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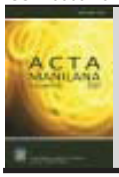

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Utilization of *Danio rerio* (zebrafish) embryos to compare the modulatory effects of *Abelmoschus esculentus* (L.) (okra), *Curcuma longa* (L.) (turmeric) and *Moringa oleifera* (Lam.) (malunggay) aqueous extracts on the development and expression of selected gene markers

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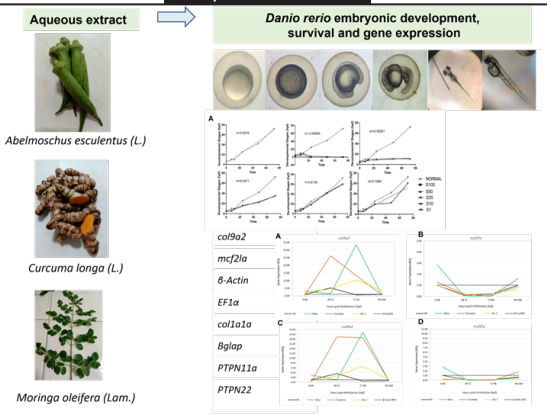
Graphical Abstract



Abstract

Abelmoschus esculentus (okra), *Curcuma longa* (turmeric) and *Moringa oleifera* (malunggay) are common and widely available sources of medicinal phytoconstituents. In this paper, we examined the feasibility of using *Danio rerio* (zebrafish) embryos in investigating the effect of the aqueous extracts from these three plant sources on the development and expression of genes associated with three metabolic conditions: osteoarthritis, diabetes and auto-immune diseases. Zebrafish embryos at different developmental stages: 48, 72, 96 and 168 hours post fertilization (hpf) were exposed to various concentrations of the aqueous extracts of okra, turmeric or malunggay and the effect on morphology, survival and gene expression was examined. Total RNA from the embryos was isolated and the gene expression was determined by SBYR green quantitative-real time PCR.

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Results showed temporal expression patterns of col9a2 and mcf2la, two osteoarthritis-associated genes in the developing zebrafish embryo upon exposure to the okra and turmeric extracts. Col1a1a and bglap that are implicated with bone and glucose metabolism, also showed temporal expression, with an increase in Col1a1a expression upon exposure to the okra seed extract, while bglap in the okra flesh extract. Also, the effect of okra and malunggay on the expression of auto-immune disease-associated genes, PTPN11a and PTPN22 was compared. An increase in gene expression of PTPN22 at the later stage and PTPN11 at an early stage was observed. However, no significant difference was observed from the gene expression patterns of PTPN11a and PTPN22 from all treatment groups. In conclusion, the zebrafish embryo and the selected genes were responsive to the effect of the aqueous extracts. Our study has explored the use of zebrafish embryos as an alternative model system in investigating the effects of natural products on the expression of metabolic disease-associated genes.

Keywords: zebrafish; *Abelmoschus*; Curcumin; *Moringa*; aqueous extract; qRT-PCR

Introduction

Danio rerio (zebrafish) has been used as a model system in development, genetics and toxicity studies due to its transparent embryo, fast rate of development and a high degree of similarity with the human genome [1]. It has been an emerging in vivo model for bioactivity screening of natural products and toxicity studies for potential pharmaceutical applications [2]. The zebrafish model was also used to investigate a number of metabolic diseases including osteoarthritis (OA), diabetes and auto immune diseases [3]. OA is a progressive degenerative joint disease that leads to loss of joint function and is the leading cause of disability in adults [4]. The development in zebrafish can be easily traced and can show manifestations of OA symptoms including bone spurs, chondrocyte proliferation, cartilage calcification and an increase in proteoglycans [5]. There were also a number of studies that reported that zebrafish is an appropriate model to study diabetes [6]. Diabetes is a disease characterized by an increase in blood glucose level. It is prevalent in the Philippines and is predicted to increase by the year 2025 [7]. In autoimmune diseases on the other hand, some studies have explored the use of zebrafish in investigating biological activities [8]. The use of zebrafish as a model in lupus or multiple sclerosis however is still limited. A number of genes are implicated in the development and progression of these metabolic diseases. For example, in OA, the collagen gene col9a2 codes for one of the three alpha helix chains of type IX collagen, a major component of hyaline cartilage. The col9a2 gene in zebrafish is expressed strongly in the otic capsule of the ear at 72 hpf [9]; At 120 hpf, the col9a2 transcripts are expressed throughout cartilaginous structures including the ventral jaw and continuously showed strong expression in the otic capsule. The mcf2la gene on the other hand is associated with the human hip/knee pain function. It regulates nerve growth factor (NGF), and treatment with an antibody against the NGF is involved in reduction of pain and knee function improvement [10]. The Mcf2la gene can also be found in zebrafish and is constantly expressed over the course of the developing embryo. The gene bglap which expresses osteocalcin, a protein produced by osteoblasts and plays a role in bone mineralization, has recently been reported to be linked to diabetes. Although bglap plays a role in bone metabolism, the uncarboxylated form of osteocalcin has shown to regulate glucose metabolism and improve insulin secretion and sensitivity [11].

Alteration of osteocalcin levels is among the mechanisms of diabetes treatment, as reported by Ferron et al. (2012). Another osteoblast-specific gene, col1a1a, was reported to increase in response to diabetic symptoms and is upregulated by antidiabetic medicines [12]. Furthermore, PTPN22 and PTPN11a are genes associated with immunity. The PTPN22 gene is expressed mostly in cells of hematopoietic lineage, and tyrosine phosphatase, which is the encoded protein, regulates tyrosine kinases that contribute in T cell activation [13]. Studies have also shown that minor T allele of PTPN22 is associated with several auto-immune diseases, including Systemic Lupus Erythematosus (SLE). This gene is highly expressed in the immune system; it dephosphorylates pAEA to yield AEA [14]. The PTPN11a gene on the other hand codes for the protein-tyrosine phosphatase, non-receptor type 11 protein of the zebrafish. This gene is expressed constitutively at a high level throughout development [15]. A number of naturally occurring plant sources have been reported to potentially treat or supplement the management of a number of metabolic diseases [16]. The extracts of okra seeds and peels for example are known to decrease glucose levels in the blood by increasing serum insulin levels [17]. Apart from its role in diabetes, okra is also found to contain significant amounts of calcium for healthy and strong bones [18]. Although okra has not been implicated much in the study of osteoarthritis, it is established to regulate blood glucose levels. There are however limited studies on how turmeric and okra can modulate gene expression related to osteoarthritis. In addition, unlike other diseases, there are few literatures that investigated the gene expression linked to glucose metabolism in fish. Also, the exact mechanism on how plant extracts prove to be effective in the prevention and management of diabetes is still limited. The zebrafish model was therefore utilized in an attempt to observe the temporal gene expression pattern of col9a2 and mcf2la, bglap, col1a1a and PTPN11a and PTPN22 upon treatment with the aqueous extracts from these plant sources. It is hypothesized that the activity of medicinal plants in reducing the severity of these metabolic diseases is due to its ability to upregulate or downregulate gene expression. The gene modulatory effect among okra, turmeric and malunggay was therefore determined. This study has defined the feasibility of using *D. rerio* embryos as a model organism to compare the expression patterns of col9a2, mcf2la, bglap, col1a1a, PTPN11a and PTPN22 at various developmental stages upon exposure to the aqueous crude extracts. Since limited studies are reported on how okra, turmeric and malunggay modulates gene expression, our study explored the use of the zebrafish model to provide insights on the mechanism on how natural products are effective in the prevention or management of metabolic diseases.

