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DIREVIEW

Effect of Pulutan (*Urena lobata*) Leaf Extract on Blood Glucose Level, Hemoglobin and Body Growth of Zebra Fish (*Danio rerio*) Exposed to Malathion

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ABSTRACT

Pulutan (*Urena lobata*) is a medicinal plant having antioxidant activity. However, the potency of herbs to inhibit the adverse effects of pesticides malathion have not been evaluated. The study aims to examine the effect of *Urena lobata* (*U. lobata*) leaves extract on blood glucose level, hemoglobin, and body growth of zebrafish (*Danio rerio*) exposed to malathion chronically.

The study used juvenile and adult of *Danio rerio* (*D.rerio*) which were divided into five groups (n=5). The leaves of *U. lobata* were extracted by the decoction method. Concentrations of 125-500 mg/L of the extract were used. The *D. rerio* was exposed to extract for 40 days concomitant with malathion 2.5-5 mg/L. Blood glucose level and hemoglobin were measured using a commercially available glucometer and Hb-meter, respectively, meanwhile body weight and length was using a balance scale and rule, respectively. All data are expressed as the mean ± SD and analyzed with one-way ANOVA followed by LSD test ($p<0.05$).

The administration of *U. lobata* leaves extract increased the body weight by about 40% - 90 % ($p<0.05$) on juveniles, meanwhile in adult *D. rerio* were not increased, whereas there was the body length increase both for the juvenile and adult *D. rerio* up to 20 %. The blood glucose level was decreased by 40% - 60% ($p<0.05$) for juveniles given *U. lobata*,

meanwhile their adult *D. rerio* were reduced by 50% - 60%. *U. lobata* extract inhibited a decrease of hemoglobin level about 10% - 40% in juvenile *D. rerio* and 10% - 30% in adult. *U. lobata* leaves extract can inhibit the decrease of body growth, hemoglobin level and prevent the increase of blood glucose level in *D. rerio*.

Keywords: endocrine disruptor, herbs, hormone, pesticides

Introduction

Malathion is one of the organophosphate pesticides having a moderate toxicity level; therefore, it is used more by people especially in the agricultural sector.[1] Malathion could enter into the body through three routes, i.e. orally, inhalation, and topical, moreover they are metabolized and produce malaoxon and free radical substance.[2] Beside as an acetylcholinesterase inhibitor, malathion could impair secretion, synthesis, action, transport, binding, and elimination of natural hormones in the body. They are responsible for homeostasis, normal cell metabolism, reproduction, growth and development.[3] In animals, malathion is a known endocrine disruptor, teratogen, and reproductive toxin.[4-5] Free radicals resulted by malathion metabolism cause oxidative stress and damage in tissue; therefore, it increases their toxicity.[6]

Pulutan (*Urena lobata*) is a plant found in Indonesia and has been empirically used to cure many diseases such as malaria, wound, and diabetes.[7] Pre-clinical study of *Urena lobata* (*U. lobata*) showed the activity as anti-diabetic by inhibition of Dipeptidyl peptidase-4 (DPP-4), broad-spectrum analgetic, and anti-anxiolytic.[8-9] Other research indicated that *U. lobata* inhibits the increase of free radicals such as superoxide radicals, hydroxyl radicals, and lipid peroxidation.[10-11] Active substances in *U. lobata* such as mangiferin, gossypetin, and quercetin are predicted as lead compounds. [7-8] There are no reports on *U. lobata* potency on blood glucose level, hemoglobin, and body growth exposed to malathion chronically.

Zebrafish (*Danio rerio*) can be used as an animal model for toxicity tests. The use of *Danio rerio* (*D. rerio*) has many advantages, such as sensitivity to poison, ease to breed and the embryo is transparent; therefore, it is easy to observe the internal organ.[12-13] Moreover, almost 70% gene encoding in a human is found in *D. rerio;* therefore, it represents more about the condition in human.[14] The report of *U. lobata* inhibiting endocrine disruptor due to pesticide is limited and not complete. The study examined the effect of *U. lobata* leaf extract on blood glucose level, hemoglobin, and body growth of *D. rerio* exposed to malathion chronically.

Material and Method

Materials

Chemical sample

Aquades (Brataco), Malathion (Riger, 2044977) Methylene Blue (E.Merck, 2005152), Tetramin (Tropical, 90001250).

Method

U. lobata leaf extract preparation

U. lobata leaf powder was obtained from Materia Medika Batu Malang on January 8th, 2019, with voucher number 074/096A/102.7/2019. Approximately 5 g of the powdered plant materials were extracted by decoction methods in 500 ml water at 90ºC for 30 minutes. *U. lobata* leaf extract was given in three doses 125 mg/L, 250 mg/L, and 500 mg/L for 40 days concomitant with malathion.

Malathion

Malathion was taken by micropipette and diluted with water and obtained a concentration of 2.5 mg/L for juvenile and 5 mg/L for adult *D.rerio*. The dose of malathion refers to Cook et al. (2005) with slightly modification. Malathion was administrated on the control group and three test groups over 40 days.

Animal and treatment

The zebrafish (*D. rerio*) was obtained from local breeding with voucher number 005/ULMKILP/UA.FPK/03/2019. The assay was based on OECD (2018)[15-16] with slight modifications. Both juvenile and adult *D. rerio* were divided into two control groups and three test groups (n=5). The *U. lobata* leaf extract was given for 40 days concomitant with malathion 2.5 - 5 mg/L.[28]

Blood glucose level

The blood sample was collected from the tail vein of *D.rerio* after overnight fasting. They were measured immediately using a commercially available glucometer and recorded in g/dL.

Hemoglobin level

The collected blood from *D.rerio* was dripped into a commercial Hb meter and recorded in g/dL.

Body Growth level

The body weight and body length were used to evaluate the growth level. Body weight was measured by balance scale and recorded in milligram (mg), meanwhile, body length was measured using a ruler and recorded in millimeter (mm).

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA. The least significant difference (LSD) test was used for mean comparisons and then p-value < 0.05 was considered to be statistically significant.

Results and Discussion

Effect of U. lobata leaf extract on blood glucose level of D. rerio exposed to malathion

The blood glucose level of *D.rerio* exposed to malathion are shown in Figure 1. Exposure to malathion increased blood glucose levels both of juvenile and adult *D. rerio* up to 60% compared to the normal group ($p<0.05$). The blood glucose level was decreased by 40%, 60%, and 40% ($p<0.05$), respectively, in a juvenile that was given *U. lobata* at the dose of 125 mg/L, 250 mg/L, and 500 mg/L, meanwhile in the adult *D. rerio* were reduced 60%, 50% and 50%, respectively ($p<0.05$).

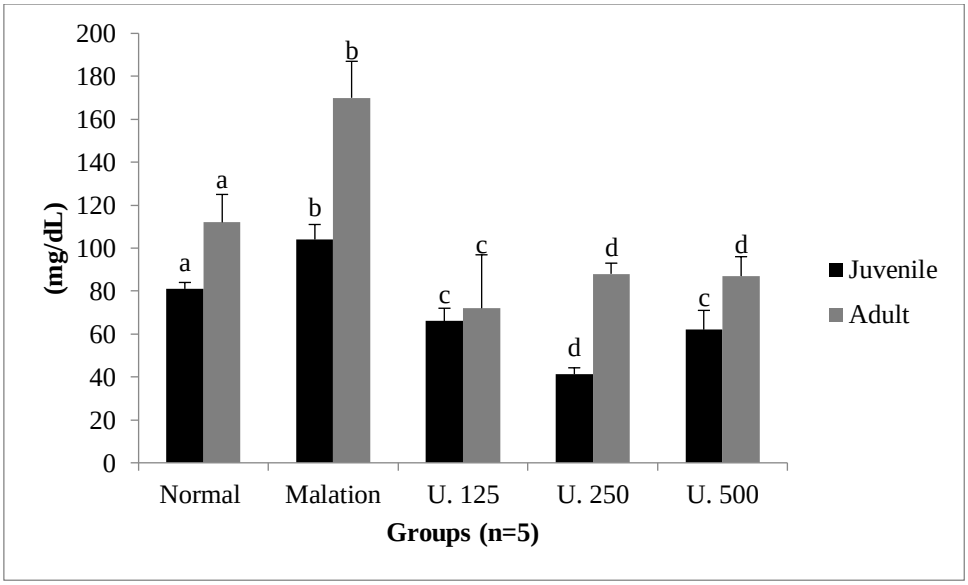

Figure 1: Effect of *U. lobata* leaf extract on blood glucose level of *D. rerio*.

Malathion disrupts the underlying endocrine mechanism, responsible for carbohydrate metabolism and causes a degenerative change in pancreatic islets through disruption of islets' mitochondrial function.[17] Administration of malathion chronically results in insulin resistance; therefore, it could increase insulin secretion by the pancreatic islet. It is shown by the increase of insulin concentration in plasma.[17] Hyperinsulinemia causes fatigue in pancreatic beta cells; therefore. the insulin production could be decreased.[18] Molecular mechanisms of insulin resistance, serine phosphorylation of insulin receptor substrate-1 and increased expression of p85-alpha, are the two sides of the coin. This condition increases blood glucose level or hyperglycemia due to interference of insulin secretion.[19]

Malathion is metabolized into malaoxon and free radicals, meanwhile acetylcholinesterase inhibitor activity of malaoxon is higher than the parent compound.[20] Inhibition of its enzyme will increase acetylcholine and stimulate muscarinic receptors; therefore, it causes bradypnea. This condition activates hypoxia-inducible factor-1 (HIF-1) and caspase-3 having a role in apoptosis of β-cells pancreas. Free radicals produced by malathion metabolism will disrupt tissue and result in cytokine pro-inflammatory, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. This cytokine stimulates apoptosis of β-cells pancreas through nuclear factor-kappa–β (NF-kB). It contributes to insulin deficiency and, moreover, increases blood glucose levels.[21] Human pancreatic islet cell destruction by cytokine involves oxygen free radicals and aldehyde production. Free radicals also decrease glucose transporter-4 (GLUT-4) through oxidative stress. GLUT-4 is a major transporter of glucose and the disruption of them causes insulin resistance.[22] GLUT-4 expression in response to oxidative stress is associated with reciprocal alterations in C/EBP alpha and delta isoform in 3T3-L1 adipocytes.

The administration of *U. lobata* leaf extract decreased blood glucose levels both in juvenile and adult *D.rerio* exposed to malathion. *U. lobata* contain active compounds such as stigmasterol, β-sitosterol, gossypetin, mangiferin, and chrysoeriol having pharmacology effects.[31] Stigmasterol and β-sitosterol inhibit DPP-4 activity; therefore, they prevent the degradation of active GLP-1 having functions in stimulating the secretion of insulin by

cAMP activation, increasing β-cell masses by MAPK pathway, and inhibiting the secretion of glucagon.[7,31,32] Mangiferin also has an anti-diabetic effect through inhibiting oxidative stress in pancreatic tissue; therefore, its damage can be prevented.[33] They contribute to decreasing the blood glucose level of *D. rerio* exposed by malathion. Gossypetin and mangiferin indicate antioxidant potency by donating an electron and scavenging free radicals.[33-35] Stigmasterol in *U. lobata* also has antioxidant activity by inhibiting lipid peroxidation or anti-peroxidative.[36] This activity protects islet pancreatic from damage caused by free radicals from malathion exposure. The protection will maintain β–cell pancreas to produce insulin hormone for controlling blood glucose levels. Chrysoeriol and β-sitosterol have potency as anti-inflammatory through inhibiting both cells and cytokine pro inflammatory.[37-38]

The anti-inflammation effect will prevent damage of tissue, including β–cell pancreas, moreover, they are able to produce insulin which is used to regulate blood glucose level.[36] In this study, the increase of dose reduces *U. lobata* activity to regulate blood glucose level both in juvenile and adult *D. rerio* generally. It is related to the desensitization of receptors if they were given substance in high dose and long term.[39] The exception for *U. lobata* 250 mg/ld. in juvenile, is that increasing of dose intensifies the potency to decrease blood glucose level. This is consistent with the pharmacology theory, that an increase in dose will elevate the activity.

Effect of U. lobata leaf extract on Hemoglobin level of D. rerio exposed to malathion

The hemoglobin level of *D. rerio* exposed to malathion is shown in Figure 2. The expose of malathion decreases hemoglobin levels in both juvenile and adult *D. rerio* up to 20% compared to the normal group ($p<0.05$). The decreased hemoglobin levels were reduced by 10%, 40%, and 20% ($p<0.05$), respectively in a juvenile that was given *U. lobata* at the dose of 125 mg/L, 250 mg/L, and 500 mg/L, meanwhile in the adult of *D. rerio* were reduced 10%, 30% and 20%, respectively ($p<0.05$).

