Trop J Nat Prod Res, June 2021; 5(6):1034-1038
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

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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ARTICLE INFO

Article history:
Received 23 January 2021
Revised 18 April 2021
Accepted 20 June 2021
Published online 01 July 2021

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]
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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Citation: Purnomo Y, Pamungkas IA, Sofiani M, Pratama S. Effect of Pulutan (*Urena lobata*) Leaf Extract on Blood Glucose Level, Hemoglobin and Body Growth of Zebra Fish (*Danio rerio*) Exposed to Malathion. Trop J Nat Prod Res. 2021; 5(6):1034-1038. doi.org/10.26538/tjnpr/v5i6.9

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

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.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

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.

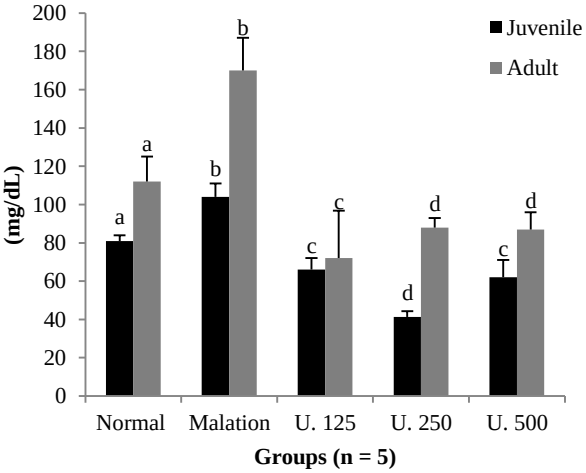

Figure 1: Effect of *U. lobata* leaf extract on blood glucose level of *D. rerio*.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

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.

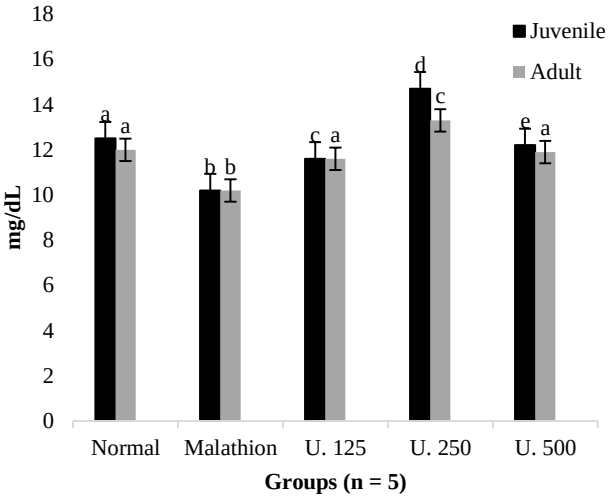

Figure 2: Effect of *U. lobata* leaf extract on hemoglobin level of *D. rerio*

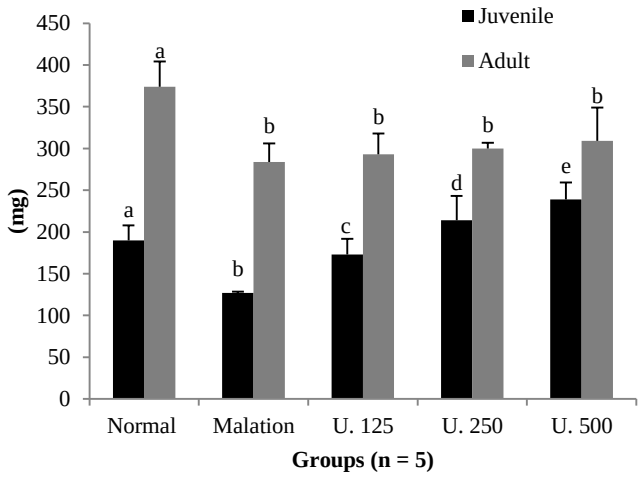

Figure 3: Bodyweight *D. rerio*

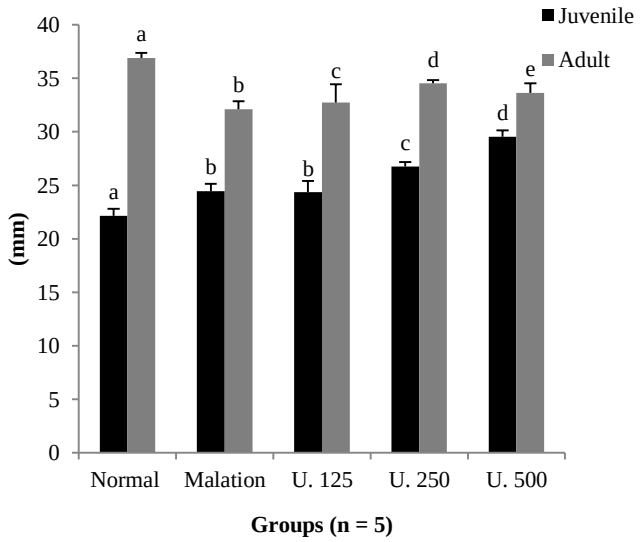

Figure 4: Body length *D. rerio*

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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