Materials and methods

Acquisition, Maintenance, and Breeding of Zebrafish. Adult male and female zebrafish, *D. rerio*, were purchased from a local pet shop and the fish were allowed to acclimate for 7 days prior to breeding. The fish were maintained following the protocol from the Zebrafish Information Network (ZFIN). The male and female breeding stocks at 12-15 months of age were placed in two separate 10 gal aquariums with dechlorinated water and circulating system that was continuously filtered and aerated. The tank temperature was maintained between 26-28°C on a 14:10 light-dark cycle and the two fish tanks were cleaned every day.

Feeding was done twice a day, morning and evening with standard commercial fish pellets. Disposal of dead fish includes wrapping them in yellow plastic bag and disposed together with other biohazardous waste. The male and female zebrafish were transferred and combined in the breeding tank with a ratio of 1:2.

The breeding tank was prepared with a net to prevent the fishes from eating the eggs. Fertilized eggs were observed the following day after natural spawning and collected at various time points using a siphon. Black containers were used to collect the embryo for clear visualization and faster collection of eggs. The fertilized eggs were washed with water to remove the debris. Continuous passage of water was performed to maintain the fertilized eggs in clear water and transferred to a petri dish for observation of embryonic development. The number and quality of collected fertilized eggs were noted. The fertilized eggs (15 /30 mL aqueous extract/treatment group) were sorted and transferred to separate dishes belonging to the normal control group (untreated) and experimental groups treated with different concentrations of the plant extracts.

Acquisition and Preparation of Turmeric, Okra and Malunggay aqueous extracts. *A. esculentus* (Okra) pods, *C. longa* (Turmeric) rhizomes and *M. oleifera* (malunggay) leaves were purchased from a local market in Manila. The preparation of the crude extracts followed the protocol of Niamsa and Sittiwet (2009) [19], with the exception of drying the turmeric and okra in the oven. Instead, chopped okra and turmeric were air-dried for 6 days. After the drying process, the okra and turmeric were pulverized using mortar and pestle. For col9a2 and mcf2l, 10 g of the powdered okra and 10 g of powdered turmeric were boiled in 500 mL of distilled water for 15 minutes. For col1a1 and bglap, the powdered okra seeds and flesh were separately boiled with distilled water under 100±1°C for 10 min with 1 g powder to 25 mL solvent ratio, then the boiled mixture was filtered using muslin cloth. The filtrates were diluted by distilled water into concentrations of 50%, 25%, 10% and 1%, and were stored in amber bottles at 4°C. The embryos were treated with the prepared extract the following day. For PTPN11a and PTPN22, the okra seeds were separated from the flesh and mucilage. The malunggay leaflets were separated from the rachis. The separated seeds, flesh and leaves were weighed and 450 g of each sample was collected. A 450 mL of distilled water was added to each 450 g of the sample employing a 1:1 ratio. Each sample was then blended prior to boiling. The samples were boiled at 100°C for 10 min [20]. The resulting product was filtered. A series of dilutions were performed after boiling to obtain different concentrations of the aqueous extract (100%, 75%, 50%, 25%, 10%, 1% and 0.5%) and the treatment of embryos with the extracts was performed on the same day or the following day.

Morphological examination and Survival test of developing embryos. The collected fertilized eggs were treated with different concentrations of the aqueous extracts. Thirty mL of the turmeric aqueous extract (TE), okra aqueous extract (OE), okra seed aqueous extract, okra flesh aqueous extract and malunggay extract each with the concentrations of 100%, 75%, 50%, 25%, 10%, 1% and 0.5% as well as in egg water for the control group were dispensed into separate petri dishes with 15-30 embryos each. This was performed to determine preliminarily the toxic effect, mortality rate and changes in morphological development of the embryos. Embryos (15 embryos/30 mL aqueous extract) were allowed to develop at room temperature on a 14L:10D cycle.

Embryo development was observed under a stereo or compound light microscope (Nikon SMZ-745N; Best Scope BS-2036C; 40x) and at 0 to 8 hpf (blastula), 8 to 12 hpf (gastrula, 75% epiboly), 12 to 24 hpf (segmentation, 5-9 somites), 24 to 48 hpf (pharyngula, prim-5), 48 to 72 hpf (hatching, long-pec), and 72 hpf to larval protruding mouth (Zebrafish Developmental, n.d.). At each time point, both the number of viable and non-viable eggs were counted and recorded. Morphological changes at different embryonic stages were observed. The abnormalities and delays in development were also noted. Four trials were performed. Pearson's correlation coefficient values were computed to determine the relationship between embryo survivals and extract concentration. The optimum concentration of the extract with the higher percentage survival with least morphological deformations was selected. A 1% concentration was selected for subsequent total RNA isolation.

