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Mangosteen *Garcinia Mangostana* L. Simplicia Effect on Bone Structure and Behaviour of Wader Fish *Rasbora Lateristriata* (Bleeker, 1854) Embryo

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ABSTRACT

Mangosteen (*Garcinia mangostana* L.) is a fruit originating from the tropics which belongs to the Clusiaceae family and has become a commodity with many enthusiasts from various countries. Previous research has shown that mangosteen peel extract has tremendous potential to be developed as an ingredient in herbal medicine. Mangosteen fruit contains anti-inflammatory, antibacterial, antifungal, antihistamine, antidiabetic, anticancer, and so on. The results of the phytochemical screening test of 95% ethanol extract on mangosteen rind showed that it contained flavonoid compounds, saponins, alkaloids, triterpenoids, tannins and polyphenols. There is no data on the effect of exposure to mangosteen pericarp simplicia, therefore the objective of the study was to examine the effect of exposure to mangosteen peel decoction on animal models of Wader Pari Fish (*Rasbora lateristriata*), with parameters such as morphology and bone structure in R. *lateristriata* larvae. The whole mount embryonic preparation method with Alcian Blue-Alizarin Red staining was used to study bone structure and visual observation with video recorded movie was implemented to study the behaviour of R. *lateristriata*. Data were analysis with SPSS ver. 25, followed with one-way analysis of variance (ANOVA) for significancy. The results showed that the embryo and larvae showed morphological abnormalities in higher concentration treatment. The cranial cartilage examination also revealed no abnormalities at the larvae, the length and the angle of the cranial cartilage did not affected by mangosteen pericarp simplicia treatment. It could be concluded that mangosteen pericarp simplicia exposure caused abnormality on the wader pari fish embryo in dosage dependent manner.

Keywords: mangosteen pericarp, *Rasbora lateristriata*, toxicity test

INTRODUCTION

Mangosteen is a fruit originating from the tropics with white flesh and thick purplish-coloured outer skin (Suwignyo, 2014). Its tree belongs to the Clusiaceae family (ITIS, 2022). Mangosteen has become a commodity with many enthusiasts from various countries (Uji, 2007) because the fruit is sweet and delicious. Previous research has shown that mangosteen peel extract has tremendous potential to be developed as an ingredient in herbal medicine. Mangosteen fruit exhibits pharmaceutical activities such as anti-inflammatory, antibacterial, antifungal, antihistamine, antidiabetic, anticancer, and so on (Nugroho, 2009; Pasaribu *et al*., 2012; Brito *et al*., 2017). The content can be in the form of xanthone active compounds including mangostenol, mangostin, mangostino A, mangostino B, tvophylin B, trapezifolixanthone, alpha mangostin, beta mangostin, garcinon B, mangostano, flavonoids epicatechin and gartanin (Rohman *et al*., 2019).

The results of the phytochemical screening test of 95% ethanol extract of mangosteen (*Garcinia mangostana* L.) rind showed that it contained flavonoid compounds, saponins, alkaloids, triterpenoids, tannins and polyphenols. Moreover, mangostin compounds have anti-inflammatory activity inhibiting the secretion of nitric oxide, TNF-, and IL-8, which are known to cause bone resorption and inhibit bone formation (Gutierrez-Orozco *et al*., 2013). Aside from demonstrating anti-inflammatory activities, those

mangostin compounds also exhibit the effect of stomach poison, which could interfere with the digestive system function (Purnomo, 2015).

Mangostin has recently been considered as one of ingredients of herbal medicines to treat inflammation and also act as an antioxidant. However, it application is usually without proper efficacy and safety test following both WHO and the Regulation of the Minister of Health of the Republic of Indonesia. Thus, the use of mangosteen rind requires a toxicity test (Mustapa *et al*., 2018).The acute toxicity test of α-mangostin on rats with a concentration of 2000 mg/kg body weight in a 48-hour treatment did not show any toxic effects or death (Nelli *et al*., 2013). A study on Wistar rats with concentrations of more than 1250 mg/kg also did not show any toxic effects, death, or changes in behaviour within 48 hours (Kumar *et al*., 2016). However, intraperitoneal injection in mice with a concentration of 150 mg/kg for 72 hours led to death (Choi *et al*., 2014). Research conducted by Suwignyo A (2014) on zebrafish embryos showed that mangosteen rind resulted in pericardial and brain abnormalities, which lead to the fish's lethality. Those aforementioned findings showed species-specific responses to the treatment with different animal models. Thus, further research on the toxic effects and its physiological manifestation of mangosteen peel extract using fish as different species is needed. *Rasbora lateristriata* is a local fish of the Cyprinidae family (ITIS, 2022) that has the ability to adapt and resist changes in extreme environmental conditions (Retnoaji *et al*., 2014). This fish has a habitat in freshwater and is often used as a bioindicator of environmental conditions (Zakeyudin *et al*., 2012). Its wide distribution and ability to produce many eggs make this fish very promising to be used as animal models in research. Each broodstock of R. *lateristriata* is known to produce 1000 eggs (Raharjeng and Retnoaji, 2020). This study aimed to determine the toxic effect and physiological effect (on fish behaviour) of mangosteen peel extract at various concentrations in R. *Lateristriata* animal model.