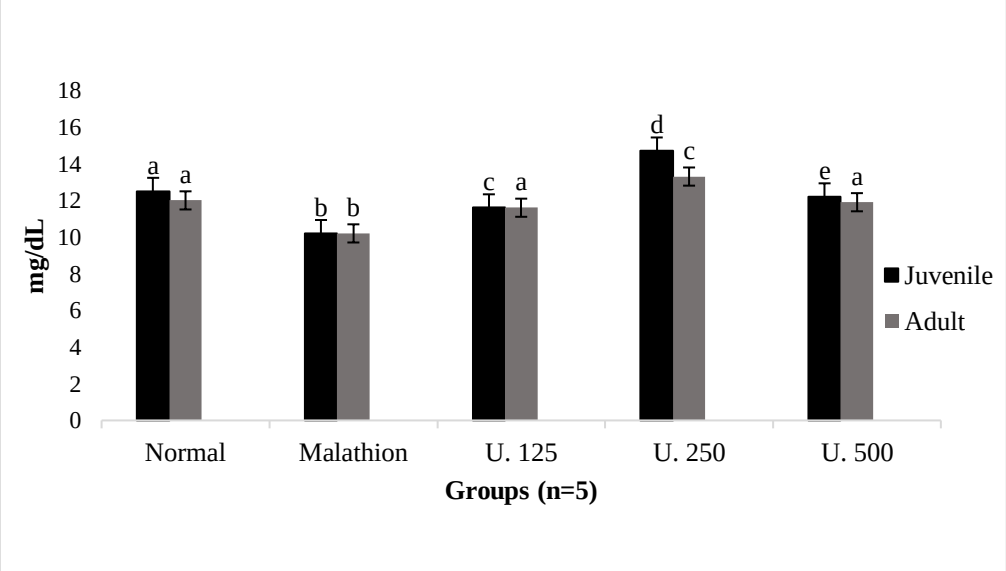

Figure 2: Effect of *U. lobata* leaf extract on hemoglobin level of *D. rerio*

Chronic malathion exposure to *D. rerio* decreases hemoglobin levels and promotes deformation of red blood cells. It is caused by malathion working as an acetylcholinesterase inhibitor and free radical compound which is produced from the metabolism process. Malaoxon causes overstimulating both of muscarinic receptor and nicotinic caused by the hypoxic condition. Chronic deoxygenation stimulates the release of Ankyrin and band 3 protein chain; therefore, followed by the release of spectrin and actin chain from erythrocyte membrane. It can increase hemolysis risk caused by the decrease of mechanical support by the cytoskeleton.[23] On the other hand, the increase of Reactive Oxygen Species (ROS) causes lipid peroxidation of erythrocyte membrane resulting from the decrease of membrane integrity. This condition increases the risk of hemolysis and, therefore, it decreases hemoglobin level.[24]

The potential of anti-anemia was shown by *U. lobata* extract by preventing the decrease of hemoglobin level of *D. rerio* exposed to malathion.[40] Quercetin is one of the compounds from *U. lobata*, a flavonoid compound that has antioxidant activity as well as being able to modulate expressions of antioxidant enzymes such as catalase and superoxide dismutase and also increases glutathione levels intracellular. Increasing of free radical level will stimulate quercetin to be oxidized and free radicals react with glutathione as well as thiol groups protein.[40] Another compound is glutathione which has a platform directly to protect protein and maintain membrane stability of red blood cells.[41] Quercetin also is a chelating agent of heavy metal. Some heavy metals will increase the rate of biochemical reaction and disrupt the stability of biological components, moreover, they must be bound by a chelating agent. Iron is an essential element in mitochondrial electron transfer; the deficiency of iron causes changes in cell metabolism and anemia. In the development of red blood cells, iron plays an important role in oxygen transport and is active in the process of proliferation and differentiation of hematopoietic stem cells.[40] Besides antioxidants, quercetin also acts as an anti-inflammatory by reducing TNF-α level; therefore, it prevents hemolysis through apoptosis pathway and inhibits a decrease of hemoglobin (Hb) level.[42] Antioxidant activity contributes to preventing rupture of the erythrocyte membrane, moreover, hemoglobin leakage is avoided outside of the erythrocyte. The antioxidant compound can stabilize the erythrocyte membrane from damage caused by free radicals; therefore, it reduces hemolysis risk and inhibits a decrease of hemoglobin level.[40]

Effect of U. lobata leaf extract on body growth of D. rerio exposed to malathion

The bodyweight of *D. rerio* exposed to malathion is shown in Figure 3, and body length in Figure 4. The exposure of malathion inhibited the increase of body weight and body length of *D. rerio* compared to the normal group ($p<0.05$); however, the body length of the juvenile was not inhibited by malathion. The administration of *U. lobata* leaf extract at the dose of 125 mg/L, 250 mg/L, and 500 mg/L increased the bodyweight about 40%, 70%, and 90% ($p<0.05$), respectively, in juvenile but showed no increase in the adult of *D. rerio* ($p>0.05$). Whereas the body length was increased both for juvenile and adult *D. rerio* up to 20% ($p<0.05$).

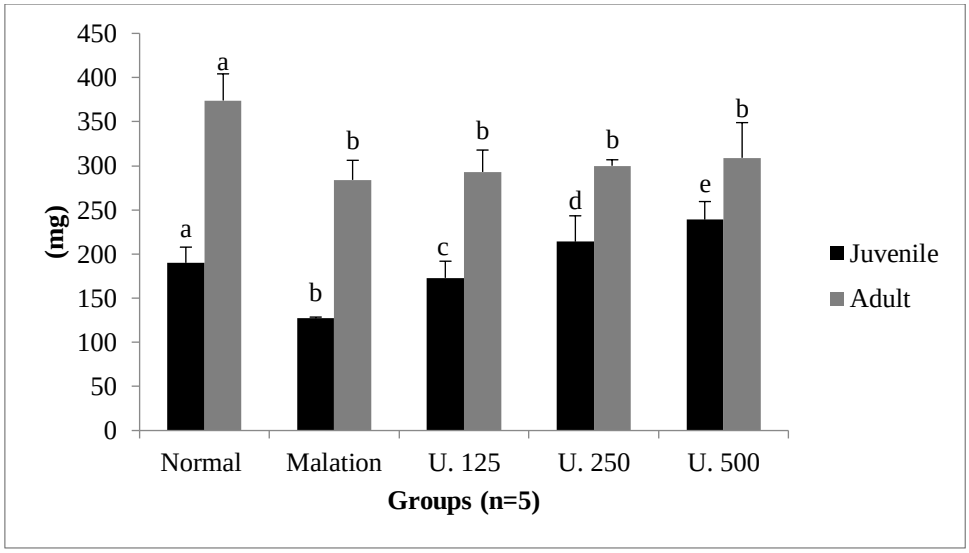

Figure 3: Bodyweight *D. rerio*

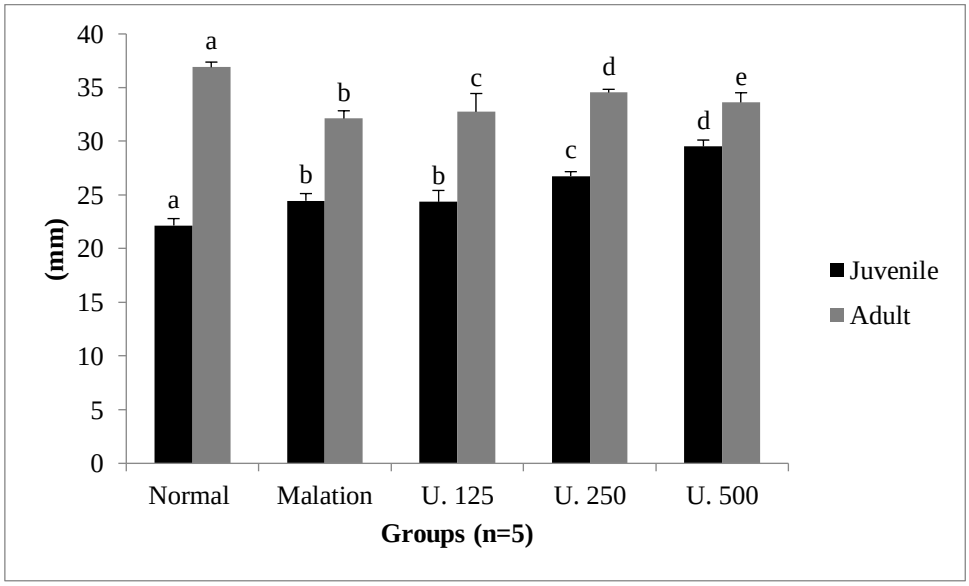

Figure 4: Body length *D. rerio*

Malathion disrupts the underlying endocrine mechanism, responsible for somatic growth. Exposure to malathion also influences the hormone that maintains the physiology of body growth. Malathion decreases both the thyroid hormone (T3 and T4) in plasma and inhibits its receptor binding, moreover, it reduces metabolic rate and body growth.[17,25-26] Perez Sanches and Lei Bail[27] have reported that hypothyroidism causes liver resistance to GH and affects hepatic insulin growth factor-1 (IGF-1) production. Growth hormone (GH) and IGF-1 decreases as a result of malathion exposure, which causes growth retardation.[17] Food consumption was reduced due to malathion exposure; therefore, it inhibits the body growth of the organism.[26] Malathion also induces lipolysis of body fat and, therefore, contributes to the bodyweight decrease.[28] Exposure to malathion results in a significantly shorter body length.[28]

GH, IGF-1, steroid, and thyroid hormone are well-known to increase growth in fishes. The growth-promoting action of GH is mediated through IGF-1; a positive correlation between

the IGF-1 and growth rate has been shown in several fish.[28-30] The significant decline in GH, IGF-1, thyroid hormone, and steroid in malathion-exposed contribute to reduced body growth and metabolism change.

U. lobata leaf extract increases the body growth of *D. rerio* exposed to malathion. Active compounds in *U. lobata* contribute to retaining the bioavailability of incretin hormone through inhibition of DPP-4 activity. Incretin hormone increases the secretion of insulin; therefore, it was able to inhibit lipolysis and controlling of body weight. Meanwhile, in the MAPK pathway, the incretin hormone increases cell proliferation, moreover, it supports the body growth of zebrafish *D. rerio* both of bodyweight and body length. Whereas, the antioxidant effect of *U. lobata* has a role to protect *D. rerio* against free radicals produced by malathion. Gossypetin in *U. lobata* leaf extract scavenges pro-oxidant substances causing oxidative damage in cells, moreover, it prevents growth retardation. Reports have indicated the effect associated with mangiferin, including antioxidant activity.[33] *U. lobata* neutralize malathion effect contributing to impaired hormone secretion, the hormone responsible for homeostasis, normal cell metabolism, reproduction, and development. Studies on laboratory animals treated by stigmasterol showed anti-inflammatory and immunomodulatory effects.[36,43] Anti-inflammatory substances of *U. lobata* inhibit cell damage which is caused by cytokine pro-inflammatory release. It is useful to support the body growth of *D. rerio* both of body weight and body length.

Conclusion

U. lobata leaf extract can inhibit the increase of blood glucose level and prevent the decrease of body growth and hemoglobin level both of juvenile and adult *D. rerio*.

Conflict of interest

The authors state that there is no conflict of interest.

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Point-to-point responses

Reviewer 1
Assistant Editor

No	Issues	Responses
Abstrak	Please add the percentage of ethanol in the title	I did not use ethanol in the extraction process, I used water for it.
	Pay attention to the tenses used, present tense or the past tense.	I have changed the tenses into past tense
	In the abstract, the word “herbs” should be replaced by “leaves “	I already replaced by “leaves”
	. There is an error in the word ‘‘iwere’’ which should be “were”.	I have fixed it
	The word “leaf” should be replaced by “leaves”	I have replaced it
introduction	It is necessary to add the urgency of research in the last paragraph.	I already added the urgency of this study
Methodology	. Give the product number/code of each chemical materials.	
	Decoction is not recommended as an extraction method because it uses high temperatures (90°), it can degrade the thermolabile compounds, including flavonoids. Why do you use the decoction as an extraction method?	I used decoction due to the method is simple, cheap and the solvent not toxic. The high temperature is used to increase extraction rate and eliminate toxic substance. However, one of the risk this method is the degradation of active compound.
	What does the amount of malathion refer to?	I used the dose of malathion refer to Cook et al, 2005 (reference number 28) I already put it in this section.
Result		
	The fish name must be written uniformly in all part of the article. For example, use "D*. rerio*” in all part, or “*Danio rerio*”, or “Zebra fish”.	I have repaired it
	There is an error in the word “125 mg/dL”, which should be “125 mg/L”.	I already fixed it
Figures	In figure 1 there is an error in the word of "Malathion".	I have replaced it

Reviewer 2

No	Issues	Responses
Abstrak	Rewrite the abstract and effect the corrections	I have repaired it
introduction	The introduction is ok. Effect the corrections	I already fixed it
Methodology	The methods chosen are appropriate for the study. Effect the corrections . Indicate standard references for the methods used	I have repaired it
Result	The figures of the results should be put after the discussion.	I have replaced it
Discussion	The discussion is ok. Effect the corrections	I have repaired it
Conclusion	The conclusion is supported by the results.	I already fixed it
Reference	Ensure that the references follow the journal format	I have repaired it
Figures, Tables	All the figures of the results should be put after the discussion section	I have repaired it

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Effect of Pulutan (*Urena lobata*) Leaf Extract on Blood Glucose Level, Hemoglobin and Body Growth of Zebra Fish (*Danio rerio*) Exposed to Malathion

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ABSTRACT

The study aims to evaluate the effect of *Urena lobata* (*U. lobata*) leaves extract on blood glucose level, hemoglobin, and body growth of zebrafish (*Danio rerio*) exposed to malathion chronically.

The study used juvenile and adult of *Danio rerio* (*D.rerio*) which were divided into five groups (n=5). The leaves of *U. lobata* were extracted by the decoction method. Concentrations of 125-500 mg/L of the extract were used. The *D. rerio* was exposed to extract for 40 days concomitant with malathion 2.5-5 mg/L. Blood glucose level and hemoglobin were measured using a commercially available glucometer and Hb-meter, respectively, meanwhile body weight and length was using a balance scale and rule, respectively. All data are expressed as the mean ± SD and analyzed with one-way ANOVA followed by LSD test ($p<0.05$).

The administration of *U. lobata* leaves extract increased the body weight by about 40% - 90 % ($p<0.05$) on juveniles, meanwhile in adult *D. rerio* were not increased, whereas there was the body length increase both for the juvenile and adult *D. rerio* up to 20 %. The blood glucose level was decreased by 40% - 60% ($p<0.05$) for juveniles given *U. lobata*, meanwhile their adult *D. rerio* were reduced by 50% - 60%. *U. lobata* extract inhibited a decrease of hemoglobin level about 10% - 40% in juvenile *D. rerio* and 10% - 30% in adult.

U. lobata leaves extract can inhibit the decrease of body growth, hemoglobin level and prevent the increase of blood glucose level in *D. rerio*.

Keywords: endocrine disruptor, herbs, hormone, pesticides

Introduction

Malathion is one of the organophosphate pesticides having a moderate toxicity level; therefore, it is used more by people especially in the agricultural sector.[1] Malathion could enter into the body through three routes, i.e. orally, inhalation, and topical, moreover they are metabolized and produce malaoxon and free radical substance.[2] Beside as an acetylcholinesterase inhibitor, malathion could impair secretion, synthesis, action, transport, binding, and elimination of natural hormones in the body. They are responsible for homeostasis, normal cell metabolism, reproduction, growth and development.[3] In animals, malathion is a known endocrine disruptor, teratogen, and reproductive toxin.[4-5] Free radicals resulted by malathion metabolism cause oxidative stress and damage in tissue; therefore, it increases their toxicity.[6]

Pulutan (*Urena lobata*) is a plant found in Indonesia and has been empirically used to cure many diseases such as malaria, wound, and diabetes.[7] Pre-clinical study of *Urena lobata* (*U. lobata*) showed the activity as anti-diabetic by inhibition of Dipeptidyl peptidase-4 (DPP-4), broad-spectrum analgetic, and anti-anxiolytic.[8-9] Other research indicated that *U. lobata* inhibits the increase of free radicals such as superoxide radicals, hydroxyl radicals, and lipid peroxidation.[10-11] Active substances in *U. lobata* such as mangiferin, gossypetin, and quercetin are predicted as lead compounds. [7-8] There are no reports on *U. lobata* potency on blood glucose level, hemoglobin, and body growth exposed to malathion chronically.