Total RNA extraction. Total RNA was isolated from zebrafish embryos at different developmental stages using ReliaPrep™ RNA Tissue Miniprep System (Promega), following the manufacturer's protocol. Embryos were collected at various time intervals for col9a1 and mcf2l: 0-48, 48-72, 72-96-, and 96-120-hours post-fertilization (hpf) and were stored in RNAlater at -20°C prior to RNA extraction (10 embryos per RNAlater tube, per time point from the same spawning group). For col1a1 and bgla screening with okra flesh and seed, embryos were collected at 0 to 12 hpf, 24 to 48 hpf, and 72 hpf to larval stages. For ptpn11a and ptpn22, embryos that developed from different time points (0-12 hpf, 12-24 hpf, 24-48 hpf and 48-72 hpf) and at 0.5% treatment of the extract were collected for RNA isolation. Whole embryos were homogenized and LBA+TG Buffer (1-Thioglycerol to LBA Buffer) was added to the tissues and total RNA was extracted as described in the manufacturer's instructions. Total RNA was eluted in 15µl of nuclease-free water. A 1µL aliquot from each sample was taken to determine the RNA purity and quantity by spectrophotometry (A260, A280 and A230). The isolated RNA from each group were diluted to a final concentration of 1 µg/µl.

cDNA Synthesis. cDNA was obtained by SensiFAST™ cDNA Synthesis Kit (Bioline, n.d.), following the manufacturer's protocol. A 20 µL mastermix was prepared on ice containing 1x TransAmp Buffer, 1 µL Reverse Transcriptase, and 1µg of total RNA. Samples were loaded to an Arktik Thermal Cycler (Thermo Scientific) set at 25°C for 10 min (primer annealing), 42°C for 15 min (reverse transcription), 48°C for 15 min (for highly-structured RNA), 85°C for 5 min (inactivation), and was chilled on ice. Obtained cDNA was stored at 4°C prior to DNA amplification.

Quantitative real-time PCR. Gene expression from the cDNA product was quantified using SensiFAST™ SYBR® No-ROX One-Step Kit (Biolone). The mastermix was prepared containing 4 µL cDNA template, 2x SensiFAST™ SYBR® No-ROX Mix (10 µL), 10 µM forward primer (0.8 µL), 10 µM reverse primer (0.8 µL), and H_2O (0.2 µL). The forward and reverse primer sequences of col9a2, mcf2la, col1a1a, bglap, ptpn11a, ptpn22 and housekeeping genes, β-Actin and EF1α are shown in Supplementary Table 1. Samples were loaded in a CFX96 Thermal Cycler (Biorad) with the step cycle program set at one cycle at 95°C for 2 minutes (polymerase activation), and for 40 cycles at 95°C for 5 seconds (denaturation), 60°C for 10 seconds (annealing), 72°C for 15 seconds (extension).

Data Visualization and Analysis. Gene expressions were computed from Ct values normalized with the reference genes, β-actin and EF1A (ΔCT) and then normalized to the untreated (normal) group at 0 hpf (ΔΔCT). Relative quantity (RQ) was determined using 2-ΔΔCt. Two-way ANOVA, with p-value of <0.05, was used to determine if there are significant differences between groups. For the toxicity test, the viable and non-viable embryos were counted per time range per extract concentration and percent survival were plotted against the time frames in a graph. The slope of the regression line was used to determine if the extracts would largely affect the survival of embryos through time. The observed developmental stages per time range per extract concentration were also tallied, and dominant developmental stages per time frame per extract concentration were also plotted in a graph. The slope of the regression line was used to analyze whether the extracts caused a delay on the development of the embryos as compared to that of the control group or not. Graphs were plotted, and slope was calculated using GraphPad Prism 8. Ct values from the RT-qPCR were analyzed by calculating mean Ct, ΔCt, ΔΔCt, and the Rq values of the samples. Rq values were plotted against the time frames in a graph and analyzed by two-way ANOVA using GraphPad Prism 8.

Results and Discussion

The zebrafish (*D. rerio*) embryos have shown to be a significant model of vertebrate development with its short reproductive cycle, ease of maintenance, optical transparency, allowing for the visualization of internal organs during development and straightforward administration of candidate compounds. It has been utilized more often as an alternative to cell culture systems in the study of toxic agents that inhibit, or otherwise interfere with, developmental processes (i.e., developmental toxins), including compounds that have probable relevance to human health [21]. However, there are only a few studies where zebrafish was utilized as a model organism to examine gene expression patterns under the exposure to various factors. In this study, we investigated the feasibility of using zebrafish embryo to test whether genes associated with osteoarthritis (col9a2 and Mcf2la), osteogenesis and diabetes (bglap and col1a1a) and immunity (PTPN22 and PTPN11a) are responsive to the effect of the extracts and to provide insights on the mechanism on how the three common plant sources (okra, turmeric and malunggay) regulate the activity of metabolic diseases.

Survival of Developing Embryos. Prior to examining the temporal gene expression pattern of developing zebrafish embryos, the percentage survival of embryos upon exposure to different concentrations of the seed and flesh extracts of okra, turmeric and malunggay was determined. Also, the maintenance and spawning condition of adult zebrafish embryos were optimized to generate enough fertilized eggs for treatment with the okra flesh, turmeric, okra seed only, okra flesh and malunggay leaves aqueous crude extracts. A ratio of 1:2 of male and female was found to be optimum to generate sufficient number of embryos and were maintained between 26-28°C on a 14:10 light-dark cycle prior to spawning. Figure 1a and 1b show the normal development of *Danio rerio* embryo. The development is rapid, and organogenesis is evident as early as 42 hours post fertilization. Figure 2 shows the morphological changes of developing embryos upon exposure to different concentrations of the okra extract (OE) (Fig. 2a) and turmeric extract (OE) (Fig. 2b) and Supplementary material 2 (S2a-c) show the morphological changes of the embryo to different concentrations of the okra seed, okra

flesh and malunggay. Results showed that 1% okra extract (OE) and 1% turmeric extract (TE) were the least lethal concentrations to use for subsequent gene expression analysis in *D. rerio* embryos and larvae. At a higher concentration (10%), although the embryos survived, significant morphological changes were observed including developmental delays, pericardial edema, curved spine, and enlarged hatching glands (Fig. 2a and 2b). As expected at 100% OE and TE concentration (Fig. 2a and Fig.2b) caused shrinkage, opacity, and death of the embryo as compared to the other concentrations. Embryos exposed to 75% concentration of OE and TE became non-viable at 24-48 hpf. Growth retardation and delay in development were also observed in extracts with 10%, 25%, and 50% concentrations. The pericardial edema can be caused by a number of factors, including defects in differentiation of cardiac progenitors, errors in the morphogenesis of cardiac tissues, and impaired heart function [22]. Although the mechanistic explanation for spinal curvature and enlarged hatching glands is still limited, exposures to higher concentration of both okra and turmeric extracts nevertheless have caused morphological changes and are even lethal, since embryos were not able to survive to the 96-168 hpf time point.