MATERIALS AND METHODS

The materials of study were R. *lateristriata* fish, mangosteen (G. *mangostana*) pericarp simplicia, egg water media (1,000 mL water, 1.5 mL salt water, and 1–2 drops methylene blue), alcohol (Genera Labora), distilled water (Genera Labora), H2O2 3% (Merck), KOH 2% (Merck), glycerine 100% (Merck), and Alizarin red and Alcian blue (ARAB) stain solution.

Fish Reproduction and egg collection

Female and male fish with mature gonads were selected and maintained at the laboratory, with adequate food and controlled environmental conditions of 28-29°C of temperature, pH of 7.0-7.5, and 14L:10D photoperiodic cycle mimicking daily light cycle (Matthews *et al*., 2000; Harper and Lawrance, 2016). Wader pari embryos were obtained from domesticated wild fish, with female to male of 1:2. Eggs were carefully collected using a pipette (Liu, 2014), and cleaned with water 3 times and placed in a petri dish.

Egg Treatment

Mangosteen peels at the decided concentration were dissolved and heated at 90°C for 30 minutes in distilled water and boiled. The fish eggs were selected for normal development under a microscope. The healthy egg was transferred to well plates containing treatment media at concentrations of 0, 0.5, 1, 5, 5, and 25 mg/mL, with three replications. Each concentration consisted of 30 embryos and each replication consisted of 10 embryos. The exposure was given from the pre-gastrulation embryo stage (3.5 – 4 hpf) to 72 hpf.

Observation of Morphologies Malformation

According to Hoyberghs *et al*. (2020), there are several characteristics of malformation that can occur during embryonic and larval development. Those characteristics may include abnormal head, ear, mouth or eye shape, absent or twisted fin, yolk, head or pericardial edema, or yolk extension, blood accumulation on tail, head, heart, yolk, or yolk extension, coagulation, indistinguishable or unrecognizable body parts, no hatching, yolk malformation, deviating pigmentation, swim bladder not inflated, non-detachment of tail, twisted or curved tail, tissue deviation on the tail, heart malformation, no blood circulation in the tail, disturbed blood circulation in the tail, and the absence of heartbeat. Morphological changes were assessed on *Rasbora lateristriata* age of 4 and 6 dpf.

Observation of Cranial Cartilage Structure

The cranial cartilage's structure were observed in 6 dpf larvae to determine the defect of the cranium. Bones and cartilages of the cranium were stained with Alizarin red–Alcian blue (Et-OH 100%, Alcian Blue, Alizarin Red, Acetic Acid, Water), following Retnoaji *et al*., (2014) with modification of KOH concentration.

The fish embryos were fixed with 96% alcohol for 3 days and then stained with Alizarin red–Alcian blue for 3 days. Larvae were bleached with 1 mL of bleach solution (H2O2 3% and KOH 2%) for 10 minutes, which was replaced with 1 mL of 0.05% KOH for 5 minutes, and transferred to 85% of glycerin solution. Image data were taken with a Leica ICC50 microscope and analyzed with ImageJ program. The measurement parameters were pq (palatoquadrate) length, m (Meckel's cartilage) length, ch (ceratohyal) angle, pq-m (palatoquadrate-Meckel's cartilage) angle, and pq-ch (palatoquadrate-ceratohyal) angle.

Fish Behavior examination

Fish behaviour was observed based on swimming behaviour and patterns activities. The fish activities were recorded with an underwater camera, and videos were analyzed for fish behaviours, mainly based on the parameters of swimming direction and acceleration and speed of the movements of the fish.

Data analysis

Quantitative data, such as percent of eggs hatchability, survival rates, and fish body ratios, were obtained from this study. Data obtained were analyzed with ANOVA statistical test ($P < 0.05$) to determine the significance between treatments.

RESULTS AND DISCUSSION

Effects of Mangosteen G*. mangostana* pericarp simplicia on Larvae Morphology

This study provides an overview of the effect of exposure to mangosteen pericarp simplicia on the development of R. *lateristriata*. In this study, treatment groups with mangosteen pericarp simplicia concentrations of 0.5, 1, 5, 25 and a control group were used. Several parameters observed in this study were larval morphology, cranial cartilage, and behaviour.