Zebrafish (*Danio rerio*) can be used as an animal model for toxicity tests. The use of *Danio rerio* (*D. rerio*) has many advantages, such as sensitivity to poison, ease to breed and the embryo is transparent; therefore, it is easy to observe the internal organ.[12-13] Moreover, almost 70% gene encoding in a human is found in *D. rerio;* therefore, it represents more about the condition in human.[14] The report of *U. lobata* inhibiting endocrine disruptor due to pesticide is limited and not complete. The study examined the effect of *U. lobata* leaf extract on blood glucose level, hemoglobin, and body growth of *D. rerio* exposed to malathion chronically.

Material and Method

Materials

Chemical sample

Aquades (Brataco), Malathion (Riger, 2044977) Methylene Blue (E.Merck, 2005152), Tetramin (Tropical, 90001250).

Method

U. lobata leaf extract preparation

U. lobata leaf powder was obtained from Materia Medika Batu Malang on January 8th, 2019, with voucher number 074/096A/102.7/2019. Approximately 5 g of the powdered plant materials were extracted by decoction methods in 500 ml water at 90ºC for 30 minutes. *U. lobata* leaf extract was given in three doses 125 mg/L, 250 mg/L, and 500 mg/L for 40 days concomitant with malathion.

Malathion

Malathion was taken by micropipette and diluted with water and obtained a concentration of 2.5 mg/L for juvenile and 5 mg/L for adult *D.rerio*. The dose of malathion refers to Cook et al. (2005) with slightly modification. Malathion was administrated on the control group and three test groups over 40 days.

Animal and treatment

The zebrafish (*D. rerio*) was obtained from local breeding with voucher number 005/ULMKILP/UA.FPK/03/2019. The assay was based on OECD (2018)[15-16] with slight modifications. Both juvenile and adult *D. rerio* were divided into two control groups and three test groups (n=5). The *U. lobata* leaf extract was given for 40 days concomitant with malathion 2.5 - 5 mg/L.[28]

Blood glucose level

The blood sample was collected from the tail vein of *D.rerio* after overnight fasting. They were measured immediately using a commercially available glucometer and recorded in g/dL.

Hemoglobin level

The collected blood from *D.rerio* was dripped into a commercial Hb meter and recorded in g/dL.

Body Growth level

The body weight and body length were used to evaluate the growth level. Body weight was measured by balance scale and recorded in milligram (mg), meanwhile, body length was measured using a ruler and recorded in millimeter (mm).

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA. The least significant difference (LSD) test was used for mean comparisons and then p-value < 0.05 was considered to be statistically significant.

Results and Discussion

Effect of U. lobata leaf extract on blood glucose level of D. rerio exposed to malathion

The blood glucose level of *D.rerio* exposed to malathion are shown in Figure 1. Exposure to malathion increased blood glucose levels both of juvenile and adult *D. rerio* up to 60% compared to the normal group ($p<0.05$). The blood glucose level was decreased by 40%, 60%, and 40% ($p<0.05$), respectively, in a juvenile that was given *U. lobata* at the dose of 125 mg/L, 250 mg/L, and 500 mg/L, meanwhile in the adult *D. rerio* were reduced 60%, 50% and 50%, respectively ($p<0.05$).

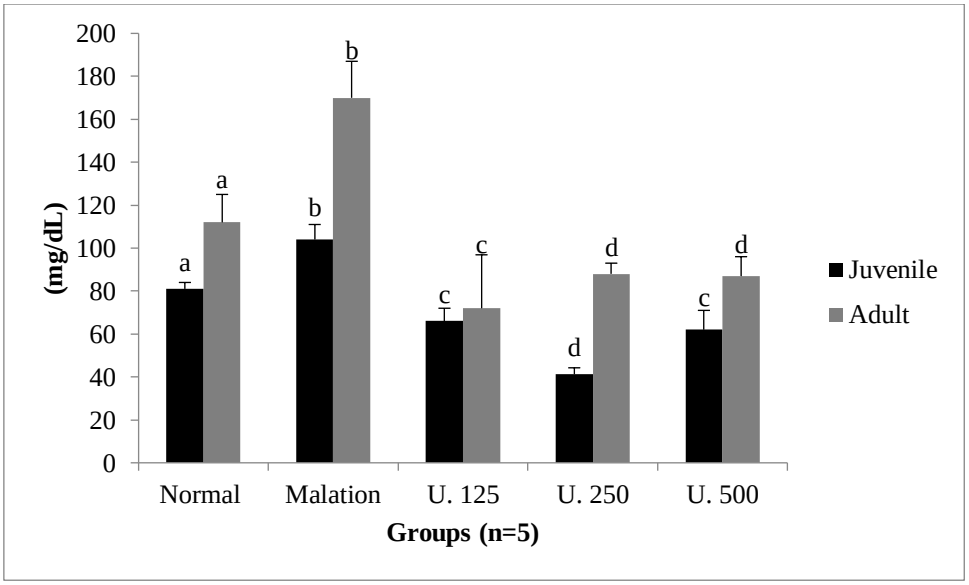

Figure 1: Effect of *U. lobata* leaf extract on blood glucose level of *D. rerio*.

Malathion disrupts the underlying endocrine mechanism, responsible for carbohydrate metabolism and causes a degenerative change in pancreatic islets through disruption of islets' mitochondrial function.[17] Administration of malathion chronically results in insulin resistance; therefore, it could increase insulin secretion by the pancreatic islet. It is shown by the increase of insulin concentration in plasma.[17] Hyperinsulinemia causes fatigue in pancreatic beta cells; therefore. the insulin production could be decreased.[18] Molecular mechanisms of insulin resistance, serine phosphorylation of insulin receptor substrate-1 and increased expression of p85-alpha, are the two sides of the coin. This condition increases blood glucose level or hyperglycemia due to interference of insulin secretion.[19]

Malathion is metabolized into malaoxon and free radicals, meanwhile acetylcholinesterase inhibitor activity of malaoxon is higher than the parent compound.[20] Inhibition of its enzyme will increase acetylcholine and stimulate muscarinic receptors; therefore, it causes bradypnea. This condition activates hypoxia-inducible factor-1 (HIF-1) and caspase-3 having a role in apoptosis of β-cells pancreas. Free radicals produced by malathion metabolism will disrupt tissue and result in cytokine pro-inflammatory, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. This cytokine stimulates apoptosis of β-cells pancreas through nuclear factor-kappa–β (NF-kB). It contributes to insulin deficiency and, moreover, increases blood glucose levels.[21] Human pancreatic islet cell destruction by cytokine involves oxygen free radicals and aldehyde production. Free radicals also decrease glucose transporter-4 (GLUT-4) through oxidative stress. GLUT-4 is a major transporter of glucose and the disruption of them causes insulin resistance.[22] GLUT-4 expression in response to oxidative stress is associated with reciprocal alterations in C/EBP alpha and delta isoform in 3T3-L1 adipocytes.

The administration of *U. lobata* leaf extract decreased blood glucose levels both in juvenile and adult *D.rerio* exposed to malathion. *U. lobata* contain active compounds such as stigmasterol, β-sitosterol, gossypetin, mangiferin, and chrysoeriol having pharmacology effects.[31] Stigmasterol and β-sitosterol inhibit DPP-4 activity; therefore, they prevent the degradation of active GLP-1 having functions in stimulating the secretion of insulin by

cAMP activation, increasing β-cell masses by MAPK pathway, and inhibiting the secretion of glucagon.[7,31,32] Mangiferin also has an anti-diabetic effect through inhibiting oxidative stress in pancreatic tissue; therefore, its damage can be prevented.[33] They contribute to decreasing the blood glucose level of *D. rerio* exposed by malathion. Gossypetin and mangiferin indicate antioxidant potency by donating an electron and scavenging free radicals.[33-35] Stigmasterol in *U. lobata* also has antioxidant activity by inhibiting lipid peroxidation or anti-peroxidative.[36] This activity protects islet pancreatic from damage caused by free radicals from malathion exposure. The protection will maintain β–cell pancreas to produce insulin hormone for controlling blood glucose levels. Chrysoeriol and β-sitosterol have potency as anti-inflammatory through inhibiting both cells and cytokine pro inflammatory.[37-38]

The anti-inflammation effect will prevent damage of tissue, including β–cell pancreas, moreover, they are able to produce insulin which is used to regulate blood glucose level.[36] In this study, the increase of dose reduces *U. lobata* activity to regulate blood glucose level both in juvenile and adult *D. rerio* generally. It is related to the desensitization of receptors if they were given substance in high dose and long term.[39] The exception for *U. lobata* 250 mg/ld. in juvenile, is that increasing of dose intensifies the potency to decrease blood glucose level. This is consistent with the pharmacology theory, that an increase in dose will elevate the activity.

Effect of U. lobata leaf extract on Hemoglobin level of D. rerio exposed to malathion

The hemoglobin level of *D. rerio* exposed to malathion is shown in Figure 2. The expose of malathion decreases hemoglobin levels in both juvenile and adult *D. rerio* up to 20% compared to the normal group ($p<0.05$). The decreased hemoglobin levels were reduced by 10%, 40%, and 20% ($p<0.05$), respectively in a juvenile that was given *U. lobata* at the dose of 125 mg/L, 250 mg/L, and 500 mg/L, meanwhile in the adult of *D. rerio* were reduced 10%, 30% and 20%, respectively ($p<0.05$).

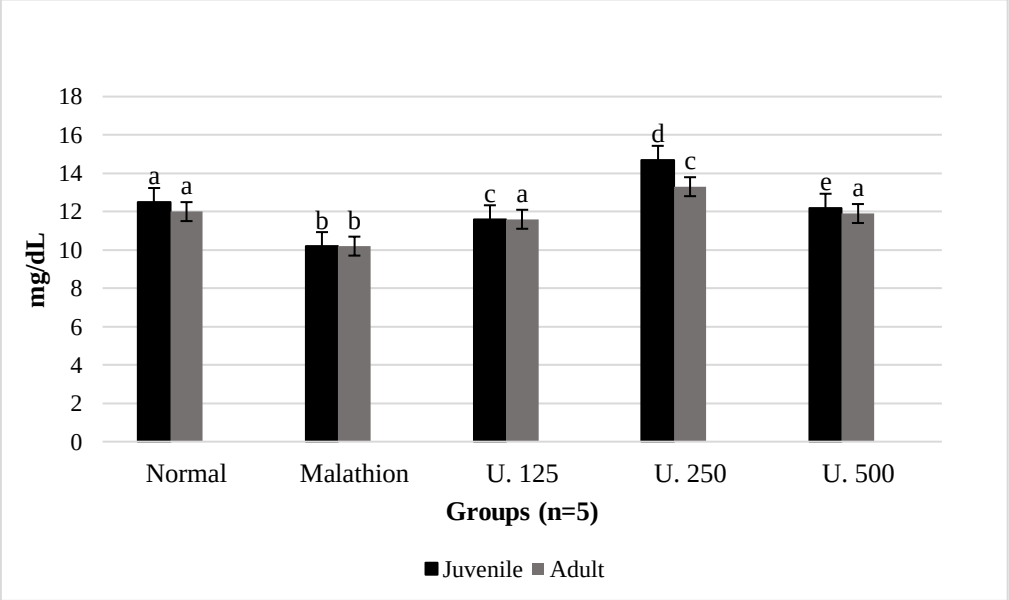

Figure 2: Effect of *U. lobata* leaf extract on hemoglobin level of *D. rerio*

Chronic malathion exposure to *D. rerio* decreases hemoglobin levels and promotes deformation of red blood cells. It is caused by malathion working as an acetylcholinesterase inhibitor and free radical compound which is produced from the metabolism process. Malaoxon causes overstimulating both of muscarinic receptor and nicotinic caused by the hypoxic condition. Chronic deoxygenation stimulates the release of Ankyrin and band 3 protein chain; therefore, followed by the release of spectrin and actin chain from erythrocyte membrane. It can increase hemolysis risk caused by the decrease of mechanical support by the cytoskeleton.[23] On the other hand, the increase of Reactive Oxygen Species (ROS) causes lipid peroxidation of erythrocyte membrane resulting from the decrease of membrane integrity. This condition increases the risk of hemolysis and, therefore, it decreases hemoglobin level.[24]

The potential of anti-anemia was shown by *U. lobata* extract by preventing the decrease of hemoglobin level of *D. rerio* exposed to malathion.[40] Quercetin is one of the compounds from *U. lobata*, a flavonoid compound that has antioxidant activity as well as being able to modulate expressions of antioxidant enzymes such as catalase and superoxide dismutase and also increases glutathione levels intracellular. Increasing of free radical level will stimulate quercetin to be oxidized and free radicals react with glutathione as well as thiol groups protein.[40] Another compound is glutathione which has a platform directly to protect protein and maintain membrane stability of red blood cells.[41] Quercetin also is a chelating agent of heavy metal. Some heavy metals will increase the rate of biochemical reaction and disrupt the stability of biological components, moreover, they must be bound by a chelating agent. Iron is an essential element in mitochondrial electron transfer; the deficiency of iron causes changes in cell metabolism and anemia. In the development of red blood cells, iron plays an important role in oxygen transport and is active in the process of proliferation and differentiation of hematopoietic stem cells.[40] Besides antioxidants, quercetin also acts as an anti-inflammatory by reducing TNF-α level; therefore, it prevents hemolysis through apoptosis pathway and inhibits a decrease of hemoglobin (Hb) level.[42] Antioxidant activity contributes to preventing rupture of the erythrocyte membrane, moreover, hemoglobin leakage is avoided outside of the erythrocyte. The antioxidant compound can stabilize the erythrocyte membrane from damage caused by free radicals; therefore, it reduces hemolysis risk and inhibits a decrease of hemoglobin level.[40]

Effect of U. lobata leaf extract on body growth of D. rerio exposed to malathion

The bodyweight of *D. rerio* exposed to malathion is shown in Figure 3, and body length in Figure 4. The exposure of malathion inhibited the increase of body weight and body length of *D. rerio* compared to the normal group ($p<0.05$); however, the body length of the juvenile was not inhibited by malathion. The administration of *U. lobata* leaf extract at the dose of 125 mg/L, 250 mg/L, and 500 mg/L increased the bodyweight about 40%, 70%, and 90% ($p<0.05$), respectively, in juvenile but showed no increase in the adult of *D. rerio* ($p>0.05$). Whereas the body length was increased both for juvenile and adult *D. rerio* up to 20% ($p<0.05$).

