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The potential of *terminalia catappa* treat to inflammation on morphological erythrocyte of *pangasius hypophthalmus* poisoned with lead (pb)

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Abstract
This reseach concentrated on exploring the deformed erythrocytes morphology due to the effects of heavy metal toxicity and the size of erythrocytes when treated with almond leaves (*Terminalia Catappa*) on *Pangasius Hypophthalmus*. The fishes were fed with food mixed with almond leaves extract with concentrations of 0.3ml, 0.6ml, and 0.9ml in 7 days. After that, lead is contaminated with an amount of 1.0mg/l for all 3 treatments to evaluate the ability to treat inflammation in catfish contaminated with lead. The results when feeding fishes with almond leaves extract shown that the size of the area and circumference of erythrocytes increased, showing the ability to treat inflammation in *Pangasius* of almond leaves extract.

Keywords: *Pangasius hypopthalmus*, almond leaves, morphology, erythrocytes, lead

Introduction
Pangasius Hypophthalmus has a long body, a dark gray back, a silver belly, a wide mouth, and 2 pairs of long antennae. Fins dark gray or black; 6 branched dorsal fin rays; gill nets develop normally. From about 2.5 kg onwards, the weight gain is faster than the increase in body length. Fish over 10 years old in the wild (in Cambodia) gain very little weight. *Pangasius* in the wild can live over 20 years. In the wild, we have encountered fishes weighing 20 kg or having fishes sample up to 1.8 m in length (Legendre *et al*, 2000) [14]. *Pangasius* in Vietnam is one of the popular high-yielding fishes varieties and *Pangasius* is also one of the factors affecting the potential environmental impacts of the industry, so we chose the *Pangasius* is an experimental object to see the treatment ability of the leaves extract on infected fishes, thereby finding a better method for the agricultural product industry in Vietnam in particular and in the country in general. Currently, human life is growingly developing, but the pollution of the environment is increasing, including the water environment - where aquatic animals live. The pollution causes aquatic life in general and fishes in particular to be infected with diseases, die in mass (Saleh, 2015) [21]. More seriously, it indirectly affects human health. So what is one of the causes of water pollution? Those are heavy metals such as Hg, Cd, Pb, Cr, Cu, Mn. mainly come from industrial waste from factories or industrial zones without treatment, from daily life activities of human (Ali *et al*, 2020) [3]. Measurement results of these heavy metals often exceed the allowable standard parameters in the aquatic environment. In which, especially lead (Pb) often has the highest toxic parameters compared to the remaining metals studied on the heavy metal index (Lee *et al*, 2019) [13]. Besides, fishes also become weaker due to the presence of toxic substances, factors such as pollution from the physiological process of fishes, innate genetics, viruses, bacteria, parasites, fungi, lice, etc., or due to the accumulation of toxic gases such as NH3, H2S, NO2, when the water is polluted that can lead to sick fishes, cause fishes to death (Sweety *et al*, 1995) [23]. Instead of using antibiotics floating on the market that easily to leave serious sequalae and only bring temporary benefits, why not use medicinal herbs such as quercetin, guaijaverin, flavonoids, tannins, lecithin to create immunity, anti-oxidant, anti-toxic, anti-inflammatory, antibacterial for fishes (Etienne *et al*, 2017)[8]. Today, scientific researchers have also paid attention to the medicinal herbs from the millennium instead of using artificial antibiotics for fishes because antibiotics will still contain bad side effects, cause environmental pollution.

And it is also because of the safety and effectiveness that the focus on the use of plant essences and essential oils to increase the immunity and resistance of animals are a research direction that is being focused. However, even so, the research topic on the ability to treat inflammation in catfish (*Pangasius hypophthalmus*) with the extract of almond leaves are still limited. Because of the above reasons, this study was aimed to assess"The potential effects of *Terminalia Catappa* on *Pangasius Hypophthalmus* erythrocytes is contaminated with lead (Pb)" was built and implemented.