Effects of Mangosteen (G*. mangostana*) Pericarp Simplicia on Larval Morphology

Observation of larval morphology was conducted based on the parameters used to determine the presence of malformations. Morphological changes are important parameters to be observed in the toxicity test. Morphological changes are caused by intense metabolisms defects, which are manifested in the form of tissue or organ abnormality. In this study, the parameters observed, included the presence of edema on the head, pericardium and yolk, blood coagulation, and head-tail malformations (Figure 1 and Table I).

The results showed that embryos treated with mangosteen pericarp simplicia at concentrations of 0, 0.5, and 1 µg/mL exhibit normal development of body, yolk sac, swim bladder, pericardium and brain. However, Embryos treated with mangosteen pericarp simplicia at a concentration of 5 µg/mL showed normal pericardium, brain and swim bladder, but exhibit yolk sac edema. Moreover, embryos treated with mangosteen pericarp simplicia at a concentration of 25 µg/mL showed heart and yolk sac edema, collapsed swim bladder, undifferentiated pericardia, and abnormal head. Interestingly, all treated fish did not exhibit any blood coagulation at the head, tail, yolk, or heart, respectively. The occurrence of edemas in the heart and yolk sac of treated embryos, also collapsed swim bladder, undifferentiated pericardia, and abnormal head, especially at higher concentration of treatment were evidence of abnormalities in the development of R. *lateristriata* larvae caused by the toxic effect mangosteen exposure. This result is in line with the research conducted by Kitipaspallop *et al*. (2021) using mangosteen on zebrafish embryos. In their research, it was known that exposure at concentrations of 3, 6, 9, and 15 µm/L caused pericardial edema, stasis of erythrocytes in the ducts of cuvier, yolk edema, axis malformation, and bent tail.

Head malformation was further assessed based on some parameters, such as development of cranial forebrain, midbrain, hindbrain and also cartilage cranium morphology. The cranial forebrain, midbrain, and hindbrain abnormality were examined based on the measurement of some components, such as forebrain length, midbrain length, and hindbrain length (Figure 1). The result showed that there was no significant difference between control and treated embryos, either in the measurement of the forebrain, midbrain, or hindbrain (Figure 2, $P < 0.05$). Further analysis to examine the possible tissues or organ malformation of fish embryo in the control group and treatment groups at concentrations of 0.5, 1, 5, and 25 µg/mL were conducted for several parameters such as the shape of the head, mouth protrusion abnormality, mandibular structure and eye development defect or deviation. The result revealed that embryos treated with mangosteen pericarp simplicia especially at a concentration of 5 and 25 µg/mL, exhibit head malformation in the form of mandibular abnormality.