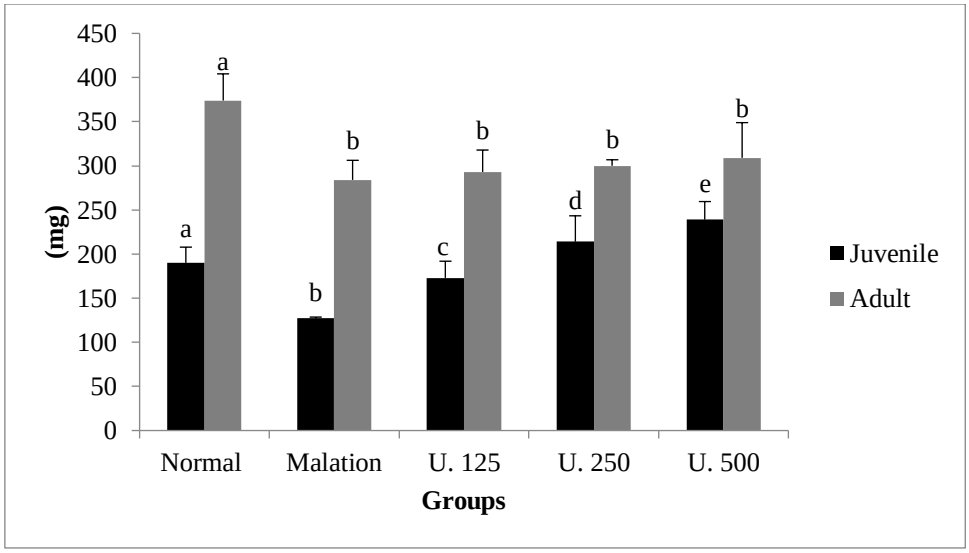

Figure 3: Bodyweight *D. rerio*

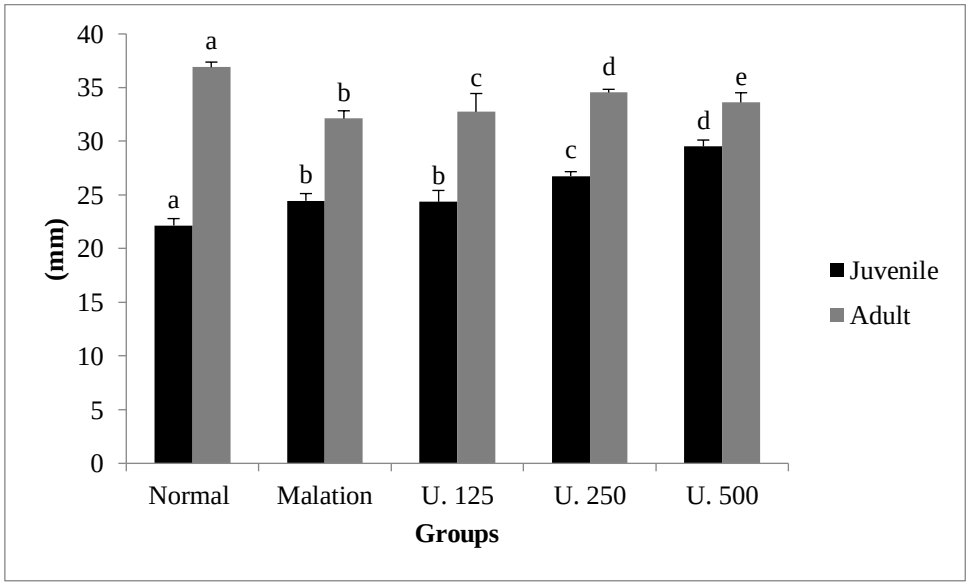

Figure 4: Body length *D. rerio*

Malathion disrupts the underlying endocrine mechanism, responsible for somatic growth. Exposure to malathion also influences the hormone that maintains the physiology of body growth. Malathion decreases both the thyroid hormone (T3 and T4) in plasma and inhibits its receptor binding, moreover, it reduces metabolic rate and body growth.[17,25-26] Perez Sanches and Lei Bail[27] have reported that hypothyroidism causes liver resistance to GH and affects hepatic insulin growth factor-1 (IGF-1) production. Growth hormone (GH) and IGF-1 decrease as a result of malathion exposure, which causes growth retardation.[17] Food consumption was reduced due to malathion exposure; therefore, it inhibits the body growth of the organism.[26] Malathion also induces lipolysis of body fat and, therefore, contributes to the bodyweight decrease.[28] Exposure to malathion results in a significantly shorter body length.[28]

GH, IGF-1, steroid, and thyroid hormone are well-known to increase growth in fishes. The growth-promoting action of GH is mediated through IGF-1; a positive correlation between

the IGF-1 and growth rate has been shown in several fish.[28-30] The significant decline in GH, IGF-1, thyroid hormone, and steroid in malathion-exposed contribute to reduced body growth and metabolism change.

U. lobata leaf extract increases the body growth of *D. rerio* exposed to malathion. Active compounds in *U. lobata* contribute to retaining the bioavailability of incretin hormone through inhibition of DPP-4 activity. Incretin hormone increases the secretion of insulin; therefore, it was able to inhibit lipolysis and controlling of body weight. Meanwhile, in the MAPK pathway, the incretin hormone increases cell proliferation, moreover, it supports the body growth of zebrafish *D. rerio* both of bodyweight and body length. Whereas, the antioxidant effect of *U. lobata* has a role to protect *D. rerio* against free radicals produced by malathion. Gossypetin in *U. lobata* leaf extract scavenges pro-oxidant substances causing oxidative damage in cells, moreover, it prevents growth retardation. Reports have indicated the effect associated with mangiferin, including antioxidant activity.[33] *U. lobata* neutralize malathion effect contributing to impaired hormone secretion, the hormone responsible for homeostasis, normal cell metabolism, reproduction, and development. Studies on laboratory animals treated by stigmasterol showed anti-inflammatory and immunomodulatory effects.[36,43] Anti-inflammatory substances of *U. lobata* inhibit cell damage which is caused by cytokine pro-inflammatory release. It is useful to support the body growth of *D. rerio* both of body weight and body length.

Conclusion

U. lobata leaf extract can inhibit the increase of blood glucose level and prevent the decrease of body growth and hemoglobin level both of juvenile and adult *D. rerio*.

Conflict of interest

The authors state that there is no conflict of interest.

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ABSTRACT

The study aims to evaluate the effect of *Urena lobata* (*U. lobata*) leaves extract on blood glucose level, hemoglobin, and body growth of zebrafish (*Danio rerio*) exposed to malathion chronically.

The study used juvenile and adult of *Danio rerio* (*D.rerio*) which were divided into five groups (n=5). The leaves of *U. lobata* were extracted by the decoction method. Concentrations of 125-500 mg/L of the extract were used. The *D. rerio* was exposed to extract for 40 days concomitant with malathion 2.5-5 mg/L. Blood glucose level and hemoglobin were measured using a commercially available glucometer and Hb-meter, respectively, meanwhile body weight and length was using a balance scale and rule, respectively. All data are expressed as the mean ± SD and analyzed with one-way ANOVA followed by LSD test ($p<0.05$).

The administration of *U. lobata* leaves extract increased the body weight by about 40% - 90 % ($p<0.05$) on juveniles, meanwhile in adult *D. rerio* were not increased, whereas there was the body length increase both for the juvenile and adult *D. rerio* up to 20 %. The blood glucose level was decreased by 40% - 60% ($p<0.05$) for juveniles given *U. lobata*, meanwhile their adult *D. rerio* were reduced by 50% - 60%. *U. lobata* extract inhibited a decrease of hemoglobin level about 10% - 40% in juvenile *D. rerio* and 10% - 30% in adult.

U. lobata leaves extract can inhibit the decrease of body growth, hemoglobin level and prevent the increase of blood glucose level in *D. rerio*.

Keywords: endocrine disruptor, herbs, hormone, pesticides

Introduction

Malathion is one of the organophosphate pesticides having a moderate toxicity level; therefore, it is used more by people especially in the agricultural sector.[1] Malathion could enter into the body through three routes, i.e. orally, inhalation, and topical, moreover they are metabolized and produce malaoxon and free radical substance.[2] Beside as an acetylcholinesterase inhibitor, malathion could impair secretion, synthesis, action, transport, binding, and elimination of natural hormones in the body. They are responsible for homeostasis, normal cell metabolism, reproduction, growth and development.[3] In animals, malathion is a known endocrine disruptor, teratogen, and reproductive toxin.[4-5] Free radicals resulted by malathion metabolism cause oxidative stress and damage in tissue; therefore, it increases their toxicity.[6]

Pulutan (*Urena lobata*) is a plant found in Indonesia and has been empirically used to cure many diseases such as malaria, wound, and diabetes.[7] Pre-clinical study of *Urena lobata* (*U. lobata*) showed the activity as anti-diabetic by inhibition of Dipeptidyl peptidase-4 (DPP-4), broad-spectrum analgetic, and anti-anxiolytic.[8-9] Other research indicated that *U. lobata* inhibits the increase of free radicals such as superoxide radicals, hydroxyl radicals, and lipid peroxidation.[10-11] Active substances in *U. lobata* such as mangiferin, gossypetin, and quercetin are predicted as lead compounds. [7-8] There are no reports on *U. lobata* potency on blood glucose level, hemoglobin, and body growth exposed to malathion chronically.

Zebrafish (*Danio rerio*) can be used as an animal model for toxicity tests. The use of *Danio rerio* (*D. rerio*) has many advantages, such as sensitivity to poison, ease to breed and the embryo is transparent; therefore, it is easy to observe the internal organ.[12-13] Moreover, almost 70% gene encoding in a human is found in *D. rerio;* therefore, it represents more about the condition in human.[14] The report of *U. lobata* inhibiting endocrine disruptor due to pesticide is limited and not complete. The study examined the effect of *U. lobata* leaf extract on blood glucose level, hemoglobin, and body growth of *D. rerio* exposed to malathion chronically.

Material and Method

Materials

Chemical sample

Aquades (Brataco), Malathion (Riger, 2044977) Methylene Blue (E.Merck, 2005152), Tetramin (Tropical, 90001250).

Method

U. lobata leaf extract preparation

U. lobata leaf powder was obtained from Materia Medika Batu Malang on January 8th, 2019, with voucher number 074/096A/102.7/2019. Approximately 5 g of the powdered plant materials were extracted by decoction methods in 500 ml water at 90ºC for 30 minutes. *U. lobata* leaf extract was given in three doses 125 mg/L, 250 mg/L, and 500 mg/L for 40 days concomitant with malathion.

Malathion

Malathion was taken by micropipette and diluted with water and obtained a concentration of 2.5 mg/L for juvenile and 5 mg/L for adult *D.rerio*. The dose of malathion refers to Cook et al. (2005) with slightly modification. Malathion was administrated on the control group and three test groups over 40 days.

Animal and treatment

The zebrafish (*D. rerio*) was obtained from local breeding with voucher number 005/ULMKILP/UA.FPK/03/2019. The assay was based on OECD (2018)[15-16] with slight modifications. Both juvenile and adult *D. rerio* were divided into two control groups and three test groups (n=5). The *U. lobata* leaf extract was given for 40 days concomitant with malathion 2.5 - 5 mg/L.[28]

Blood glucose level

The blood sample was collected from the tail vein of *D.rerio* after overnight fasting. They were measured immediately using a commercially available glucometer and recorded in g/dL.

Hemoglobin level

The collected blood from *D.rerio* was dripped into a commercial Hb meter and recorded in g/dL.

Body Growth level

The body weight and body length were used to evaluate the growth level. Body weight was measured by balance scale and recorded in milligram (mg), meanwhile, body length was measured using a ruler and recorded in millimeter (mm).

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA. The least significant difference (LSD) test was used for mean comparisons and then p-value < 0.05 was considered to be statistically significant.

Results and Discussion

Effect of U. lobata leaf extract on blood glucose level of D. rerio exposed to malathion

The blood glucose level of *D.rerio* exposed to malathion are shown in Figure 1. Exposure to malathion increased blood glucose levels both of juvenile and adult *D. rerio* up to 60% compared to the normal group ($p<0.05$). The blood glucose level was decreased by 40%, 60%, and 40% ($p<0.05$), respectively, in a juvenile that was given *U. lobata* at the dose of 125 mg/L, 250 mg/L, and 500 mg/L, meanwhile in the adult *D. rerio* were reduced 60%, 50% and 50%, respectively ($p<0.05$).

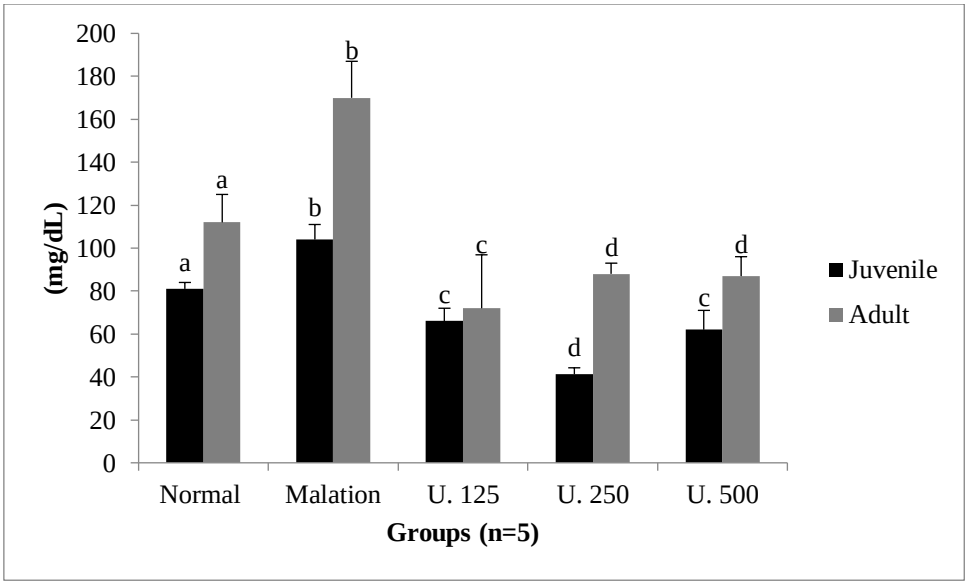

Figure 1: Effect of *U. lobata* leaf extract on blood glucose level of *D. rerio*.