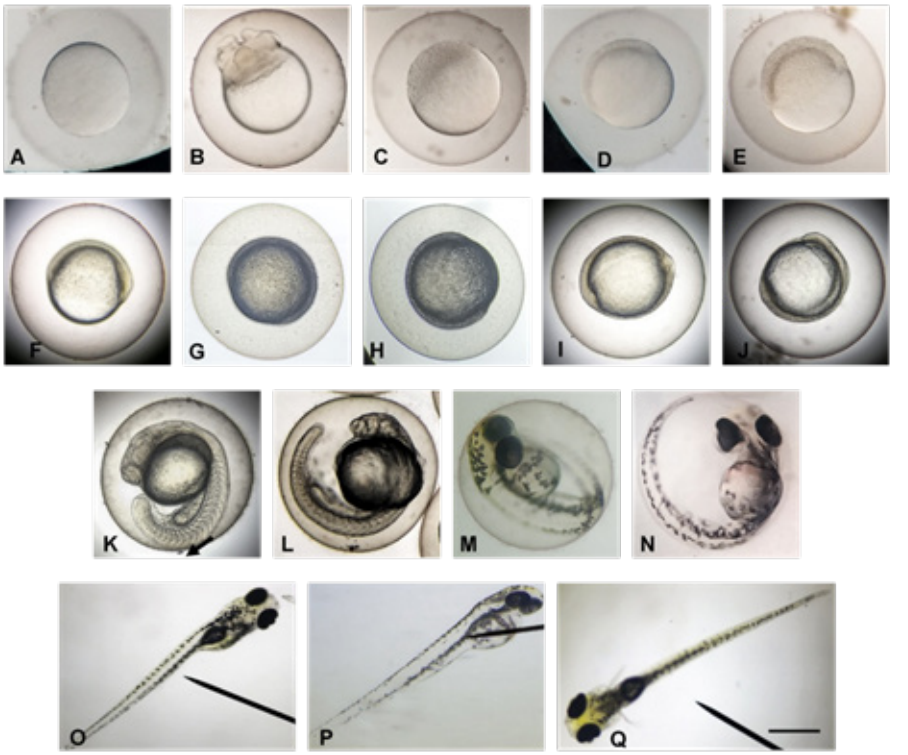

Fig. 1a. Normal developmental stages of *D. rerio*. A: Zygote B: Four-cell stage (1 h). C: Blastula period: 256-cell stage (2.5 h). D-H: Gastrula period. D: 50%-epiboly stage (5.25 h). E: Germ ring stage (5.7 h). F: Shield stage (6 h). G, H: 70%-epiboly stage (7.7 h). I-K: Segmentation period. I: Ventral view of two-cell somite stage (10.7 h). J: Four-somite stage (11.3 h). K: Twenty-somite stage (19 h). L-O: Pharyngula period. L: Prim-5 stage (24 h). M, N: Prim-20 period. Melanophores can be seen along the axis dorsal to the yolk and on the dorsal part of the yolk. O: High-pec stage (42 h). Melanophores can be seen on the whole length of the embryo. P, Q: Hatching period of embryogenesis. P: Pec-fin stage (60 h). Q: Protruding-mouth stage (72 h). Progressive filling of melanophores into the lateral stripe and increase in yellow pigmentation can be seen. Magnification = 40x. Scale bar = 250 μm

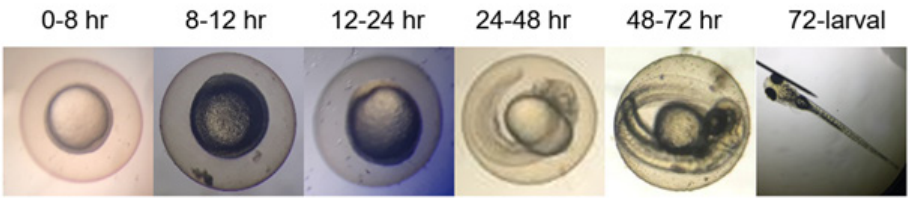

Figure 1b. Representative stages for normal development per time frame. Developmental stages are Shield (0-8 hr), Bud (8-12 hr), Bud (12-24 hr), Prim-6 (24-48), High pec (48-72), Protruding mouth (72-larval). Magnification = 40x.

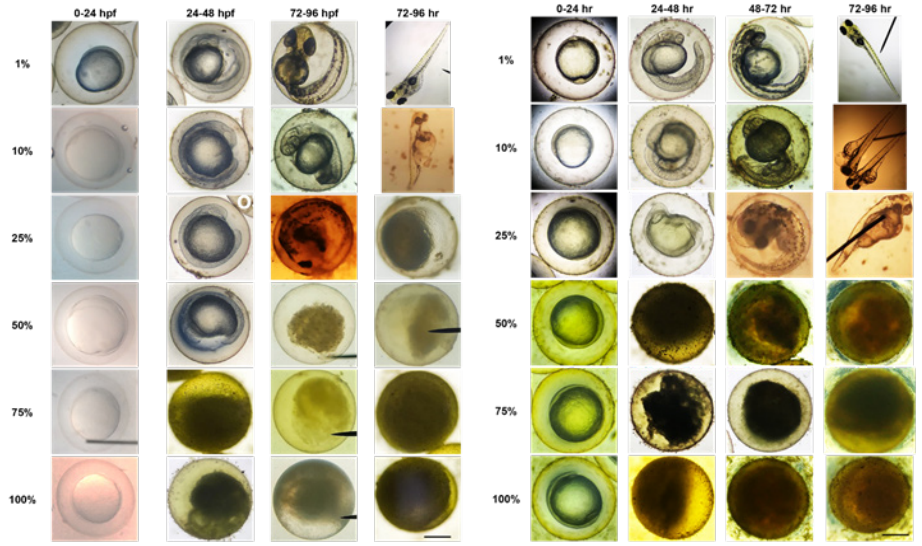

Fig. 2. Morphological characteristics of zebrafish embryo exposed to different concentrations of A. *A. esculentus* (okra) and B. *C. longa* (turmeric) at different time points. Magnification = 40x. Scale bar = 250 µm.