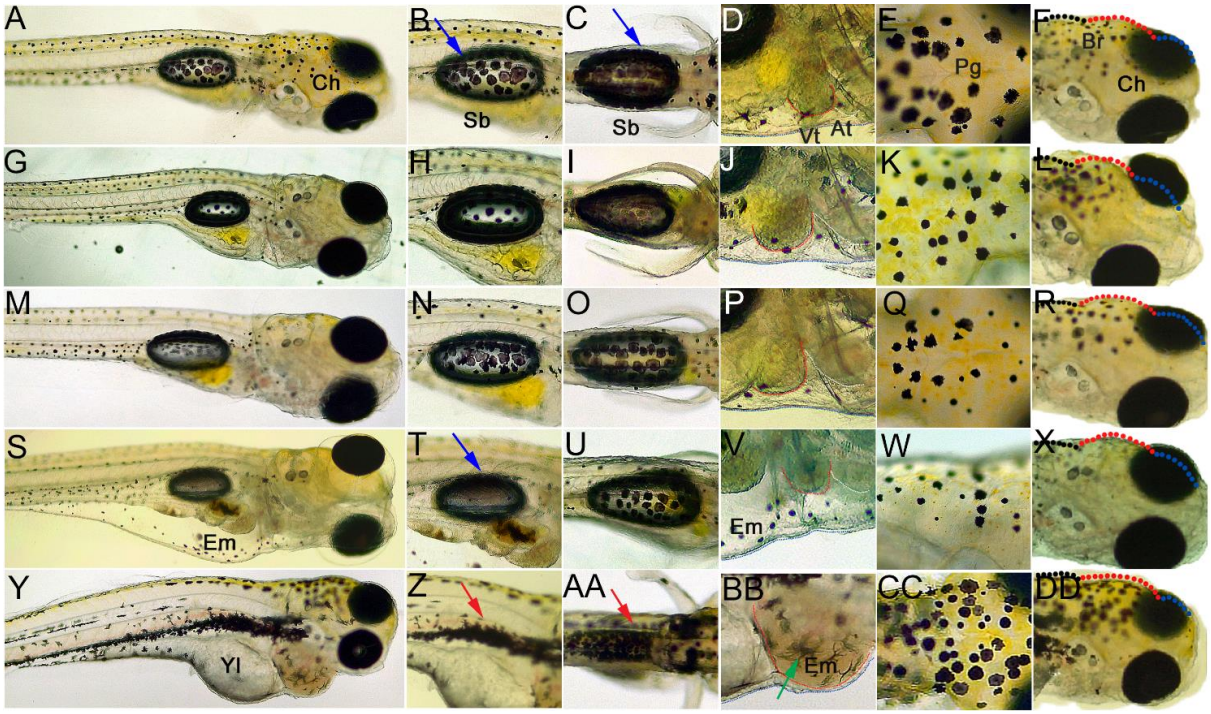

Figure 1. Morphologies of 96 hpf R. *lateristriata* showing malformation assessment of all treated embryos. (A-F) Control. (G-L) Embryos treated with mangosteen pericarp simplicia concentration 0.5 µg/mL. (M-R) Embryos treated with mangosteen pericarp simplicia concentration 1 µg/mL. (S-X) Embryos treated with mangosteen pericarp simplicia concentration 5 µg/Ml. (Y-DD) Embryos treated with mangosteen pericarp simplicia concentration 25 µg/mL. (Ch) Chondrocranium (Sb) swim bladder. (Vt) Ventricle. (At) Atrium. (Pg) Pigmentation. (Br) Brain. (Em) Edema. (Yl) Yolk Pneumatocyst. (Blue arrow) normal swim bladder. (Red arrow) Collapsed swim bladder. (Green arrow) Undifferentiated pericard. (Black dots) Hindbrain. (Red dots) Midbrain. (Blue dots) Forebrain

However, no any other head abnormalities parameters or abnormal phenotype were found (Table I). The result suggest that mangosteen treatment only took effect on specific tissues or organ of fish embryos at certain level of concentration. This finding is in line with the research result conducted by Jose *et al*. (2016), which reported the occurrence of Growth retardation, movement abnormalities, and, malformation of zebrafish embryos head after exposure to mangosteen leaves and stem-bark lyophilized water extract. In addition, research conducted by Fazry *et al*. (2018) also showed the presence of a bent tail in the zebrafish embryos treated with α-mangostin. This indicates that some of the components contained in mangosteen pericarp or other parts of the plant could cause different effects on the embryonic development of different fish species.

Effects of Mangosteen (G. *mangostana*) pericarp simplicia on Fish Pigmentation

Fish, amphibians, and reptiles exhibit colouration through a combination of pigment-based and structural colours, which are highly adaptable and sensitively affected by environmental factors. Therefore, it is important to implement the pigmentation intensity and pattern as a toxicity test parameter. The result showed the embryo treated with mangosteen pericarp simplicia at concentrations of 0.5, 1, 5 µg/mL, and that in the control group exhibited mild pigmentation intensity. On the other hand, embryos treated with mangosteen pericarp simplicia at a concentration of 25 µg/mL showed considerably increased melanocyte around the body, or body hyperpigmentation compared to those with other treatments (Table I).

