Besides, the bioavailability of the substances is higher with intraperitoneal administration than oral route because they do not enter the gastrointestinal organs. Therefore, the damage by digestive and biotransformation enzymes is avoided[34]. The bioactivity of herbs in the organism is prolonged, meanwhile, the accumulation and toxicity are increased[35]. Biotransformation is needed to decrease the toxicity of xenobiotics such as drugs, herbs, and chemical substances[34]. However, the biotransformation process may produce a more active metabolite or the same activity with the parent drug. Moreover, it can modulate the activity of the compounds as well as the toxicity[15,36].

The LD-50 of *U. lobata* is higher in mice than in the rats. It is postulated that the organ capacity to eliminate xenobiotic is limited in mice compared to the rat, thus, the accumulation of active metabolite and toxicity risk are increased[37]. Elimination processes, including metabolism and excretion, aims to decrease the bioactivity of active substances[15]. The liver is a major organ of metabolisms, while kidney, gastrointestinal lumen, blood, and lung eliminate the substances[34,37].

Acute Toxicity Level of *Urena lobata* Extract:

Toxicity level of *U. lobata* leaf extract on embryo, juvenile and adult of zebrafish were shown in Table 3, 4 and 5. LC-50 values of *U. lobata* leaf extract were the lowest in embryo (2548.79mg/L), indicating moderate toxic level, whereas on juvenile (8748.45mg/L) and adult zebrafish (8088.11mg/L), the toxicity level was mild.

Table 3: Acute toxicity level of *U. lobata* leaf extract on embryo *D. rerio*

Concentration (mg/L)	n	Number of death (%)
1000	3	0.00 ± 0.00
1500	3	3.33 ± 0.58
2500	3	50.00 ± 0.58
3000	3	70.00 ± 1.15
4000	3	100.00 ± 0.58
		LC-50 = 2548.79 mg/L Level Toxicity = Moderate

Table 4: Acute toxicity level of *U. lobata* leaf extract on juvenile *D. rerio*

Concentration (mg/L)	n	Number of death (%)
2000	3	0.00 ± 0.00
6000	3	10.00 ± 0.00
8000	3	30.00 ± 0.00
10000	3	70.00 ± 0.00
12000	3	83.33 ± 5.77
		LC-50 = 8748.45 mg/L Level Toxicity = Mild

Table 5: Acute toxicity level of *U. lobata* leaf extract on adult *D. rerio*

Concentration (mg/L)	n	Number of death (%)
500	3	0.00 ± 0.00
2000	3	0.00 ± 0.00
6000	3	40.00 ± 0.00
8000	3	43.33 ± 0.58
12000	3	80.00 ± 0.00
		LC-50 = 8088.11 mg/L Level Toxicity = Mild

The toxicity level of *U. lobata* leaf extract on embryo is higher compared to juvenile and adult of zebrafish. The embryo is more sensitive to the active constituent of *U. lobata* leaf extract due to the lack of metabolism enzyme and the immaturity of metabolism or excretion organ system thus increasing the toxicity risk[15,38]. There is relatively lower level of plasma protein in the embryo, and this caused a higher level of the free drugs circulating in the blood, leading to the increased toxicity risk. Detoxifying enzymes, like glucuronidase, is also limited in an embryo; thus, the active compounds are less inactivated by the metabolic processes[34,39]. This results in increased bioactivity and toxicity risk also. With regards to the excretion process, glomerular filtration rate is lower in embryo compared to the adult organism due to the under-developed excretion organs[40]. Hence, the performance of the organ systems to eliminate toxic metabolites is reduced[15]. Whereas for the adult organisms, both their metabolism and excretion organs have grown better compared to juvenile and

embryo. This explains our observation that toxicity level of *U. lobata*, defined by LC-50 value on embryo, is lower in juvenile than adults. Therefore, the use of *U. lobata* in pregnancy and infant should be considered carefully based on these safety issues[41,42]. Dose adjustment of this herb should be considered to adjust for its bioactivity, adverse reaction, and toxicity among the vulnerable groups.

According to *in silico* study, alkaloids like mangiferin and phytosterols like stigmasterol and β-sitosterol in *U. lobata* leaf extract are predicted to be toxic. These substances have the potential to interact with each other, thereby increasing the toxicity of the extract[38]. The interaction between the active compounds in this herb could also modulate their biological activity[15,31]. The compounded effects of the cytotoxic, pro-oxidant and damage to the pancreatic from the active compounds in *U. lobata* should be considered together in explaining its toxicity[28,43].

CONCLUSION:

Stigmasterol and β-sitosterol are predicted to be toxic in *U. lobata* leaf extract. The embryo is more sensitive to xenobiotic agents due to the lack of metabolic enzymes and immaturation of the metabolic and excretion organs. Therefore, *U. lobata* leaf extract could have teratogenic effects. Application of this extract among pregnant women and children should be cautioned.

ACKNOWLEDGEMENT:

This work was funded by Research Grant of Directorate General of Higher Education Indonesia.

CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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