Similar delay in embryonic development from embryos treated with the okra seed, okra flesh and malunggay was observed (Supplementary figure 1; S1). Most embryos survived at 1% concentration of the seed alone and flesh alone, although more embryos survived when treated with the flesh extract than the seed extract (Fig. 3 a and b). The plant part of okra therefore, either the flesh or the seed aqueous extract have different effects. Also, more embryos have developmental delays when treated with the seed extract than the flesh extract (Figure 4). Although most embryos survived at 10% to 50% concentrations, development was delayed as early as 24-48 hours post fertilization (Fig. S1). Delay of development also increased as the concentrations of both extracts increased. There was an evident chorion collapse and no 48-hour to larval stage embryos in both 100% concentration extracts. At 50% and 100% no embryos in any extract reaching the protruding mouth stage and caused mortality of the embryos as early as 24-48 hours and 48-72 hours respectively. A number of studies revealed that the aqueous extracts of okra has the presence of flavonoids, terpenoids, cardiac glycosides, saponins and tannins which interrupt the physiological processes of the embryos, ultimately causing a higher mortality rate and delay in the development of the embryos [23, 24, 25].

In addition, the study of Alafiatayo, et al. showed that exposure to high concentration of extracts of zebrafish embryos caused a decrease in survival rates and delayed development and hatching [26]. Aside from the aforementioned compounds, specific components from the aqueous extracts such as rhamnogalacturonan I and type II arabinogalactan, galacturonic acid and monosaccharides at high concentration might affect embryonic development. Also, the properties of the extract, for example, extract viscosity, excessive amount of nutrients and fat in the more concentrated extracts, or contamination of the more concentrated extracts with higher amounts of toxic metals while growing the crop such as arsenic, which is insoluble by boiling [27,28] may have contributed also to toxicity at higher concentrations. Although there are limited reports on the saturation point for tocixity of okra, the turmeric and malunggay extracts have reported concentration ranges that render toxicity to embryos. For example, a 0.3-6 µg/ml concentration range and an LC50 of 1.5 µg/mL at 36 h of the malunggay hot water leaf extract were reported. For the aqueous extract curcuma on the other hand, a concentration range of 0–10,000 µg/mL and LC50 of 748.6 µg/mL (48 hpf) and 703.7 µg/mL (96 hpf) were reported [29].

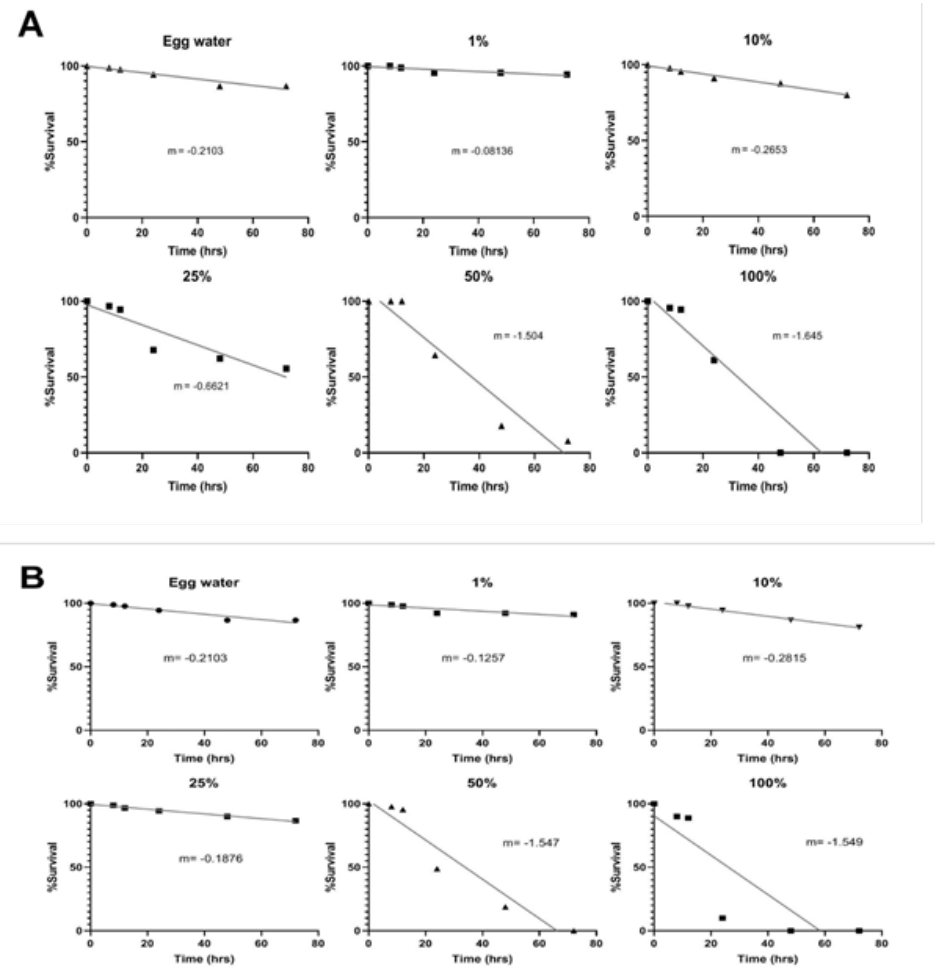

Figure 3. Percentage survival of zebrafish embryos at different concentrations of okra (a) seed and (b) flesh extracts. n=15