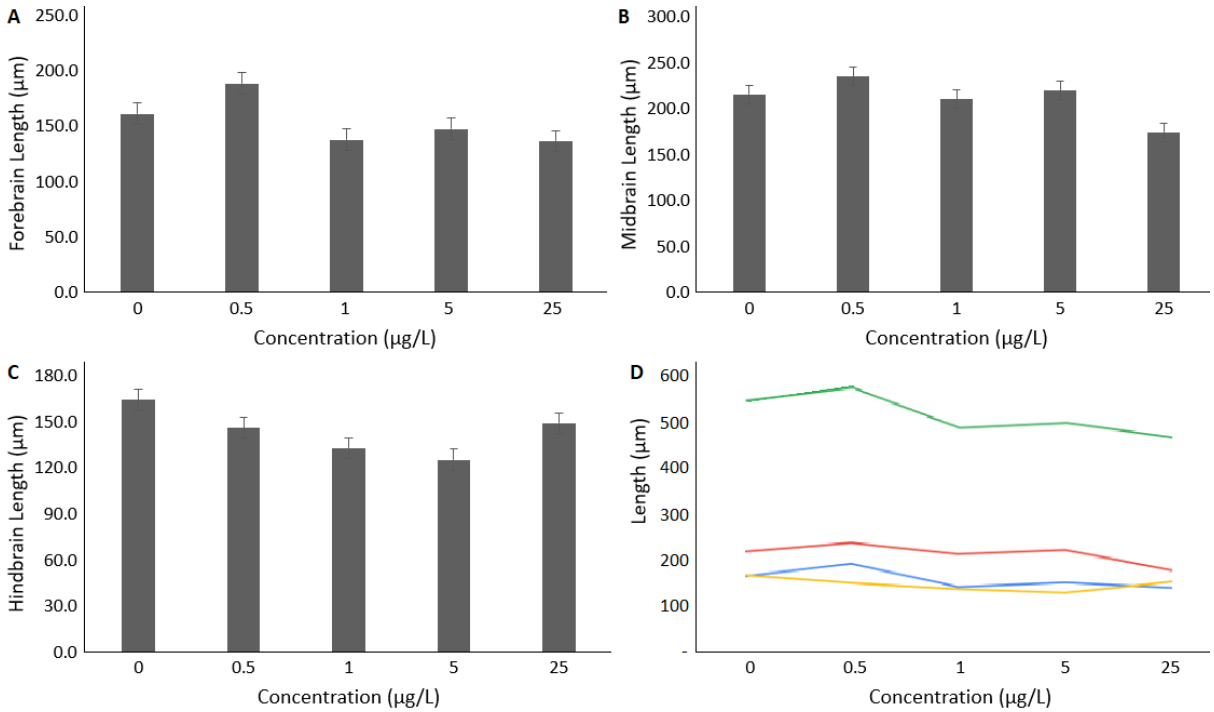

Figure 2. Assessment of the cranial forebrain, midbrain, and hindbrain by measuring the length

Table I. Malformation assessment in *R. lateristriata* morphology

Malformation Parameter	Treatment				
	Control	Mangosteen 0.5 µg/mL	Mangosteen 1 µg/mL	Mangosteen 5 µg/mL	Mangosteen 25 µg/mL
Head Malformation	×	×	×	✓	✓
Edema	×	×	×	✓	✓
Tail Malformation	×	×	×	×	×
Blood Coagulation	×	×	×	×	×
Yolk Malformation	×	×	×	✓	✓
Pigmentation	+++	+++	++++	++++	+++++

× : showing no abnormalities; ✓: showing abnormalities; + : showing pigmentation

Fish pigment usually contained of Pteridine- and carotenoid-based yellow and red pigments of xanthophores and erythrophores, as well as the melanin-based pigment of melanophores. Carotenoid vesicles and melanosomes, two membrane-bound organelles, are responsible for pigment production and storage, respectively. A diversity of colour patterns, particularly abundant in fish, results from variations in the basic colour-forming unit. The ability of pigment cells to change colour physiologically, which results in an adjustable body appearance, is one of the most important aspects and the melanophores change is a result of respond to neurological and hormonal stimuli (Singh and Nusslein-Volhard, 2015).

The hyperpigmentation occurrence at the concentration of 25 µg/mL suggests the effect of mangosteen treatment on pigment regulation and melatonin distribution. Since hyperpigmentation may be caused by an increase in the number and size of melanocytes (Hultman *et al*., 2007). Therefore, it is possible that mangosteen pericarp simplicia affect to the neurological and hormonal system of the fish in a dose-dependent manner, which stimulate and promotes melanin synthesis and tyrosinase activity, leading to the of embryonic hyperpigmentation (Hamid, *et al*., 2012).

Effects of Mangosteen (G. *mangostana*) pericarp simplicia on Bone Structure

In this study, R. *lateristriata* embryos aged 6 dpf were prepared for bone staining and stained with Alizarin red-Alcian blue staining (Figure 3). The results showed the whole structural composition and part of bones in the cranium. The staining uncover that all cranial bone components were consisted of cartilage, which is stained as blue in colour. Detail observation showed that there were several cranial structures, including palatoquadrate (pq), Meckel's cartilage (m), ceratohyal (ch), and (cb) ceratobranchial cartilage, respectively.

The cranial bone structural observations, and the identification of cranial bone components, showed that the mangosteen treatment did not affect the cranial bone component and arrangement. Moreover, there were no defects on the bone shape and number missing at various treatment concentrations.

Cartilage Cranium Examination

Observation of cranial cartilage is an important parameter in the study of teratological effects on the development of *R. lateristriata*. It is known that skeleton formation in fish begins to appear at the age of 48-52 hpf (Gavaia *et al*., 2006). This stage is a crucial time window for the development of *R. lateristriata* embryos. Observing cranial cartilage of *R. lateristriata* embryos at the age of skeleton development, reflecting the possible abnormalities caused by mangosteen pericarp simplicia. There are several parameters used in observing cranial cartilage, such as the length of the cranium, the length and angle of the cartilage in the cranium, and the completeness of the cartilage within the cranium (Sandi *et al*., 2022). The results of this examination showed no difference in the cartilage cranium (Figure 3-4) between the control and the mangosteen pericarp simplicial treatment groups.

Further analysis to examine the possible cranial structural defect was measurements of the cartilage component size and orientation. Some of these components were head length, pq length, m angle, ch angle, pq-m angle, and pq-ch angle. Several measurement parameters for cartilage bone were head length, PQ length, M angle, CH angle, PQ-M angle, and PQ-CL angle, which could be assessed at 6 dpf embryos stage.