Materials and Methods

The study was carried out on the peripheral blood of *Pangasius* about 2.5 months old, with an average weight of 28±3.15g/fish. Catfish (*Pangasius Hypopthalmus*) were purchased at the fish hatchery, Binh Chanh district, Ho Chi Minh City. Fishes were reared for 2 weeks to stabilize the lead-free water as a control. Before stocking fishes, water was pumped from tap water, sun-dried for 2 days to reduce chlorine in the water, then aerated for 1 day to have enough dissolved oxygen available. During the rearing period, the fishes were fed with commercial feed, fed twice a day according to the fish's needs. Fishes aere reared and water is changed every 4 days. The water is continuously aerated to ensure sufficient dissolved oxygen in the water. There is a net on the mouth of the aquarium to avoid the impact of outside animals on the fishes and prevent the fishes from waving out. Catfish were stocked in a tanks, with the size of each tank is 100cm x 90cm x 70cm. The fishes were randomly divided into 4 treatments with the number of 10 individuals/treatment. In which, 3 tanks were fed with food containing almond leaves extract and contaminated with lead, while the remaining aquariums were not fed food containing almond leaves extract and not contaminated with lead, as a control. To evaluate the ability to treat inflammation in catfish with the extract of the leaves, we need to have fresh almond leaves collected from the botanical garden of Nguyen Hien high school, VietNam. The fresh leaves were washed with plain water, cut off the petioles, then cut them into small pieces and put them in a blender. We use a filter bag to filter the blended mixture to filter out the solution. When it is not in use, the leaves extract is stored in the refrigerator. Immunization of the fishes were performed first with the extract of the leaves mixed with the feed, the ratio of the respective extracts of the leaves were 0.3ml, 0.6ml, 0.9ml to determine the ability to control the disease. Treatment of lead inflammation after feeding within 7 days. Then, to cause heavy metal contamination of *Pangasius*, the study used lead nitrate ($Pb(NO_3)_2$) with equal lead (Pb) concentration of 1.0mg/l after 5 days of study (Vo *et al*, 2019) [25]. This is because lead is one of the heavy metals of concern and has concentrations that are significantly harmful to aquatic life (Burton *et al*, 1972) [4]. We dilute the leaves extract with a little water and then mix it with the commercial feed corresponding to the experimental treatments. Fishes blood was collected from the tail vein by aspiration injection. To prevent coagulation, the blood collection needle is impregnated with heparin at a concentration of 10 IU/ml. Blood is stored in specialized blood tubes pre-impregnated with heparin purchased from Hong Thien My Medical Supplies Joint Stock Company. Next, fishes blood was viewed under the microscope by Giemsa smear method (Hrubec TC, Cardinale JL and Smith SA, 2000) [10], (Natt MP and Herrick CA, 1952) [18], (Humason GL, 1962) [11] to detect the deformation of erythrocytes of *Pangasius* when contaminated with lead. Then, due to the size of the area and the erythrocytes cycle, in this method we use the S-EYE erythrocytes viewing software and have the ability to measure the erythrocytes size and besides the erythrocytes size. The demand of *Pangasius* is also measured more carefully by manual method, which is the circumference and area. Blood samples were collected 5 days after lead contamination, obtained using the fishes blood collection method as described above. Blood samples were observed and randomly measured the size of 500 erythrocytes per cell smear. The dimensions of the major axis (a) and the minor axis (b) were measured for each erythrocytes. The circumference (P) and area (S) are calculated from the dimensions of the major and minor axes using the following formula (Chernyavskikh SD, Quyet DH and Thanh VV, 2018) [5], (Frolova *et al*, 2017) [9]:

$$P = 2\pi\sqrt{\frac{\left(\frac{a}{2}\right)^2+\left(\frac{b}{2}\right)^2}{2}} \quad \text{and} \quad S = \pi ab$$

Terminalia Catappa is a tropical species widely distributed in the Indo-Malayan region, extending into the Philippines; It has been widely cultivated throughout the tropics, often naturally converted. It was found mainly along coastal lands forming part of coastal forest communities. The leaves extract has been studied and tested through GC-MS methods and gives results on the components in the leaves: magnesium, calcium, phosphorous, iron, tannin, these are the ingredients. a significant and therapeutic effect for fishes (Asgary S, Naderi G and Askari N, 2005) [2]. Guajaverin,Alkaloids, tannins, and flavonoids in the leaves have anti-inflammatory properties (Prabu GR, Gnanamani, and Sadulla S, 2006 and Katiki, 2017) [19,12] and have high antioxidant, antitoxic properties (Mohale *et al*, 2008 [15]. Among them, Tannin is being considered as a biologically active compound in nutritional science, capable of removing toxic substances from seawater (Das *et al*, 2020) [6]. In addition, Flavonoids have been shown to have antioxidant, anticancer, and anti-inflammatory activities (Sadzuka *et al*, 1997) [20]. The extract of the leaves has the ability to resist toxicity from bacterial species that cause inflammatory diseases in fish (Nair R, and Chanda S, 2008) [17]. When feeding fish with food combined with almond leaves extract, it not only does not adversely affect the health of the fish but also benefits, creates the immune system, increases the fish's ability to withstand toxicity when living in the toxic environment. For these reasons, it was shown that the leaves extract may be resistant to lead (Pb) toxicity.