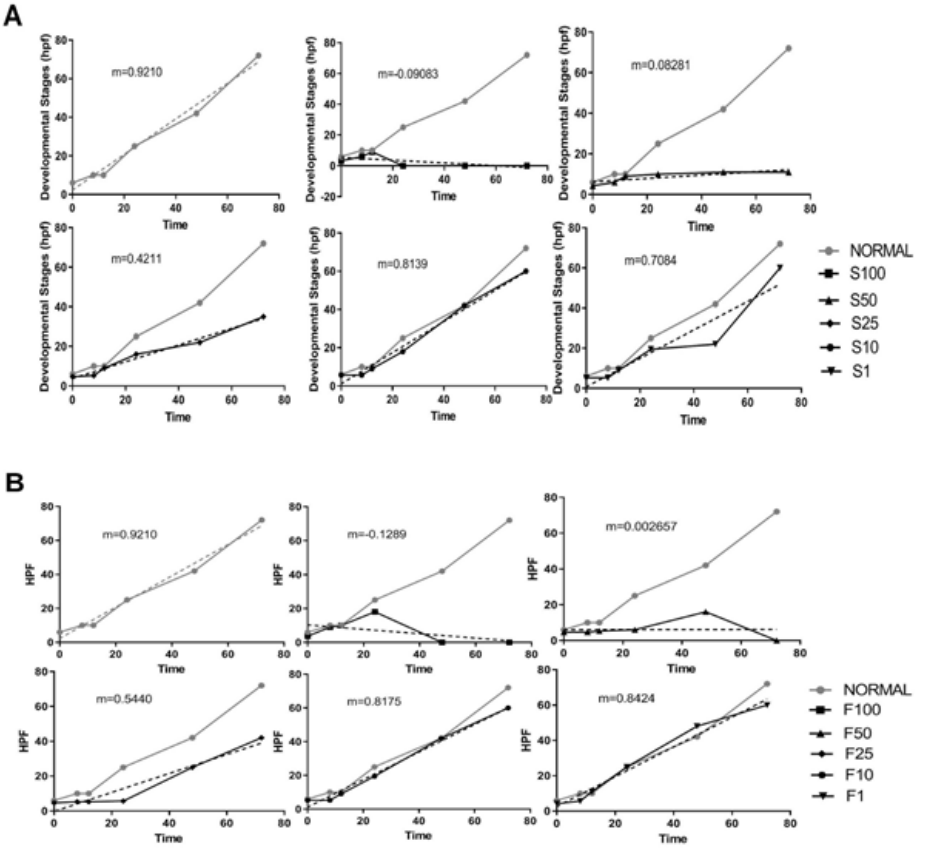

Figure 4. Developmental Stages per Time Frame per Concentration of *A. esculentus* (okra) (a) seed and (b) flesh extracts; S100 to S1-100-1% seed extract; F100 to F1- 100%-1% flesh extract. n=15

For the malunggay leaf extract (MLE), growth retardation or slow development started to be observed in most extract concentrations starting at 10% (Supplementary Figure S2). Coagulated embryos were already observed in 0-12 hpf embryos starting from the 0.5% until the highest concentration (75%). In 12-24 hpf, normal development was observed in 0.5%, 1%, and 10% of MLE. However, growth retardation or slow development became evident in 10% and 25% concentrations starting from 24-48 hpf. The remaining concentrations with normal development during this timepoint were the 0.5% and 1% MLE. However, in 48-72 hpf, slow development and delayed hatching process was observed in most embryos in 25% and higher of the extract. At a 25% concentration and higher, it caused deformed yolk and significant retardation of growth and hatching from the chorion than those of the control. The chorions of the zebrafish embryo were not manually removed, to guarantee minimum manipulation of embryos [30]. Furthermore, the natural ability to hatch from the chorion after 48 hpf was one of the developmental phases used to evaluate developmental delays. Based on the endpoints used by Busquet et al., growth delay was evaluated by considering these different parameters such as the global size of the fish egg, the position of the eye, the degree of pigmentation, if the tail is not detached and the frequency of spontaneous movements exhibited by the zebrafish embryo [31].

Whereas a separate list of ‘‘development’’ endpoints were proposed (e.g., formation of somites, development of eyes, heartbeat). These ‘‘development’’ endpoints were used by Bachmann [] to calculate an EC50 for developmental delay at 24 and 48 hpf in some chemicals. However, we only based it on obvious morphological characteristics to confirm if the embryos are developing in the right timepoints. Comparing the effect from the okra seed, okra flesh and malunggay aqueous extracts, the okra flesh and mucilage extract showed more surviving and developed embryos even at the later stage (at 50% concentration, the embryo still reached the 48-72 hpf developmental stage) as compared to the okra seed and malunggay extract. These results provide insights that the okra flesh and mucilage render less toxicity to the developing embryo even at higher concentration as compared to the seed and malunggay leaf extracts. For the gene expression analysis, the expression of col9a2, mcf2la, bglap, col1a1a, PTPN22 and PTPN11a occur in a temporal manner during the normal development of the zebrafish. Our results showed that an exposure of the embryos to even low concentration and non-toxic dose of the extracts can affect the expression of candidate metabolic genes.

Expression of col9a2 and mcf2la gene expression (RQ) of col9a2 and mcf2la were compared from different experimental groups: untreated (NT) group, 1% okra extract (OE), 1% turmeric extract (TE), and 10 mg/mL Vitamin C (Vit. C). Fig. 5A and 5B show the temporal expression of col9a2 in the untreated control groups (NT) normalized to the housekeeping genes EF1α and β-Actin. Col9a2 gene expression starts to be expressed as early as 24 hours with its peak of gene expression observed at 48-72 hpf. On the succeeding timepoints however, at 72-96 hours up to the 168 hours, the gene expression of col9a2 decreased. In the turmeric treated group however, Col9a2 expression increased and is higher as compared to the untreated groups. Also, it appears that the presence of turmeric sustained Col9a2 gene expression until 96-168 hours. In the okra turmeric group, a delay in col9a2 expression was observed and started to be expressed at 48 hours and peaked at 72-96 hours and eventually declined at 168 hours.

The expression of col9a2 is highest in the okra treated group and col9a2 expression was sustained at the later stages of development as compared to the turmeric and Vitamin C groups. In the Vit. C treated group, the expression of col9a2 was found to be highest during the 72-96 hpf. In our study, the peak expression of col9a2 was observed at 72-96 hpf with low expression at 48-72 hpf while in a study of Mitchell et al, the gene col9a2 in zebrafish starts to express at its peak at 48-72 hpf and 72- 96 hpf in the areas of the otic capsule of the ear [9]. In this study however, we can observe the temporal pattern of gene expression and how the extracts can affect its expression.

Furthermore, the housekeeping genes EF1α and β-Actin expressions were consistent and remained stable in the control untreated groups with RQ values of 1.00 throughout the development of the embryo, however, the presence of the okra extract, turmeric and Vit. C has affected the gene expression which initially increased at the early time point (0-48 hours) but reduced expression at the succeeding time points. Between the two housekeeping genes, the EF1α showed a more stable expression and is least affected by various treatments (OE, TE and Vit. C) as compared to β-Actin. It can be surmised that these extracts can possibly have an effect on gene expression.