The result showed that embryos treated with 0.5, 1, and 5 µg/mL mangosteen pericarp simplicia all survive up to 6 hpf stage. However, there was massive mortality in the mangosteen treated group at a concentration of 25 µg/mL, which occur before the embryo reached 6 dpf stage. Indicates that mangosteen pericarp simplicia at higher concentration can cause adverse toxic effects or lethal to embryo before the bone formation to complete. This finding is in line with the principle of toxicity, where any organic substance has a toxic potential and can cause a toxic effect at a certain dose (George, 2011). Due to the massive deaths in *R. lateristriata* before 6 dpf, the effect of mangosteen pericarp simplicia treatment at a concentration of 25 µg/mL on their larval morphology and cranial cartilage could not be proceed. Exposure to mangosteen pericarp simplicia in lower to medium concentration caused no changes in the cranium of R. *lateristriata* larvae. Moreover, the treatment of mangosteen pericarp simplicia caused no significant difference ($P < 0.05$) in the length and size of the cranial cartilage angle in R. *lateristriata*.

Single bone angel or articulation measurement showed the result that the head length of embryos treated with 0.5, 1, and 5 µg/mL mangosteen pericarp simplicia showed no significant difference compared to the control group (Figure 4A). Moreover, PQ length (Figure 4B). M angle (Figure 4C). CH angle (Figure 4D). PQ-M angle (Figure 4E). PQ-CH angle of the embryos treated with 1.5, 1, and 5 µg/mL simplicia showed no significant difference compared to the control group (Figure 4F, $P < 0.05$), respectively.

Interestingly embryos treated with mangosteen pericarp simplicia at concentrations of 25 µg/mL had reduced pharyngeal arches and also shown mandibles defect (Table I). Pharyngeal arches in teleost's are the developmental precursors of the mouth, gills, cranial muscles, and cranial nerve, which are anatomical structures for feeding and breathing. All three germ layers form pharyngeal arches, and subsequent pharyngeal cartilage development depends on precisely controlled interactions between the various tissue types (Knight & Schilling, 2006). The cranial neural crest cells independently generate dorsal and ventral condensations around the mesoderm core in each pharyngeal arch, which results in the production of corresponding cartilage parts. The recurrent development of pharyngeal endodermal pouches also serves to isolate the segmented pharyngeal arches from one another. Studies on several mutations have shown that the pharyngeal endoderm is crucial for regulating cartilage development (Yelick & Schilling, 2002).