Results

This experiment focused on the morphology and size of *Pangasius* erythrocytes when contaminated with lead metal at the concentration of 1.0 mg/l and treated with the extract of the leaves of the catfish at the concentration of 0.3ml; 0.6 ml; 0.9 ml.The results of the deformation of erythrocytes due to the effects of lead toxicity are shown in the following images:

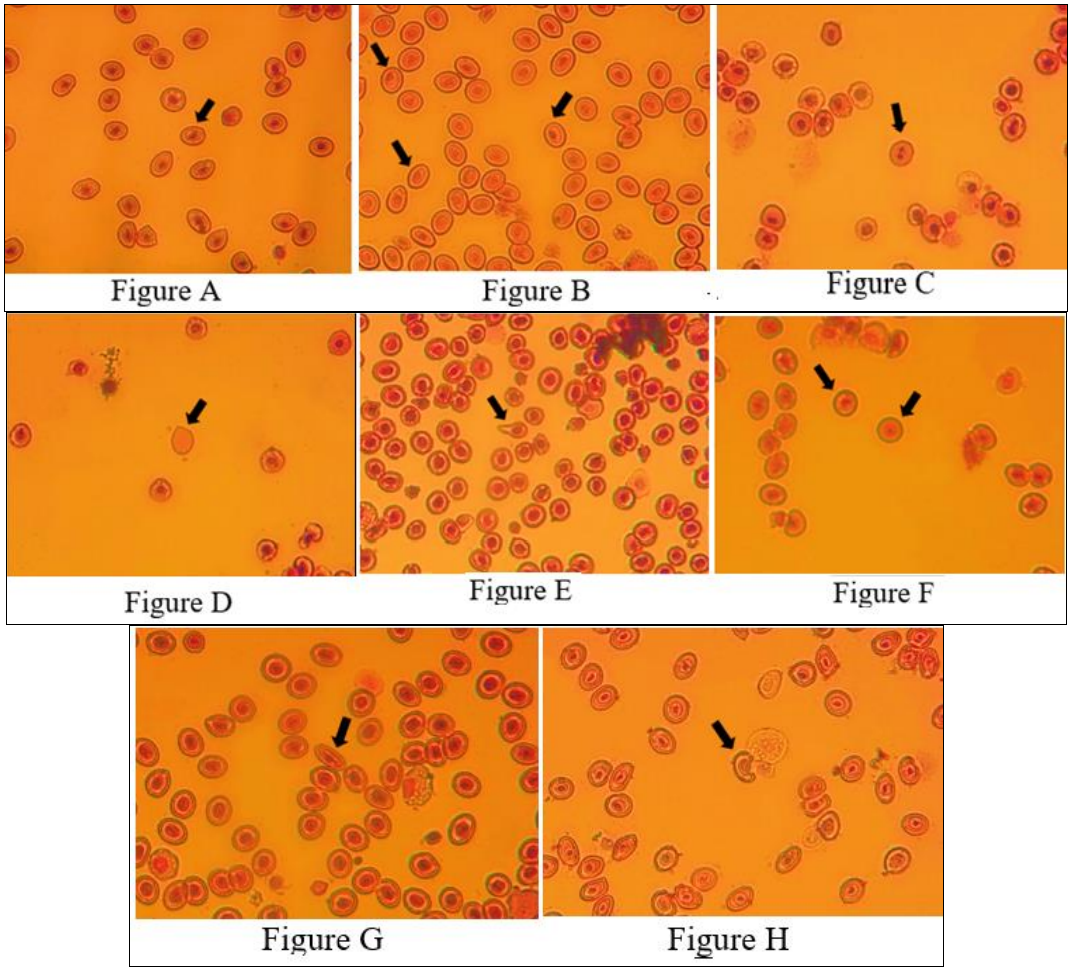

Fig 1: Eccentric erythrocytes (Figure B), dinuclear erythrocytes also known as polymorphonuclear erythrocytes (Figure C), nonnucleated RBCs (Figure D), teardrop-shaped RBCs (Figure E), progenitor erythrocytes mature (Figure F), hammer (Figure G) and sickle (Figure H) erythrocytess. Tables