Malathion disrupts the underlying endocrine mechanism, responsible for carbohydrate metabolism and causes a degenerative change in pancreatic islets through disruption of islets' mitochondrial function.[17] Administration of malathion chronically results in insulin resistance; therefore, it could increase insulin secretion by the pancreatic islet. It is shown by the increase of insulin concentration in plasma.[17] Hyperinsulinemia causes fatigue in pancreatic beta cells; therefore. the insulin production could be decreased.[18] Molecular mechanisms of insulin resistance, serine phosphorylation of insulin receptor substrate-1 and increased expression of p85-alpha, are the two sides of the coin. This condition increases blood glucose level or hyperglycemia due to interference of insulin secretion.[19]

Malathion is metabolized into malaoxon and free radicals, meanwhile acetylcholinesterase inhibitor activity of malaoxon is higher than the parent compound.[20] Inhibition of its enzyme will increase acetylcholine and stimulate muscarinic receptors; therefore, it causes bradypnea. This condition activates hypoxia-inducible factor-1 (HIF-1) and caspase-3 having a role in apoptosis of β-cells pancreas. Free radicals produced by malathion metabolism will disrupt tissue and result in cytokine pro-inflammatory, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. This cytokine stimulates apoptosis of β-cells pancreas through nuclear factor-kappa–β (NF-kB). It contributes to insulin deficiency and, moreover, increases blood glucose levels.[21] Human pancreatic islet cell destruction by cytokine involves oxygen free radicals and aldehyde production. Free radicals also decrease glucose transporter-4 (GLUT-4) through oxidative stress. GLUT-4 is a major transporter of glucose and the disruption of them causes insulin resistance.[22] GLUT-4 expression in response to oxidative stress is associated with reciprocal alterations in C/EBP alpha and delta isoform in 3T3-L1 adipocytes.

The administration of *U. lobata* leaf extract decreased blood glucose levels both in juvenile and adult *D.rerio* exposed to malathion. *U. lobata* contain active compounds such as stigmasterol, β-sitosterol, gossypetin, mangiferin, and chrysoeriol having pharmacology effects.[31] Stigmasterol and β-sitosterol inhibit DPP-4 activity; therefore, they prevent the degradation of active GLP-1 having functions in stimulating the secretion of insulin by

cAMP activation, increasing β-cell masses by MAPK pathway, and inhibiting the secretion of glucagon.[7,31,32] Mangiferin also has an anti-diabetic effect through inhibiting oxidative stress in pancreatic tissue; therefore, its damage can be prevented.[33] They contribute to decreasing the blood glucose level of *D. rerio* exposed by malathion. Gossypetin and mangiferin indicate antioxidant potency by donating an electron and scavenging free radicals.[33-35] Stigmasterol in *U. lobata* also has antioxidant activity by inhibiting lipid peroxidation or anti-peroxidative.[36] This activity protects islet pancreatic from damage caused by free radicals from malathion exposure. The protection will maintain β–cell pancreas to produce insulin hormone for controlling blood glucose levels. Chrysoeriol and β-sitosterol have potency as anti-inflammatory through inhibiting both cells and cytokine pro inflammatory.[37-38]

The anti-inflammation effect will prevent damage of tissue, including β–cell pancreas, moreover, they are able to produce insulin which is used to regulate blood glucose level.[36] In this study, the increase of dose reduces *U. lobata* activity to regulate blood glucose level both in juvenile and adult *D. rerio* generally. It is related to the desensitization of receptors if they were given substance in high dose and long term.[39] The exception for *U. lobata* 250 mg/ld. in juvenile, is that increasing of dose intensifies the potency to decrease blood glucose level. This is consistent with the pharmacology theory, that an increase in dose will elevate the activity.

Effect of U. lobata leaf extract on Hemoglobin level of D. rerio exposed to malathion

The hemoglobin level of *D. rerio* exposed to malathion is shown in Figure 2. The expose of malathion decreases hemoglobin levels in both juvenile and adult *D. rerio* up to 20% compared to the normal group ($p<0.05$). The decreased hemoglobin levels were reduced by 10%, 40%, and 20% ($p<0.05$), respectively in a juvenile that was given *U. lobata* at the dose of 125 mg/L, 250 mg/L, and 500 mg/L, meanwhile in the adult of *D. rerio* were reduced 10%, 30% and 20%, respectively ($p<0.05$).

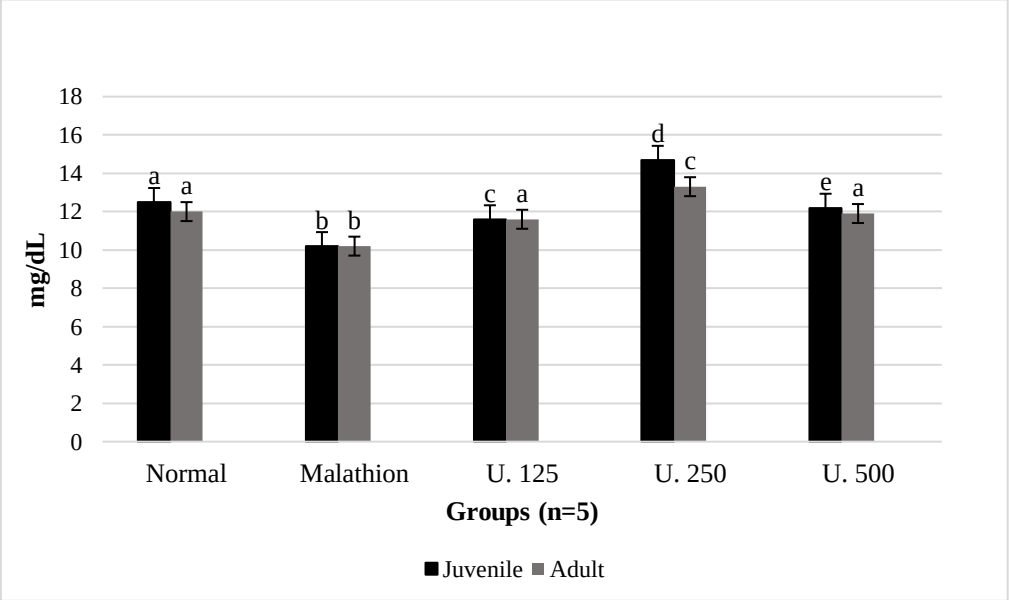

Figure 2: Effect of *U. lobata* leaf extract on hemoglobin level of *D. rerio*

Chronic malathion exposure to *D. rerio* decreases hemoglobin levels and promotes deformation of red blood cells. It is caused by malathion working as an acetylcholinesterase inhibitor and free radical compound which is produced from the metabolism process. Malaoxon causes overstimulating both of muscarinic receptor and nicotinic caused by the hypoxic condition. Chronic deoxygenation stimulates the release of Ankyrin and band 3 protein chain; therefore, followed by the release of spectrin and actin chain from erythrocyte membrane. It can increase hemolysis risk caused by the decrease of mechanical support by the cytoskeleton.[23] On the other hand, the increase of Reactive Oxygen Species (ROS) causes lipid peroxidation of erythrocyte membrane resulting from the decrease of membrane integrity. This condition increases the risk of hemolysis and, therefore, it decreases hemoglobin level.[24]

The potential of anti-anemia was shown by *U. lobata* extract by preventing the decrease of hemoglobin level of *D. rerio* exposed to malathion.[40] Quercetin is one of the compounds from *U. lobata*, a flavonoid compound that has antioxidant activity as well as being able to modulate expressions of antioxidant enzymes such as catalase and superoxide dismutase and also increases glutathione levels intracellular. Increasing of free radical level will stimulate quercetin to be oxidized and free radicals react with glutathione as well as thiol groups protein.[40] Another compound is glutathione which has a platform directly to protect protein and maintain membrane stability of red blood cells.[41] Quercetin also is a chelating agent of heavy metal. Some heavy metals will increase the rate of biochemical reaction and disrupt the stability of biological components, moreover, they must be bound by a chelating agent. Iron is an essential element in mitochondrial electron transfer; the deficiency of iron causes changes in cell metabolism and anemia. In the development of red blood cells, iron plays an important role in oxygen transport and is active in the process of proliferation and differentiation of hematopoietic stem cells.[40] Besides antioxidants, quercetin also acts as an anti-inflammatory by reducing TNF-α level; therefore, it prevents hemolysis through apoptosis pathway and inhibits a decrease of hemoglobin (Hb) level.[42] Antioxidant activity contributes to preventing rupture of the erythrocyte membrane, moreover, hemoglobin leakage is avoided outside of the erythrocyte. The antioxidant compound can stabilize the erythrocyte membrane from damage caused by free radicals; therefore, it reduces hemolysis risk and inhibits a decrease of hemoglobin level.[40]

Effect of U. lobata leaf extract on body growth of D. rerio exposed to malathion

The bodyweight of *D. rerio* exposed to malathion is shown in Figure 3, and body length in Figure 4. The exposure of malathion inhibited the increase of body weight and body length of *D. rerio* compared to the normal group ($p<0.05$); however, the body length of the juvenile was not inhibited by malathion. The administration of *U. lobata* leaf extract at the dose of 125 mg/L, 250 mg/L, and 500 mg/L increased the bodyweight about 40%, 70%, and 90% ($p<0.05$), respectively, in juvenile but showed no increase in the adult of *D. rerio* ($p>0.05$). Whereas the body length was increased both for juvenile and adult *D. rerio* up to 20% ($p<0.05$).

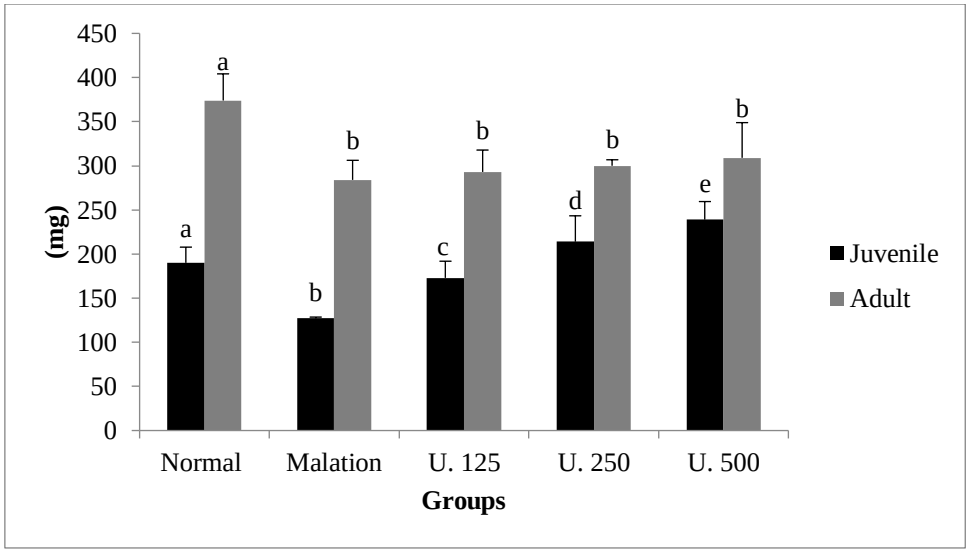

Figure 3: Bodyweight *D. rerio*

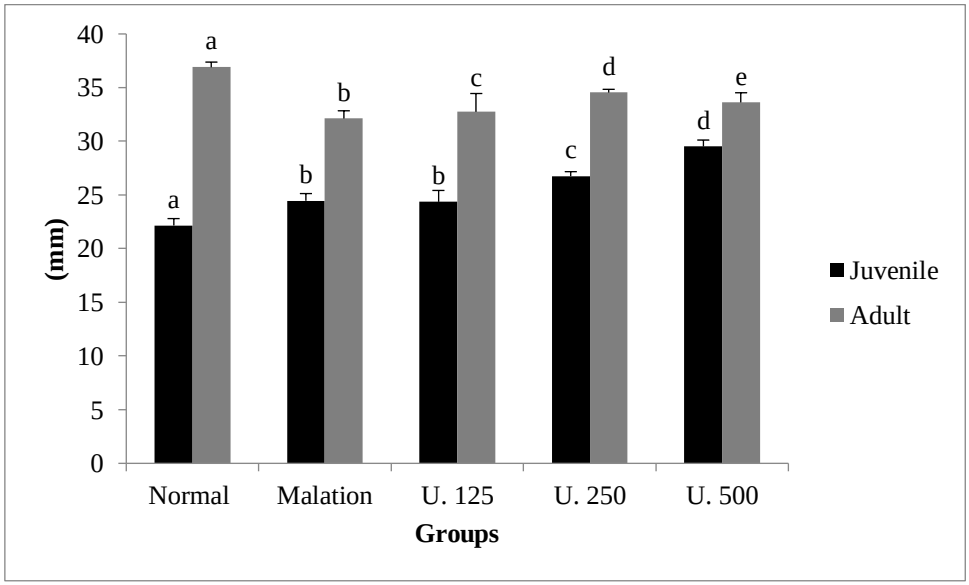

Figure 4: Body length *D. rerio*

Malathion disrupts the underlying endocrine mechanism, responsible for somatic growth. Exposure to malathion also influences the hormone that maintains the physiology of body growth. Malathion decreases both the thyroid hormone (T3 and T4) in plasma and inhibits its receptor binding, moreover, it reduces metabolic rate and body growth.[17,25-26] Perez Sanches and Lei Bail[27] have reported that hypothyroidism causes liver resistance to GH and affects hepatic insulin growth factor-1 (IGF-1) production. Growth hormone (GH) and IGF-1 decrease as a result of malathion exposure, which causes growth retardation.[17] Food consumption was reduced due to malathion exposure; therefore, it inhibits the body growth of the organism.[26] Malathion also induces lipolysis of body fat and, therefore, contributes to the bodyweight decrease.[28] Exposure to malathion results in a significantly shorter body length.[28]

GH, IGF-1, steroid, and thyroid hormone are well-known to increase growth in fishes. The growth-promoting action of GH is mediated through IGF-1; a positive correlation between

the IGF-1 and growth rate has been shown in several fish.[28-30] The significant decline in GH, IGF-1, thyroid hormone, and steroid in malathion-exposed contribute to reduced body growth and metabolism change.