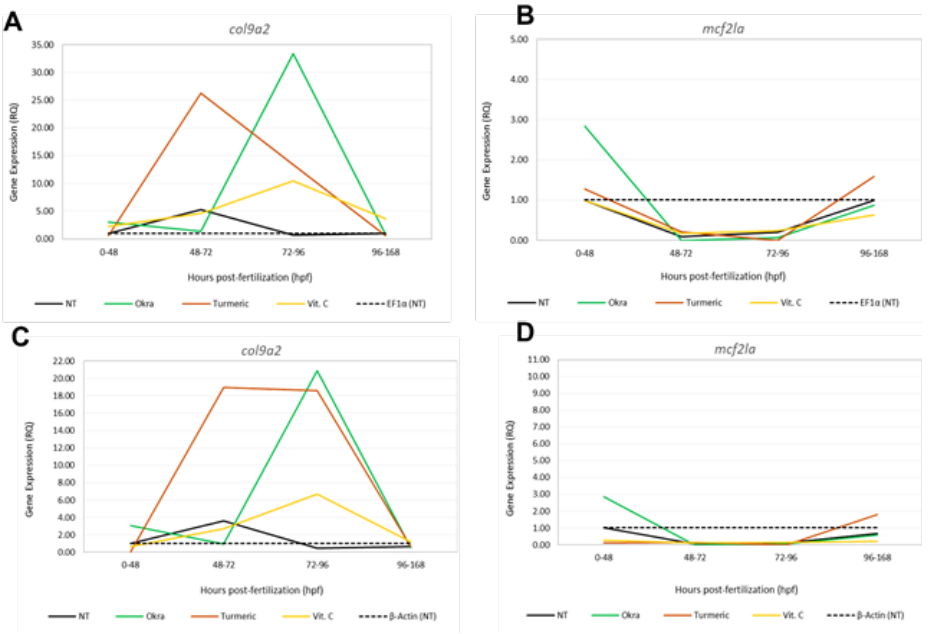

Fig. 5. Gene expression (RQ) of col9a2 and mcf2la on *D. rerio* embryos treated with 1% *A. esculentus* and *C. longa* crude aqueous extracts. A: Expression of col9a2 normalized with housekeeping gene EF1α. B: Expression of mcf2la normalized with the housekeeping gene EF1α. C: Expression of col9a2 normalized with the housekeeping gene β-Actin. D: Expression of mcf2la normalized with the housekeeping gene β-Actin.

Theoretically, a stable mRNA expression level should be maintained for an ideal reference gene, without changing between different developmental stages or experimental conditions [32]. A consistent normalization reference gene should be used wherein it has stable expressions with minimal fluctuations in expression levels to obtain accurate results from qRT-PCR assays associated to zebrafish development [32]. Although for col9a2 expression for example, using both reference genes, no significant difference in gene expression of col9a2 between groups was observed: col9a2 (EF1α) = 0.21 col9a2 (β-Actin) = 0.10. It may therefore be recommended to identify other housekeeping genes for gene expression studies in zebrafish treated with different plant extracts.

The expression of mcf2la at the early stages of the developing embryo is low as compared to col9a2 with the peak expression between 0-48 hours for all groups and decreased expression at the later developmental stages (Fig. 5C, D). The okra extract however increased mcf2la expressed as compared to the turmeric and vit. C treated group. Gene expression of mcf2la in the control group, OE group, TE group, and Vit. C group all yielded low RQ values (RQ ≤ 2) with the highest RQ value being 2.85 (OE group, 0-48 hpf). Meanwhile for the OE group, no gene expression was observed during the 48-72 time point and a lower gene expression was observed at the later time points (72-96 & 96-168).

At the later time point however (72-96 hrs), the expression of mcf2la increased again and is observed to be higher in the turmeric treated group as compared to the okra, vitamin C and untreated groups. Two-way ANOVA shows the p-values for both mcf2la (EF1α) = 0.009 mcf2la (β-Actin) = 0.22 and are above the set critical value of 0.05. No significant difference in gene expression of mcf2la between groups was observed. Similar with the pattern of expression in col9a2 detection, the presence of the okra extract, turmeric and Vit. C has affected the gene expression of the house keeping genes which initially increased at the early time point (0-48 hours) but reduced expression at the succeeding time points.

As of this writing, there is limited information on the gene expression of mcf2la on the developing embryo as well as the effect of both *A. esculentus* and *C. longa* extracts on gene expression. Indeed, the extracts can modulate mcf2la expression as compared to the control group. Okra increased gene expression at 3 different time points (0-48, 48-72, 72-96 hpf) as compared to the turmeric and the NT group. As compared to previous studies, the mcf2la expression in zebrafish is strongest at 72-96 hpf time points which is delayed in expression in our study. Studies should be made to further validate if the non-expression of genes in the OE 48-72 hpf and TE 72-96 hpf were caused by qPCR confounding factors such as amount of isolated RNA, quality of the isolated RNA, and optimized annealing condition during qPCR. It is also possible that the gene was simply not expressed or that it was delayed in expression, since there was an increase in gene expression for the last time point (96-168 hpf). Further studies are needed to determine why this phenomenon was observed since the gene was studied to be strongly expressed during 72-96 hpf in the study of Mitchell et al. [9].

Expression of col1a1a and bglap. The expression of col1a1a and bglap were observed from zebrafish embryos at various time points treated with the okra seed and flesh aqueous extracts. In Figure 6, it was observed that there was an increase of col1a1a and bglap as the embryo develops (0 hpf to 72 hpf). For col1a1a with ef1a as the housekeeping gene, it can be observed that there is a slight increase in the expression of the gene treated with the okra seed and flesh as compared to the untreated group. Meanwhile, the bglap gene was significantly expressed in both the okra flesh and seed treated groups as compared to the untreated one. These results showed that the genes are responsive to the modulatory effect of the okra aqueous extracts.

In addition, Col1a1a was found to be highly expressed in embryos exposed to *A. esculentus* seed extract, while bglap is highly expressed in embryos exposed to flesh extract. Also, as the development of the embryo progressed, bglap and col1a1a expression was found to be increasing. Therefore, lower concentrations of *A. esculentus* seed and flesh crude aqueous extracts can increase the levels of expression of bone-forming genes. Bglap and col1a1a are genes expressed by bone forming cells and are essential for bone homeostasis. These genes are also reported to regulate glucose metabolism and putatively linked to diabetes. There are limited studies, however, linking bglap and col1a1 to diabetes and how it is responsive to anti-diabetic drugs or natural products. The use of the zebrafish embryo model may serve as a useful tool to understand the role of these bone markers to diabetes.