Under the microscope, the erythrocytes in the blood were mostly observed in the normal state as the characteristic elliptic erythrocytes (figure A) and this was the steady state of the fish. After experimenting and causing lead contamination in water, fishes weree poisoned by lead toxiciety, which changed the blood circulation system. In the long term, heavy metal contamination of fishes causes changes in blood circulation, leading to the appearance of malformed erythrocytes in the peripheral blood of fishes. The presence of more pre-mature erythrocytes usually indicates that the fishes are suffering from anemia. The presence of pre-mature erythrocytes were often a symptom of anemia and that the number of pre-mature erythrocytes increases when fish are infected. However, there was another observation from the study that the presence of premature erythrocytes were not always confirm anemia but also the presence of these premature erythrocytes react to harmful substances from the environment. In addition, the deformation of erythrocytes is also caused by environmental temperature factors, which are always fluctuating and govern the obvious changes in hematological indicators as well as the morphology of erythrocytes. Through the process of observing the specimen on the microscope, it was shown that in the control treatment mainly normal erythrocytes. In 0.3ml there were the most abundant premature and malformed RBCs, when abnormal and malformed erythrocytes occupied the upper part of the specimen. In 0.6ml and 0.9ml there were normal-shaped RBCs and abnormal-shaped RBCs, however, the number of abnormal erythrocytes were lower than in 0.3ml. Between 0.6ml and 0.9ml, there were similar numbers of abnormal and malformed erythrocytes, so there was no statistical difference. Abnormal fishes hematologic morphology associated with erythrocytes includes polymorphonuclear erythrocytes, erythrocytes morphology deformed, erythrocytes denuclearized, or erythrocytes with only nuclei with no cytoplasm remaining. The cause of premature erythrocytes gradually replaced for mature cells is explained by the author as possibly due to a toxic agent affecting the erythrocytes production organ leading to an impaired cell cycle shorten. Cytoplasm and nuclei in erythrocytes are deformed leading to deformation of erythrocytes when observed under the microscope, even erythrocytes lose their nucleus or rupture during formation leading to hemorrhagic conditions. In the study show, the erythrocytes in a normal state are elliptical in shape that is most favorable for transport in the circulatory system. The process of action of lead makes erythrocytes bigger and rounder, no longer keeping the original elliptical shape, to block blood vessels when the blood cells are transported, besides, the appearance of sickle cells can lead to hematoma (blood cancer).

That is the reason why we only used the almond leaves extract to study the ability to treat inflammation in *Pangasius* caused by heavy metals, specifically in this study, lead and the results of erythrocytes morphology change. when treated with bladder fluid to show in the table below:

Table 1: *Pangasius* erythrocytes size according to the concentration of the leaves extract

	Concentration of leaves extract			
	Control (0 ml)	**NT1 (0.3 ml)**	**NT2 (0.6 ml)**	**NT3 (0.9 ml)**
RBC major axis (µm)	10.046±0.280[a]	7.858±0.096[b]	8.545±0.180[c]	9.531± 0.109[d]
erythrocytes minor axis (µm)	5.171±0.398[a]	6.193±0.266[b]	6.339±0.185[c]	6,102±0.112[c]
RBC circumference (µm)	25.099±1.501[a]	22.225±1,870[b]	23,635±1, 123[c]	25,140±1,371[d]
Area of erythrocytes (µm ^{2})	40.799±7.146[a]	38.221±7,320[b]	42,542±6, 102[c]	43.154±7,044[d]