U. lobata leaf extract increases the body growth of *D. rerio* exposed to malathion. Active compounds in *U. lobata* contribute to retaining the bioavailability of incretin hormone through inhibition of DPP-4 activity. Incretin hormone increases the secretion of insulin; therefore, it was able to inhibit lipolysis and controlling of body weight. Meanwhile, in the MAPK pathway, the incretin hormone increases cell proliferation, moreover, it supports the body growth of zebrafish *D. rerio* both of bodyweight and body length. Whereas, the antioxidant effect of *U. lobata* has a role to protect *D. rerio* against free radicals produced by malathion. Gossypetin in *U. lobata* leaf extract scavenges pro-oxidant substances causing oxidative damage in cells, moreover, it prevents growth retardation. Reports have indicated the effect associated with mangiferin, including antioxidant activity.[33] *U. lobata* neutralize malathion effect contributing to impaired hormone secretion, the hormone responsible for homeostasis, normal cell metabolism, reproduction, and development. Studies on laboratory animals treated by stigmasterol showed anti-inflammatory and immunomodulatory effects.[36,43] Anti-inflammatory substances of *U. lobata* inhibit cell damage which is caused by cytokine pro-inflammatory release. It is useful to support the body growth of *D. rerio* both of body weight and body length.

Conclusion

U. lobata leaf extract can inhibit the increase of blood glucose level and prevent the decrease of body growth and hemoglobin level both of juvenile and adult *D. rerio*.

Conflict of interest

The authors state that there is no conflict of interest.

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Original Research Article

Effect of Pulutan (*Urena lobata*) Leaf Extract on Blood Glucose Level, Hemoglobin and Body Growth of Zebra Fish (*Danio rerio*) Exposed to Malathion

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ABSTRACT

Pulutan (*Urena lobata*) is a medicinal plant having antioxidant activity. However, their potency to inhibit the adverse effects of malathion has not been evaluated. The study aims to examine *Urena lobata* (*U. lobata*) leaves extract on blood glucose level, hemoglobin, and body growth of *Danio rerio* (*D. rerio*) exposed to malathion. The study used juvenile and adult of *D. rerio* which were divided into five groups (n=5). The leaves of *U. lobata* were extracted by the decoction method. The *D. rerio* was administered with extract 125-500 mg/L for 40 days concomitantly with malathion 2.5-5 mg/L. Blood glucose level and hemoglobin were measured using a commercially available glucometer and Hb-meter, respectively. Meanwhile body weight and length was measured using a balance scale and a ruler, respectively. All data are expressed as the mean ± SD and analyzed with one-way ANOVA followed by LSD test ($p<0.05$).
The administration of *U. lobata* extract increased the body weight by about 40% - 90 % ($p<0.05$) on juveniles *D. rerio*, while no changes were observed in adult, whereas there was a 20% increase in body length for both juvenile and adult *D. rerio*. The blood glucose level was decreased by 40% - 60% ($p<0.05$) for juveniles given *U. lobata*, meanwhile in adult *D. rerio*, it was reduced by 50% - 60%. *U. lobata* reduced the decrease of hemoglobin levels by 10% - 40% in juvenile *D.rerio* and 10% - 30% in adult. *U. lobata* extract reduced the decrease in body growth and hemoglobin level, and prevented blood glucose level increase.

***Keywords*:** Endocrine disruptor, Herbs, Hormone, Pesticides.

Introduction

Malathion is one of the organophosphate pesticides having a moderate toxicity level; therefore, it is used more by people especially in the agricultural sector.[1] Malathion could enter into the body through three routes, i.e. orally, inhalation, and topical. In the body, Malathion are metabolized into malaoxon and free radical substances.[2] While it is known to act as an acetylcholinesterase inhibitor, malathion could also impair secretion, synthesis, action, transport, binding, and elimination of natural hormones in the body. These hormones are responsible for homeostasis, normal cell metabolism, reproduction, growth and development.[3] In animals, malathion is a known endocrine disruptor, teratogen, and reproductive toxin.[4,5] Free radicals resulted by malathion metabolism cause oxidative stress and damage in tissue; therefore, it increases their toxicity.[6] Pulutan (*Urena lobata*) is a plant found in Indonesia and has been empirically used to cure many diseases such as malaria, wound, and diabetes.[7] Pre-clinical study of *Urena lobata* (*U. lobata*) showed anti-diabetic activity, and acts by inhibiting Dipeptidyl peptidase-4 (DPP-4), is a broad-spectrum analgetic, and has anti-anxiolytic properties.[8,9]
Other research indicated that *U. lobata* inhibits the increase of free radicals such as superoxide radicals, hydroxyl radicals, and lipid peroxidation.[10-11] Active substances in *U. lobata* such as mangiferin, gossypetin, and quercetin are predicted as lead compounds.[7,8]

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There are no reports on *U. lobata* potency on blood glucose level, hemoglobin, and body growth exposed to malathion chronically.
Zebrafish (*Danio rerio*) can be used as an animal model for toxicity tests. The use of *Danio rerio* (*D. rerio*) has many advantages, such as sensitivity to poison, ease to breed and the embryo is transparent; therefore, it is easy to observe the internal organ.[12,13] Moreover, almost 70% gene encoding in a human is found in *D. rerio;* therefore, it represents more about the condition in human.[14] Currently, reports regarding efficacy of *U. lobata* in preventing damage, such as endocrine disruption, due to pesticides are limited and has not been completed. The study examined the effect of *U. lobata* leaf extract on blood glucose level, hemoglobin, and body growth of *D. rerio* exposed to malathion chronically.

Material and Methods

Chemical sample
Aquades (Brataco), Malathion (Riger, 2044977) Methylene Blue (E. Merck, 2005152), Tetramin (Tropical, 90001250).

U. lobata leaf extract preparation
U. lobata leaf powder was obtained from Materia Medika Batu Malang on January 8th, 2019, with voucher number 074/096A/102.7/2019. Approximately 5 g of plant materials (in powder form) were extracted by decoction methods in 500 ml water at 90ºC for 30 minutes. *U. lobata* leaf extract was given in three doses 125 mg/L, 250 mg/L, and 500 mg/L for 40 days concomitant with malathion.

Malathion
Malathion was diluted using water to a concentration of 2.5 mg/L and 5 mg/L, which are the doses for juvenile and adult *D. rerio*, respectively. The doses selected of malathion exposure was based on Cook et al. (2005) with sligh modification. Malathion was administrated on the control group and three test groups over 40 days.

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Animal and treatment
The zebrafish (*D. rerio*) was obtained from a local fish breeding establishment with a determination registration number 005/ULMKILP/UA.FPK/03/2019. The assay used was based on OECD (2018)[15-16] with slight modifications. Both juvenile and adult *D. rerio* were divided into two control groups and three test groups (n=5). The *U. lobata* leaf extract was given for 40 days concomitant with malathion 2.5 - 5 mg/L.[28]

Blood glucose level
The blood sample was collected from the tail vein of *D. rerio* after overnight fasting. They were measured immediately using a commercially available glucometer and recorded in g/dL.

Hemoglobin level
The collected blood from *D. rerio* was dripped into a commercial Hb meter and recorded in g/dL.

Body growth level
The body weight and body length were used to evaluate the growth level. Body weight was measured by balance scale and recorded in milligram (mg), meanwhile, body length was measured using a ruler and recorded in millimeter (mm).

Statistical analysis
All data are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA. The least significant difference (LSD) test was used for mean comparisons and then p-value < 0.05 was considered to be statistically significant.

Results and Discussion

Effect of U. lobata leaf extract on blood glucose level of D. rerio exposed to malathion
The blood glucose level of *D. rerio* exposed to malathion are shown in Figure 1. Exposure to malathion increased blood glucose levels both in juvenile and adult *D. rerio* up to 60% compared to the normal group ($p<0.05$). In juvenile *D. rerio*, the blood glucose level was decreased by 40% ($p<0.05$) after administration of *U. lobata* at a dose of 125 mg/L and 500 mg/L, while at a dose of 250 mg/L, the blood glucose level was decreased by 60% ($p<0.05$). In adult *D. rerio*, *U. lobata* at 125 mg/L decreased blood glucose level by 60% ($p<0.05$), while both 250 mg/L and 500 mg/L reduced it to 50% ($p<0.05$).
Malathion disrupts the underlying endocrine mechanism, responsible for carbohydrate metabolism and causes a degenerative change in pancreatic islets through disruption of islets' mitochondrial function.[17] Long-term exposure to malathion is known to increase insulin secretion by the pancreatic island and therefore resulting in insulin resistance, shown by the increase of insulin concentration in plasma.[17] Hyperinsulinemia causes fatigue in pancreatic beta cells; therefore the insulin production could be decreased.[18] Molecular mechanisms of insulin resistance, serine phosphorylation of insulin receptor substrate-1 and increased expression of p85-alpha, are the two sides of the coin. This condition increases blood glucose level or hyperglycemia due to interference of insulin secretion.[19]
Malathion is metabolized into malaoxon and free radicals, meanwhile acetylcholinesterase inhibitor activity of malaoxon is higher than the parent compound.[20] Inhibition of its enzyme will increase acetylcholine and stimulate muscarinic receptors; therefore, it causes bradypnea. This condition activates hypoxia-inducible factor-1 (HIF-1) and caspase-3 having a role in apoptosis of *β*-cells pancreas. Free radicals produced by malathion metabolism will disrupt tissue and result in cytokine pro-inflammatory, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1*β*. This cytokine stimulates apoptosis of β-cells pancreas through nuclear factor-kappa–β (NF-kB). It contributes to insulin deficiency and, moreover, increases blood glucose levels.[21] Human pancreatic islet cell destruction by cytokine involves oxygen free radicals and aldehyde production. Free radicals also decrease glucose transporter-4 (GLUT-4) through oxidative stress. GLUT-4 is a major transporter of glucose and the disruption of them causes insulin resistance.[22] GLUT-4 expression in response to oxidative stress is associated with reciprocal alterations in C/EBP alpha and delta isoform in 3T3-L1 adipocytes.
The administration of *U. lobata* leaf extract decreased blood glucose levels both in juvenile and adult *D. rerio* exposed to malathion. *U. lobata* contain active compounds such as stigmasterol, *β*-sitosterol, gossypetin, mangiferin, and chrysoeriol having pharmacology effects.[31] Stigmasterol and *β*-sitosterol inhibits DPP-4 activity, and therefore, prevents the degradation of activated GLP-1, which has a function of stimulating insulin secretion via cAMP activation, increasing β-cell masses via MAPK pathway, and inhibiting the secretion of glucagon.[7,31,32] Mangiferin also has an anti-diabetic effect by inhibiting oxidative stress in pancreatic tissue; therefore, the damage caused by oxidative stress can be prevented.[33] Furthermore, Mangiferin also contributes to the decrease of blood glucose level of *D. rerio* exposed to malathion. Gossypetin and mangiferin acts as antioxidants by donating an electron and scavenging free radicals.[33-35] Stigmasterol in *U. lobata* also has antioxidant activity by inhibiting lipid peroxidation or anti-peroxidative.[36] This activity protects islet pancreatic from damage caused by free radicals from malathion exposure. The protection will maintain *β*-cell pancreas to produce insulin hormone for controlling blood glucose levels. Chrysoeriol and *β*-sitosterol acts an anti-inflammatory agent through inhibiting both pro inflammatory cells and cytokines.[37-38]
The anti-inflammation effect will prevent damage of tissue, including β–cell pancreas, moreover, they are able to produce insulin which is used to regulate blood glucose level.[36] In this study, the increase of dose reduces *U. lobata* activity to regulate blood glucose level both in juvenile and adult *D. rerio* generally. One possible explanation is that it is due to desensitization of receptors when a substance is continutially administered in high dose and long term.[39] The exception for *U. lobata* 250 mg/ld. in juvenile, where increasing of dose would intensify the potency to decrease blood glucose level. This is consistent with the pharmacology theory, that an increase in dose will elevate the activity.

Effect of U. lobata leaf extract on Hemoglobin level of D. rerio exposed to Malathion
The hemoglobin level of *D. rerio* exposed to malathion is shown in Figure 2. Exposure of malathion decreases hemoglobin levels in both juvenile and adult *D. rerio* up to 20% compared to the normal group ($p < 0.05$). This decrease was reduced by 10%, 40%, and 20% ($p < 0.05$), respectively in a juvenile that was given *U. lobata* at the dose of 125 mg/L, 250 mg/L, and 500 mg/L. Meanwhile, in adult *D. rerio*, the decrease were reduced by 10%, 30% and 20%, at the same doses, respectively ($p < 0.05$).
Chronic malathion exposure to *D. rerio* decreases hemoglobin levels and promotes deformation of red blood cells. It is caused by malathion working as an acetylcholinesterase inhibitor and free radical compound which is produced from the metabolism process.