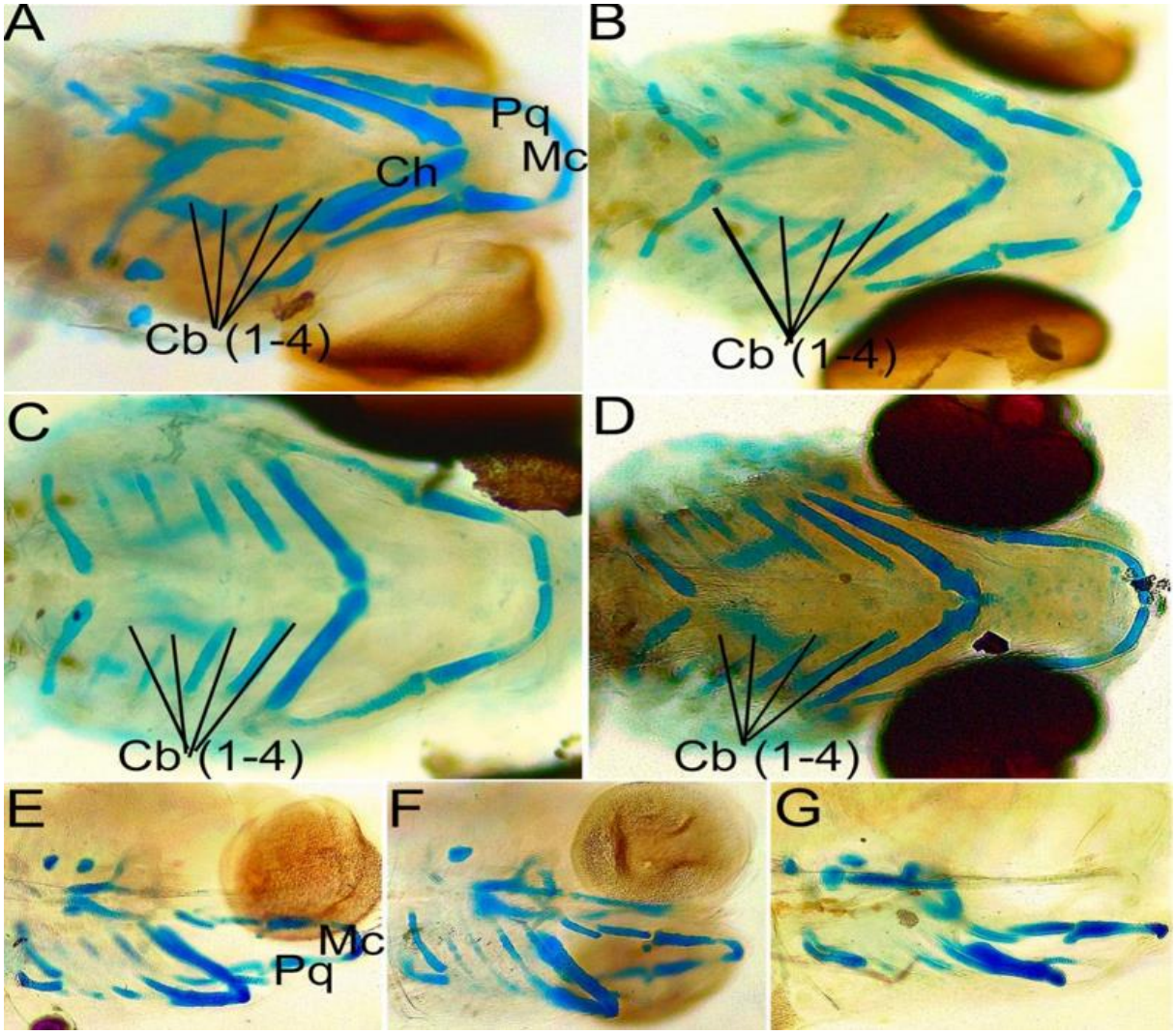

Figure 3. The bone structure of 6 dpf *Rasbora lateristriata* can be seen ventrally and laterally. (A) Control. (B) Mangosteen pericarp simplicia concentration 0.5 µg/mL. (C) Mangosteen pericarp simplicia concentration 1 µg/mL. (D) Mangosteen pericarp simplicia concentration 5 µg/mL. (E-G) Lateral view. (Pq) Palatoquadrate. (Mc) Meckel's cartilage. (Ch) Ceratohyal. 10× magnification.

Interruption of the nodal signalling system prevents endoderm growth, which in turn prevents pharyngeal cartilage from developing in zebrafish (David *et al*., 2002). The low concentration of mangosteen pericarp simplicia caused neither pharyngeal arch nor mandibular abnormalities. These results suggest that mangosteen pericarp simplicia may affect pharyngeal arches and mandible formation and development of the structures, only at a higher concentration of simplicia at or above of 25 µg/mL.

Effects of Mangosteen G. mangostana pericarp simplicia on Fish Behaviour

Fish embryonic behaviour analysis usually were examined through assessment of parameters such as swimming activity or rheotactic behaviour. Therefore, we conducted observations on the swimming behaviour and pattern on the 96 hpf of R. *lateristriata* embryos treated with 0.5, 1, 5 µg/mL mangosteen pericarp simplicia and those in the control group. Result showed that fish embryos from all treatment exhibit very active swimming movement.