Note: a, b, c, d- the difference is statically significant with $p<0.05$

Experiment to study the change in erythrocytes size in *Pangasius* under the anti-inflammatory effect of sage leaves extract: control: 0 ml; 0.3ml; 0.6ml; 0.9ml after 9 days of immunostimulation. The study results shown that the erythrocytes size of *Pangasius* in the condition of not feeding with almond leaves extract and not contaminated with lead (10.046±0.280µm) and the size of erythrocytes of each type of fishes were different. Differences in erythrocytes size may be influenced by conditions, food sources, and fishes stock. In addition, swimming speed, activity, shape and age of fishes can affect the size of erythrocytes in the fish's peripheral blood. The results from the table shown that the size at the major axis of erythrocytes increases gradually at different concentrations of the leaves extract 0.3ml, 0.6ml, 0.9ml: from 7.858±0.096µm up to 9.531±0.109µm and all 3 treatments had lower major axis size than the control. Specifically, at the concentration of 0.3ml, the large axis size decreased compared to the control by 2.188±0.19µm. in the large axis size of 0.6ml concentration increased compared to 0.3ml large axis 0.687±0.084µm and decreased 1.501±0.1µm compared to the control. At 0.9ml large axis, the size increased compared to 0.6ml large axis size was 0.986±0.018µm and decreased 0.515±0.082µm compared to the control. All of those differences are statistically significant with $p<0.001$. The tendency to change the size of the minor axis of erythrocytes were opposite to that of the major axis. At concentrations of leaves extract of 0ml and 0.6ml, the minor axis size increased from 5.171±0.398µm to 6.339±0.185µm compared to the control. Specifically, at the concentration of 0.3ml of the leaves extract, the increase compared to the control was 1.022±0.073µm. There continued to be an increase in the size of the small axis at the concentration of 0.6ml, which was higher than that of the control 1.168±0.154µm and 0.146±0.081µm compared with the concentration of 0.3ml leaves extract. At the concentration of 0.9ml extract, the small axis size decreased compared to the concentration of 0.3ml and 0.6ml, but still increased in size compared to the control. Specifically, at the concentration of 0.9ml, the small axis size decreased compared to the small axis size at 0.6ml concentration was 0.237±0.088µm, decreased compared to the small axis size at 0.3ml concentration was 0.091±0.007µm and increased compared to the 0.6ml concentration control is 0.931±0.066µm. The trend of changing the circumference and area of erythrocytes was relatively similar. The details of the change were as follows: decrease in concentration of 0.3ml leaves extract compared with control and gradually increase in concentration of erythrocytes extract in 0.6ml and 0.9ml.

All 3 treatments gave the same lead concentration and the concentration of the leaves extract was different: 0.3ml, 0.6ml, 0.9ml. The circumference and area of erythrocytes in 0.3ml were the lowest in size compared to the control because 0.3ml shown lead poisoning, but feeding the fish with a concentration of the leaves extract was only 0.3ml. Although 0.6m and 0.9ml had a lower area and circumference than the control, they gradually increased in size and circumference compared to 0.3ml, thereby demonstrating the ability of almond leaves extract to treat inflammation in catfish. The results were shown that the concentration of the leaves extract was proportional to the ability to resist lead toxicity. All of those differences are statistically significant with $p<0.001$. Almond leaves extract had a positive effect on erythrocytes of both catfish. Almond leaves extract has been studied and tested by various methods and shows high antioxidant and antitoxic activity. Especially the extract of the leaves had the ability to treat inflammation on fishes due to the impact of heavy metals. When feeding fishes at concentrations of 0.3ml, 0.6ml, and 0.9ml of leaves extract, the number and size of erythrocytes were increase, indicating that the fishes had the ability to treat inflammation from almond leaves extract.

Discussion

It is necessary to arrange a longer study period with more treatments. It is necessary to use other toxic substances such as bacteria, etc. to evaluate the effectiveness of the fishes immune system after feeding with the leaves extract. It can be applied to industrial fishes farming to reduce fishes diseases and contribute to increased productivity.

Conclusion

Pangasius was inflamed due to lead poisoning even though it was fed with extracts of the leaves to increase its resistance to toxicity.e Under the influence of lead concentration of 1.0mg/l, the affected fishes reduced the number of erythrocytes and the erythrocytes were deformed, indicating that the fishes were inflamed. When fishes were fed diets containing fenugreek leaves extract at concentrations of 0.3ml, 0.6ml and 0.9ml, the size and circumference of erythrocytes increased, indicating the ability to treat inflammation on *Pangasius* by extract of the leaves and gradually brought the fishes back to the same balance as the control fishes without lead contamination. Through the research process, the hypothesis that the group put forward is scientifically grounded, confirmed from the experimental results of the topic. This topic is being continued by our research team on actual river water and some areas with polluted water on a larger scale to test the applicability and community development. We will have the results reported as soon as possible in the next time.

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