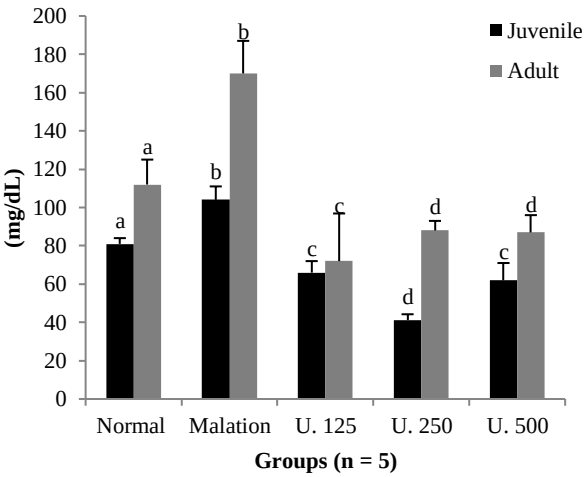

Figure 1: Effect of *U. lobata* leaf extract on blood glucose level of *D. rerio.*

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Malaoxon causes overstimulation of both muscarinic and nicotinic receptors by stimulating hypoxic conditions on these receptors. Chronic deoxygenation stimulates the release of Ankyrin and band 3 protein chain and is followed by the release of spectrin and actin chain from erythrocyte membrane. It can increase hemolysis risk caused by the decrease of mechanical support by the cytoskeleton.[23] On the other hand, the increase of Reactive Oxygen Species (ROS) causes lipid peroxidation of erythrocyte membrane which results in the the decrease of cell membrane integrity. This condition increases the risk of hemolysis and thus decreases hemoglobin level.[24]

The potential of anti-anemia was shown by *U. lobata* extract by preventing the decrease of hemoglobin level of *D. rerio* exposed to malathion.[40] Quercetin is one of the compounds from *U. lobata*, a flavonoid compound that has antioxidant activity as well as being able to modulate expressions of antioxidant enzymes such as catalase and superoxide dismutase and also increases glutathione levels intracellular. The increase of free radicals will be offset by quercetin by oxidation, and free radicals would also react with glutathione as well as other proteins having thiol groups.[40] Another compound is glutathione which has a platform directly to protect protein and maintain membrane stability of red blood cells.[41] Quercetin also is a chelating agent of heavy metal. Some heavy metals will increase the rate of biochemical reaction and disrupt the stability of biological components, moreover, they must be bound by a chelating agent. Iron is an essential element in mitochondrial electron transfer; the deficiency of iron causes changes in cell metabolism and anemia. In the development of red blood cells, iron plays an important role in oxygen transport and is active in the process of proliferation and differentiation of hematopoietic stem cells.[40] Besides acting as antioxidants, quercetin can also acts as an anti-inflammatory by reducing TNF-α level, preventing hemolysis through apoptosis pathway and results in the reduction hemoglobin (Hb) level decrease.[42] Antioxidant activity contributes to preventing rupture of the erythrocyte membrane, moreover, hemoglobin leakage is avoided outside of the erythrocyte. The antioxidant compounds can stabilize the erythrocyte membrane from damage caused by free radicals and reduce the risk of hemolysis and inhibits a decrease of hemoglobin level.[40]

Effect of U. lobata leaf extract on body growth of D. rerio exposed to malathion

The bodyweight of *D. rerio* exposed to malathion is shown in Figure 3, and body length in Figure 4. The exposure of malathion inhibited the increase of body weight and body length of *D. rerio* compared to the normal group ($p<0.05$); however, the body length of the juvenile was not inhibited by malathion. The administration of *U. lobata* leaf extract at the dose of 125 mg/L, 250 mg/L, and 500 mg/L increased the bodyweight about 40%, 70%, and 90% ($p<0.05$), respectively, in juvenile but showed no increase in the adult of *D. rerio* ($p>0.05$). Whereas the body length was increased both for juvenile and adult *D. rerio* up to 20% ($p<0.05$).

Malathion disrupts the underlying endocrine mechanism, responsible for somatic growth. Exposure to malathion also influences the hormone that maintains the physiology of body growth. Malathion decreases both the thyroid hormone (T3 and T4) in plasma and inhibits its receptor binding, moreover, it reduces metabolic rate and body growth.[17,25,26] Perez Sanches and Lei Bail[27] have reported that hypothyroidism causes liver resistance to GH and affects hepatic insulin growth factor-1 (IGF-1) production. Growth hormone (GH) and IGF-1 decreases as a result of malathion exposure, which causes growth retardation.[17] Food consumption was reduced due to malathion exposure, causing the growth retardation.[26] Malathion also induces lipolysis of body fat and, therefore, contributes to the bodyweight decrease.[28] Exposure to malathion results in a significantly shorter body length.[28]

GH, IGF-1, steroid, and thyroid hormone are well-known to increase growth in fishes. The growth-promoting action of GH is mediated through IGF-1; a positive correlation between the IGF-1 and growth rate has been shown in in vivo studies.[28-30] The significant decline in GH, IGF-1, thyroid hormone, and steroid in malathion-exposed contribute to reduced body growth and metabolism change.

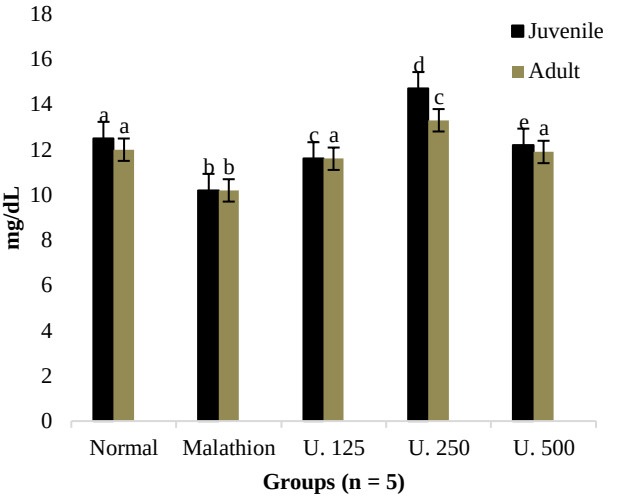

Figure 2: Effect of *U. lobata* leaf extract on hemoglobin level of *D. rerio*

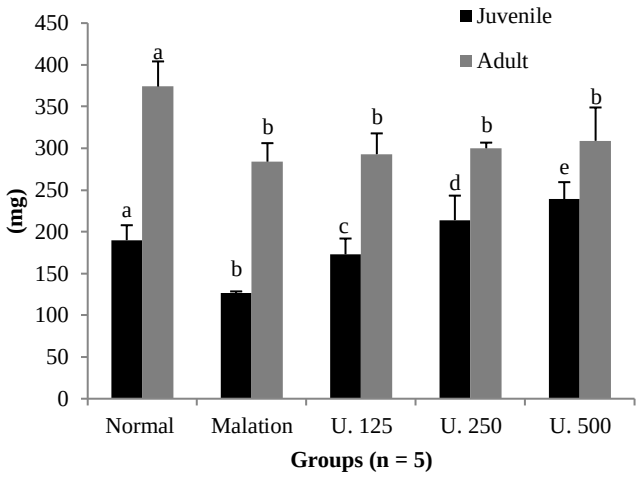

Figure 3: Bodyweight *D. rerio*

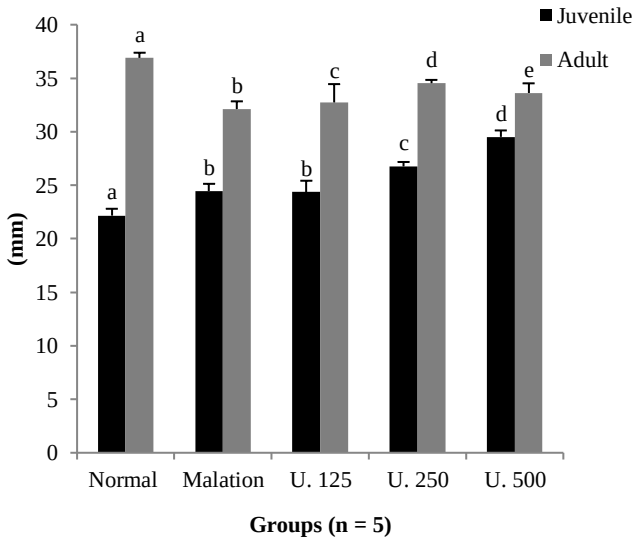

Figure 4: Body length *D. rerio*

U. lobata leaf extract increases the body growth of *D. rerio* exposed to malathion. Active compounds in *U. lobata* contribute to retaining the bioavailability of incretin hormone through inhibition of DPP-4 activity. Incretin hormone increases the secretion of insulin causing lipolysis inhibition and regulating body weight. Meanwhile, in the MAPK pathway, the incretin hormone increases cell proliferation, moreover, it supports the body growth of zebrafish *D. rerio* both of bodyweight and body length. Whereas, the antioxidant effect of *U. lobata* has a role to protect *D. rerio* against free radicals produced by malathion. Gossypetin in *U. lobata* leaf extract scavenges pro-oxidant substances causing oxidative damage in cells, moreover, it prevents growth retardation. Reports have indicated the effect associated with mangiferin, including antioxidant activity.[33] *U. lobata* neutralize malathion effect contributing to impaired hormone secretion, the hormone responsible for homeostasis, normal cell metabolism, reproduction, and development. Studies on laboratory animals treated by stigmasterol showed anti-inflammatory and immunomodulatory effects.[36,43] Anti-inflammatory substances of *U. lobata* inhibit cell damage which is caused by cytokine pro-inflammatory release. It is useful to support the body growth of *D. rerio* both of body weight and body length.

Conclusion

U. lobata leaf extract can inhibit the increase of blood glucose level and prevent the decrease of body growth and hemoglobin level both in juvenile and adult *D. rerio*.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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Original Research Article

Effect of Pulutan (*Urena lobata*) Leaf Extract on Blood Glucose Level, Hemoglobin and Body Growth of Zebra Fish (*Danio rerio*) Exposed to Malathion

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ABSTRACT

Pulutan (*Urena lobata*) is a medicinal plant having antioxidant activity. However, their potency to inhibit the adverse effects of malathion has not been evaluated. The study aims to examine *Urena lobata* (*U. lobata*) leaves extract on blood glucose level, hemoglobin, and body growth of *Danio rerio* (*D. rerio*) exposed to malathion. The study used juvenile and adult of *D. rerio* which were divided into five groups (n=5). The leaves of *U. lobata* were extracted by the decoction method. The *D. rerio* was administered with extract 125-500 mg/L for 40 days concomitantly with malathion 2.5-5 mg/L. Blood glucose level and hemoglobin were measured using a commercially available glucometer and Hb-meter, respectively. Meanwhile, the body weight and length was measured using a balance scale and a ruler, respectively. All data were expressed as the mean ± SD and analyzed with one-way ANOVA followed by LSD test.
The administration of *U. lobata* extract increased the body weight by about 40-90% ($p<0.05$) on juveniles *D. rerio*, while no changes were observed in adult, whereas there was a 20% increase in body length for both juvenile and adult *D. rerio*. The blood glucose level was decreased by 40-60% ($p<0.05$) for juveniles given *U. lobata*, meanwhile in adult *D. rerio*, it was reduced by 50-60%. *U. lobata* reduced the decrease of hemoglobin levels by 10-40% in juvenile *D. rerio* and 10-30% in adult. *U. lobata* extract reduced the decrease in body growth and hemoglobin level, and prevented blood glucose level increase.

***Keywords*:** Endocrine disruptor, Herbs, Hormone, Pesticides.

Introduction

Malathion is one of the organophosphate pesticides having a moderate toxicity level; therefore, it is used more by people especially in the agricultural sector.[1] Malathion could enter into the body through three routes, i.e. orally, inhalation, and topical. In the body, Malathion are metabolized into malaoxon and free radical substances.[2] While it is known to act as an acetylcholinesterase inhibitor, malathion could also impair secretion, synthesis, action, transport, binding, and elimination of natural hormones in the body. These hormones are responsible for homeostasis, normal cell metabolism, reproduction, growth and development.[3] In animals, malathion is a known endocrine disruptor, teratogen, and reproductive toxin.[4,5] Free radicals resulted by malathion metabolism cause oxidative stress and damage in tissue; therefore, it increases their toxicity.[6] Pulutan (*Urena lobata*) is a plant found in Indonesia and has been empirically used to cure many diseases such as malaria, wound, and diabetes.[7] Pre-clinical study of *Urena lobata* (*U. lobata*) showed anti-diabetic activity, and acts by inhibiting Dipeptidyl peptidase-4 (DPP-4), is a broad-spectrum analgesic, and has anti-anxiolytic properties.[8,9]
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There are no reports on *U. lobata* potency on blood glucose level, hemoglobin, and body growth exposed to malathion chronically.
Zebrafish (*Danio rerio*) can be used as an animal model for toxicity tests. The use of *Danio rerio* (*D. rerio*) has many advantages, such as sensitivity to poison, ease to breed and the embryo is transparent; therefore, it is easy to observe the internal organ.[12,13] Moreover, almost 70% gene encoding in a human is found in *D. rerio;* therefore, it represents more about the condition in human.[14] Currently, reports regarding efficacy of *U. lobata* in preventing damage, such as endocrine disruption, due to pesticides are limited and has not been completed. The study examined the effect of *U. lobata* leaf extract on blood glucose level, hemoglobin, and body growth of *D. rerio* exposed to malathion chronically.

Material and Methods

Chemical sample
Aquades (Brataco), Malathion (Riger, 2044977) Methylene Blue (E. Merck, 2005152), Tetramin (Tropical, 90001250).

U. lobata leaf extract preparation
U. lobata leaf powder was obtained from Materia Medika Batu Malang on January 8th, 2019, with voucher number 074/096A/102.7/2019. Approximately 5 g of plant materials (in powder form) were extracted by decoction methods in 500 ml water at 90ºC for 30 minutes. *U. lobata* leaf extract was given in three doses 125 mg/L, 250 mg/L, and 500 mg/L for 40 days concomitant with malathion.

Malathion
Malathion was diluted using water to a concentration of 2.5 mg/L and 5 mg/L, which are the doses for juvenile and adult *D. rerio*, respectively. The doses selected of malathion exposure was based on Cook et al. (2005) with slight modification. Malathion was administrated on the control group and three test groups over 40 days.

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Animal and treatment
The zebrafish (*D. rerio*) was obtained from a local fish breeding establishment with a determination registration number 005/ULMKILP/UA.FPK/03/2019. The assay used was based on OECD (2018)[15-16] with slight modifications. Both juvenile and adult *D. rerio* were divided into two control groups and three test groups (n=5). The *U. lobata* leaf extract was given for 40 days concomitant with malathion 2.5 - 5 mg/L.[28]

Blood glucose level
The blood sample was collected from the tail vein of *D. rerio* after overnight fasting. They were measured immediately using a commercially available glucometer and recorded in mg/dL.

Hemoglobin level
The collected blood from *D. rerio* was dripped into a commercial Hb meter and recorded in mg/dL.

Body growth level
The body weight and body length were used to evaluate the growth level. Body weight was measured by balance scale and recorded in milligram (mg), meanwhile, body length was measured using a ruler and recorded in millimeter (mm).

Statistical analysis
All data are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA. The least significant difference (LSD) test was used for mean comparisons and then p-value < 0.05 was considered to be statistically significant.