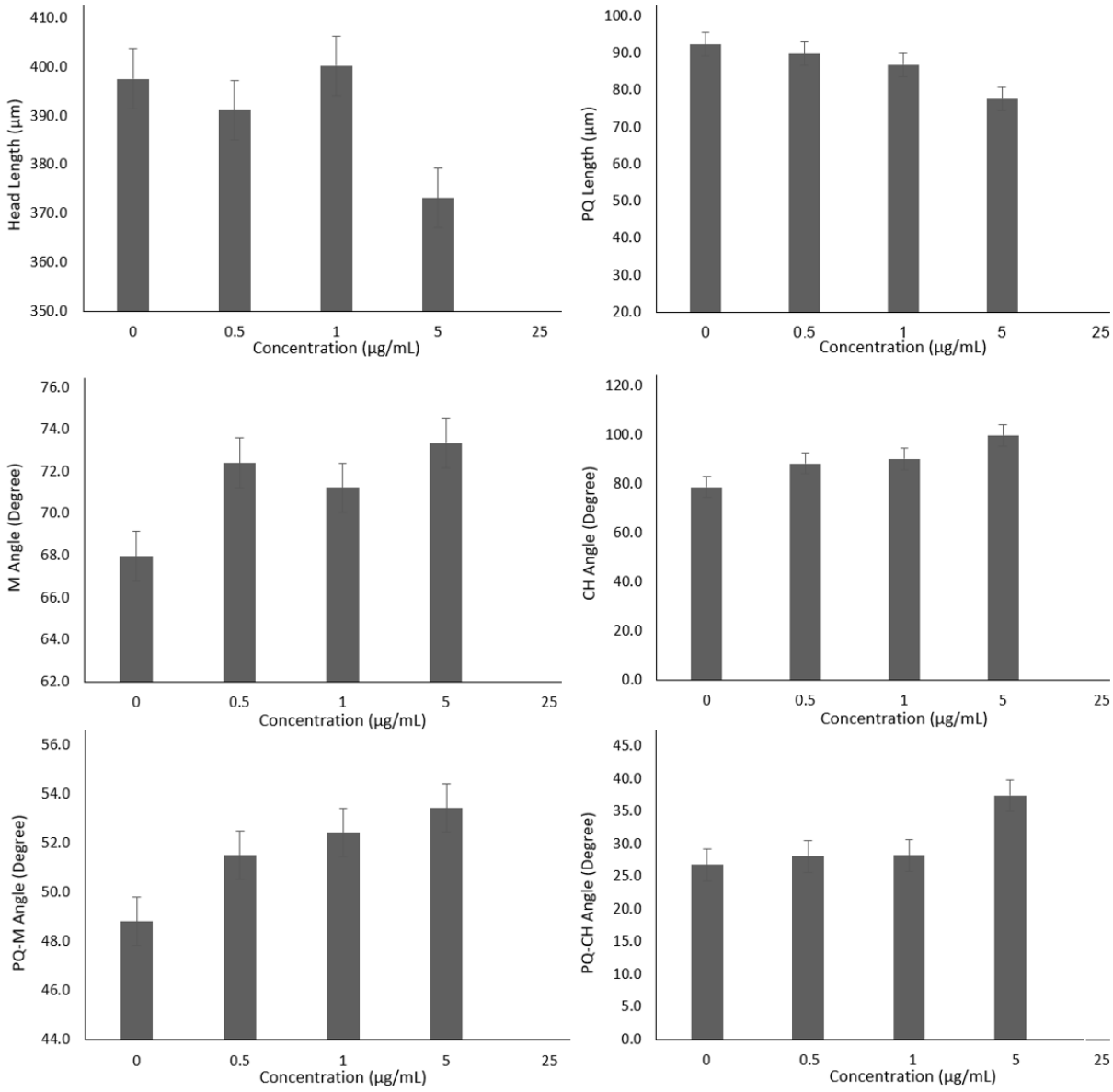

Figure 4. Assessment of the structure of the cranium cartilage by measuring the angles and lengths of each cranium cartilage

Table II. Effects of Mangosteen G. *mangostana* pericarp simplicia on Fish Behaviour

Treatment				
Control	**Mangosteen 0.5 µg/mL**	**Mangosteen 1 µg/mL**	**Mangosteen 5 µg/mL**	**Mangosteen 25 µg/mL**
swimming forward, to the right and left, also up and down	swimming forward, to the right and left, also up and down	swimming forward, to the right and left, also up and down	swimming forward, to the right and left, also up and down	swimming slowly with some fish showing swimming sideways

The embryos showed agility in swimming forward, to the right and left, and also up and down (Zigzag pattern), exploring the entire container area. On the other hand, 96 hpf R. *lateristriata* embryos treated with 25 µg/mL mangosteen pericarp simplicia exhibit different swimming activity and pattern. Fish larvae were swam very slowly, less reactive, had no zigzag pattern and some fish were seen swimming sideways. Moreover, the swimming pattern were very sluggish and coverage area were very limited.

The swimming abnormality shown by 96 hpf R. *lateristriata* embryos, is possibly related with the finding on the morphological defect on fish swim bladder at a treatment concentration of 25 µg/mL of mangosteen pericarp simplicia. One of possibly caused of abnormal swimming behaviour and pattern exhibit by the embryo was the occurrence of swim bladder defect.

The swim bladder is a fish-specific organ, which has a vital role in fish air-breathing, and for the regulation of buoyancy. Therefore, the defect or absence of a swim bladder caused by mangosteen exposure affects the fish's swim and buoyancy ability, resulting in behavioural abnormalities of R. *lateristriata*. Moreover, It is reported that the fish exhibited swimming sideways typically caused by swim bladder abnormality of malformations (Good *et al*., 2014; Pelster, 2021).

CONCLUSION

The study result showed the toxicity effect of mangosteen pericarp simplicia on wader pari fish. The exposure of 5 µg/mL and higher concentration were caused yolk sac, pericardium and eyes edemas, reduced pharyngeal arches and mandible, collapsed swim bladder, abnormal pigmentation, and behavioural change in R. *lateristriata* larvae. Therefore, the use of mangosteen for ingredients in herbal medicine should be very carefully implemented considering its toxicity potential.

AUTHOR CONTRIBUTION

Bambang Retnoaji designing experiments, assisting in experiment, visualizing data, compiling, editing and finishing paper. Pradnya Paramita and Luthfia Uswatun Khasanah designed and conducted experiments, conducted statistical analysis, visualized data, and compiled papers. All authors have read and approved the final manuscript.

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