Results and Discussion

Effect of U. lobata leaf extract on blood glucose level of D. rerio exposed to malathion
The blood glucose level of *D. rerio* exposed to malathion are shown in Figure 1. Exposure to malathion increased blood glucose levels both in juvenile and adult *D. rerio* up to 60% compared to the normal group ($p<0.05$). In juvenile *D. rerio*, the blood glucose level was decreased by 40% ($p<0.05$) after administration of *U. lobata* at a dose of 125 mg/L and 500 mg/L, while at a dose of 250 mg/L, the blood glucose level was decreased by 60% ($p<0.05$). In adult *D. rerio*, *U. lobata* at 125 mg/L decreased blood glucose level by 60% ($p<0.05$), while both 250 mg/L and 500 mg/L reduced it to 50% ($p<0.05$).
Malathion disrupts the underlying endocrine mechanism, responsible for carbohydrate metabolism and causes a degenerative change in pancreatic islets through disruption of islets' mitochondrial function.[17] Long-term exposure to malathion is known to increase insulin secretion by the pancreatic island and therefore resulting in insulin resistance, shown by the increase of insulin concentration in plasma.[17] Hyperinsulinemia causes fatigue in pancreatic beta cells; therefore the insulin production could be decreased.[18] Molecular mechanisms of insulin resistance, serine phosphorylation of insulin receptor substrate-1 and increased expression of p85-alpha, are the two sides of the coin. This condition increases blood glucose level or hyperglycemia due to interference of insulin secretion.[19]
Malathion is metabolized into malaoxon and free radicals, meanwhile acetylcholinesterase inhibitor activity of malaoxon is higher than the parent compound.[20] Inhibition of its enzyme will increase acetylcholine and stimulate muscarinic receptors; therefore, it causes bradypnea. This condition activates hypoxia-inducible factor-1 (HIF-1) and caspase-3 having a role in apoptosis of *β*-cells pancreas. Free radicals produced by malathion metabolism will disrupt tissue and result in cytokine pro-inflammatory, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1*β*. This cytokine stimulates apoptosis of β-cells pancreas through nuclear factor-kappa–β (NF-kB). It contributes to insulin deficiency and, moreover, increases blood glucose levels.[21] Human pancreatic islet cell destruction by cytokine involves oxygen free radicals and aldehyde production. Free radicals also decrease glucose transporter-4 (GLUT-4) through oxidative stress. GLUT-4 is a major transporter of glucose and the disruption of them causes insulin resistance.[22] GLUT-4 expression in response to oxidative stress is associated with reciprocal alterations in C/EBP alpha and delta isoform in 3T3-L1 adipocytes.
The administration of *U. lobata* leaf extract decreased blood glucose levels both in juvenile and adult *D. rerio* exposed to malathion. *U. lobata* contain active compounds such as stigmasterol, *β*-sitosterol, gossypetin, mangiferin, and chrysoeriol having pharmacology effects.[31] Stigmasterol and *β*-sitosterol inhibits DPP-4 activity, and therefore, prevents the degradation of activated GLP-1, which has a function of stimulating insulin secretion via cAMP activation, increasing β-cell masses via MAPK pathway, and inhibiting the secretion of glucagon.[7,31,32] Mangiferin also has an anti-diabetic effect by inhibiting oxidative stress in pancreatic tissue; therefore, the damage caused by oxidative stress can be prevented.[33] Furthermore, Mangiferin also contributes to the decrease of blood glucose level of *D. rerio* exposed to malathion. Gossypetin and mangiferin acts as antioxidants by donating an electron and scavenging free radicals.[33-35] Stigmasterol in *U. lobata* also has antioxidant activity by inhibiting lipid peroxidation or anti-peroxidative.[36] This activity protects islet pancreatic from damage caused by free radicals from malathion exposure. The protection will maintain *β*-cell pancreas to produce insulin hormone for controlling blood glucose levels. Chrysoeriol and *β*-sitosterol acts an anti-inflammatory agent through inhibiting both pro inflammatory cells and cytokines.[37-38]
The anti-inflammation effect will prevent damage of tissue, including β–cell pancreas, moreover, they are able to produce insulin which is used to regulate blood glucose level.[36] In this study, the increase of dose reduces *U. lobata* activity to regulate blood glucose level both in juvenile and adult *D. rerio* generally. One possible explanation is that it is due to desensitization of receptors when a substance is continually administered in high dose and long term.[39] The exception for *U. lobata* 250 mg/ld. in juvenile, where increasing of dose would intensify the potency to decrease blood glucose level. This is consistent with the pharmacology theory, that an increase in dose will elevate the activity.

Effect of U. lobata leaf extract on Hemoglobin level of D. rerio exposed to Malathion
The hemoglobin level of *D. rerio* exposed to malathion is shown in Figure 2. Exposure of malathion decreases hemoglobin levels in both juvenile and adult *D. rerio* up to 20% compared to the normal group ($p < 0.05$). This decrease was reduced by 10%, 40%, and 20% ($p < 0.05$), respectively in a juvenile that was given *U. lobata* at the dose of 125 mg/L, 250 mg/L, and 500 mg/L. Meanwhile, in adult *D. rerio*, the decrease was reduced by 10%, 30% and 20%, at the same doses, respectively ($p < 0.05$).
Chronic malathion exposure to *D. rerio* decreases hemoglobin levels and promotes deformation of red blood cells. It is caused by malathion working as an acetylcholinesterase inhibitor and free radical compound which is produced from the metabolism process.

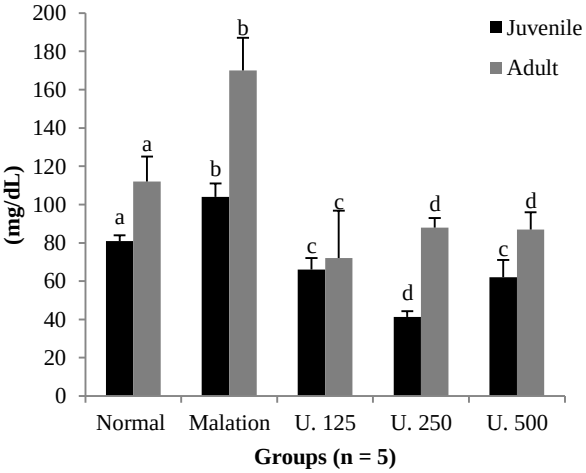

Figure 1: Effect of *U. lobata* leaf extract on blood glucose level of *D. rerio*.

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Malaoxon causes overstimulation of both muscarinic and nicotinic receptors by stimulating hypoxic conditions on these receptors. Chronic deoxygenation stimulates the release of Ankyrin and band 3 protein chain and is followed by the release of spectrin and actin chain from erythrocyte membrane. It can increase hemolysis risk caused by the decrease of mechanical support by the cytoskeleton.[23] On the other hand, the increase of Reactive Oxygen Species (ROS) causes lipid peroxidation of erythrocyte membrane which results in the decrease of cell membrane integrity. This condition increases the risk of hemolysis and thus decreases hemoglobin level.[24]

The potential of anti-anemia was shown by *U. lobata* extract by preventing the decrease of hemoglobin level of *D. rerio* exposed to malathion.[40] Quercetin is one of the compounds from *U. lobata*, a flavonoid compound that has antioxidant activity as well as being able to modulate expressions of antioxidant enzymes such as catalase and superoxide dismutase and also increases glutathione levels intracellular. The increase of free radicals will be offset by quercetin by oxidation, and free radicals would also react with glutathione as well as other proteins having thiol groups.[40] Another compound is glutathione which has a platform directly to protect protein and maintain membrane stability of red blood cells.[41] Quercetin also is a chelating agent of heavy metal. Some heavy metals will increase the rate of biochemical reaction and disrupt the stability of biological components, moreover, they must be bound by a chelating agent. Iron is an essential element in mitochondrial electron transfer; the deficiency of iron causes changes in cell metabolism and anemia. In the development of red blood cells, iron plays an important role in oxygen transport and is active in the process of proliferation and differentiation of hematopoietic stem cells.[40] Besides acting as antioxidants, quercetin can also acts as an anti-inflammatory by reducing TNF-α level, preventing hemolysis through apoptosis pathway and results in the reduction hemoglobin (Hb) level decrease.[42] Antioxidant activity contributes to preventing rupture of the erythrocyte membrane, moreover, hemoglobin leakage is avoided outside of the erythrocyte. The antioxidant compounds can stabilize the erythrocyte membrane from damage caused by free radicals and reduce the risk of hemolysis and inhibits a decrease of hemoglobin level.[40]

Effect of U. lobata leaf extract on body growth of D. rerio exposed to malathion

The bodyweight of *D. rerio* exposed to malathion is shown in Figure 3, and body length in Figure 4. The exposure of malathion inhibited the increase of body weight and body length of *D. rerio* compared to the normal group ($p<0.05$); however, the body length of the juvenile was not inhibited by malathion. The administration of *U. lobata* leaf extract at the dose of 125 mg/L, 250 mg/L, and 500 mg/L increased the bodyweight about 40%, 70%, and 90% ($p<0.05$), respectively, in juvenile but showed no increase in the adult of *D. rerio* ($p>0.05$). Whereas the body length was increased both for juvenile and adult *D. rerio* up to 20% ($p<0.05$).

Malathion disrupts the underlying endocrine mechanism, responsible for somatic growth. Exposure to malathion also influences the hormone that maintains the physiology of body growth. Malathion decreases both the thyroid hormone (T3 and T4) in plasma and inhibits its receptor binding, moreover, it reduces metabolic rate and body growth.[17,25,26] Perez Sanches and Lei Bail[27] have reported that hypothyroidism causes liver resistance to GH and affects hepatic insulin growth factor-1 (IGF-1) production. Growth hormone (GH) and IGF-1 decreases as a result of malathion exposure, which causes growth retardation.[17] Food consumption was reduced due to malathion exposure, causing the growth retardation.[26] Malathion also induces lipolysis of body fat and, therefore, contributes to the bodyweight decrease.[28] Exposure to malathion results in a significantly shorter body length.[28]

GH, IGF-1, steroid, and thyroid hormone are well-known to increase growth in fishes. The growth-promoting action of GH is mediated through IGF-1; a positive correlation between the IGF-1 and growth rate has been shown in in vivo studies.[28-30] The significant decline in GH, IGF-1, thyroid hormone, and steroid in malathion-exposed contribute to reduced body growth and metabolism change.

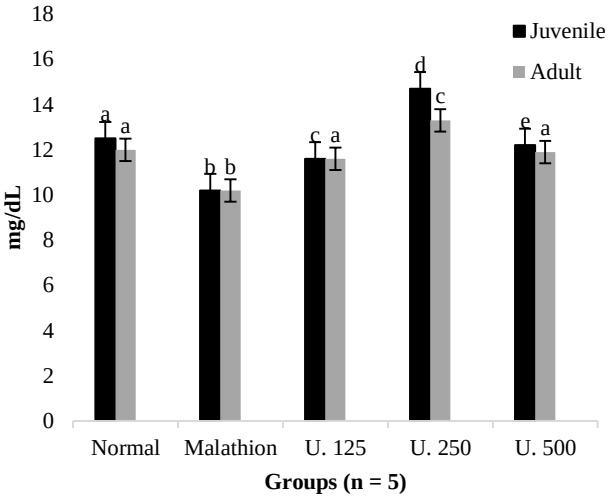

Figure 2: Effect of *U. lobata* leaf extract on hemoglobin level of *D. rerio*

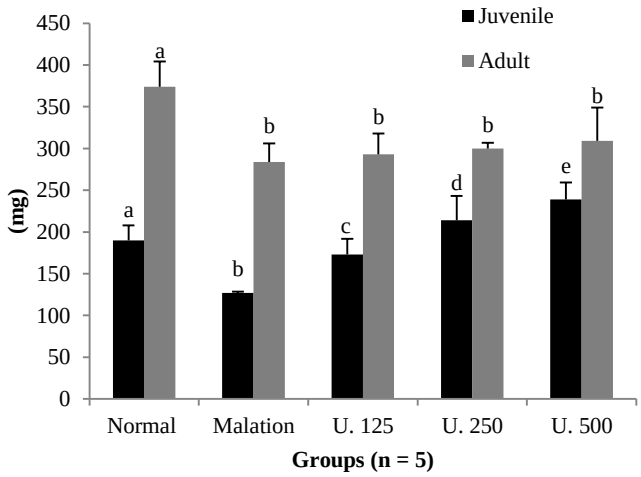

Figure 3: Bodyweight *D. rerio*

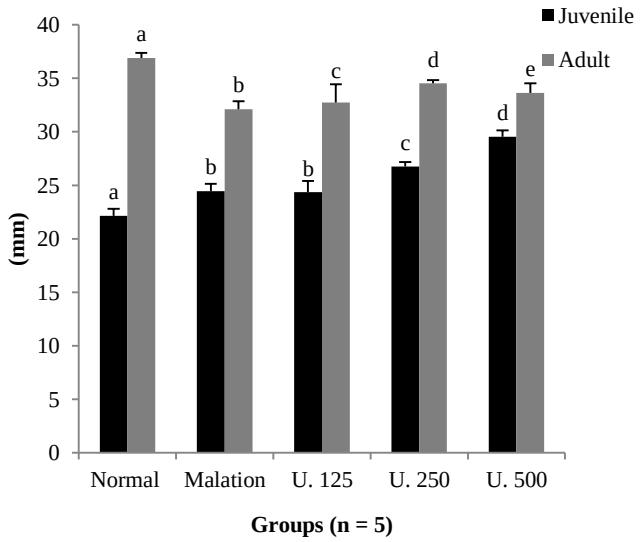

Figure 4: Body length *D. rerio*

U. lobata leaf extract increases the body growth of *D. rerio* exposed to malathion. Active compounds in *U. lobata* contribute to retaining the bioavailability of incretin hormone through inhibition of DPP-4 activity. Incretin hormone increases the secretion of insulin causing lipolysis inhibition and regulating body weight. Meanwhile, in the MAPK pathway, the incretin hormone increases cell proliferation, moreover, it supports the body growth of zebrafish *D. rerio* both of bodyweight and body length. Whereas, the antioxidant effect of *U. lobata* has a role to protect *D. rerio* against free radicals produced by malathion. Gossypetin in *U. lobata* leaf extract scavenges pro-oxidant substances causing oxidative damage in cells, moreover, it prevents growth retardation. Reports have indicated the effect associated with mangiferin, including antioxidant activity.[33] *U. lobata* neutralize malathion effect contributing to impaired hormone secretion, the hormone responsible for homeostasis, normal cell metabolism, reproduction, and development. Studies on laboratory animals treated by stigmasterol showed anti-inflammatory and immunomodulatory effects.[36,43] Anti-inflammatory substances of *U. lobata* inhibit cell damage which is caused by cytokine pro-inflammatory release. It is useful to support the body growth of *D. rerio* both of body weight and body length.

Conclusion

U. lobata leaf extract can inhibit the increase of blood glucose level and prevent the decrease of body growth and hemoglobin level both in juvenile and adult *D. rerio*.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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