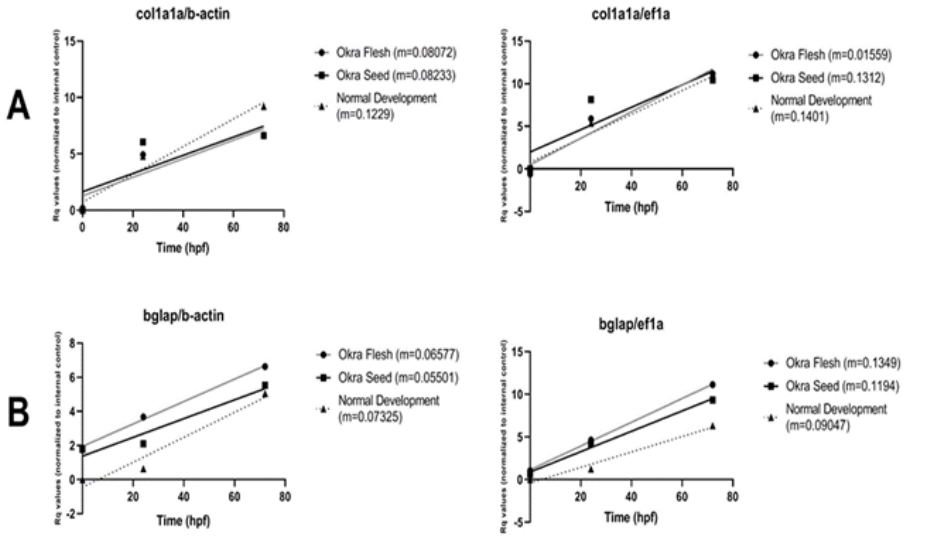

Figure 6. Gene expression (RQ) of (a) col1a1a and (b) bglap per time point (hours post fertilization-hpf) normalized with β-actin and ef1α housekeeping genes on *D. rerio* embryos treated with 1% *A. esculentus* A. seed and B. flesh aqueous extracts.

Expression of PTPN11a and PTPN22. Gene expression of PTPN11a and PTPN22 was also determined from embryos treated with the okra seed, okra flesh and malunggay aqueous extracts. Figure 7 A and C show the relative expression of the target genes PTPN11A using β-actin and EF1α as housekeeping genes. The gene PTPN11A was observed to be expressed at the early stage of development, during 0-12 hpf and 12-24 hpf and declined at later time points. The presence of the extract appears to decrease the level of PTPN11A expression. The okra flesh aqueous extract showed a more stable downregulation of gene expression of PTPN11A as compared to malunggay. The PTPN11a gene codes for the protein-tyrosine phosphatase, non-receptor type 11 protein of the zebrafish. Although it has been reported that the gene is expressed constitutively at a high level throughout development [15], expression however increased upon treatment with the extract, particularly malunggay than the okra flesh. We further recommend to repeat this finding and identify the cause of the downregulation of this gene in our system.

For the gene PTPN22, the gene begins to express at a later time point starting from 24-48 hours and continue to increase up to the 48-72 hours (Fig. 7). In the presence of the okra flesh, the PTPN22 expression increased as compared to the normal and other treatment groups. These results showed that there is a slight modulatory effect of the okra aqueous extracts to these inflammatory genes. The PTPN22 gene is expressed mostly in cells of hematopoietic lineage, and tyrosine phosphatase, which is the encoded protein, regulates tyrosine kinases that contribute in T cell activation. Although a slight increase of these gene was observed in the malunggay and okra seed extract, it is further recommended to identify the probable mechanism of the increase of this gene upon treatment with the okra flesh extract.

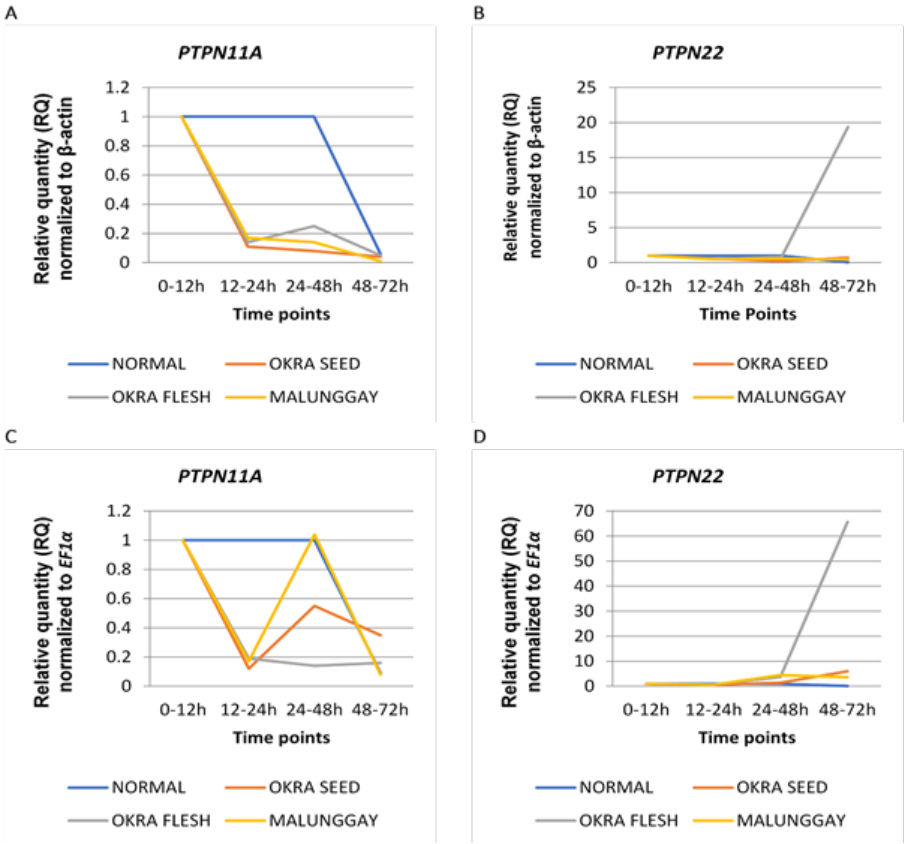

Figure 7. Gene expression (RQ) of PTPN11A and PTPN22 per time point based on β-actin housekeeping genes on *D. rerio* embryos treated with 1% *A. esculentus* (okra) seed and flesh and *M. oleifera* (malunggay) aqueous extracts.

Conclusion

The zebrafish embryos can be used as a model system to investigate the expression patterns of metabolic genes and are responsive to the effect of okra, turmeric and malunggay aqueous extracts. It was found that col9a2, mcf2la, bglap, col1a1a, PTPN22 and PTPN11a gene expression responded to the treatment of the aqueous extracts. These genes were expressed in a specific developmental stage and expressions are modulated upon exposure to the optimum, non-lethal dose of the aqueous extracts. Among the aqueous extracts, that of the okra flesh treated showed higher survival of embryos and modulated gene expression during development. The utilization of zebrafish embryos may serve as a cheaper and practical alternative as an in-vivo model in investigating the mechanism on how natural products render therapeutic or preventive potentials to several diseases.

Author Contributions

All authors contributed equally to the development of the concept, methods and conduct of the experiments.

Institutional Review Board Statement

Not applicable.

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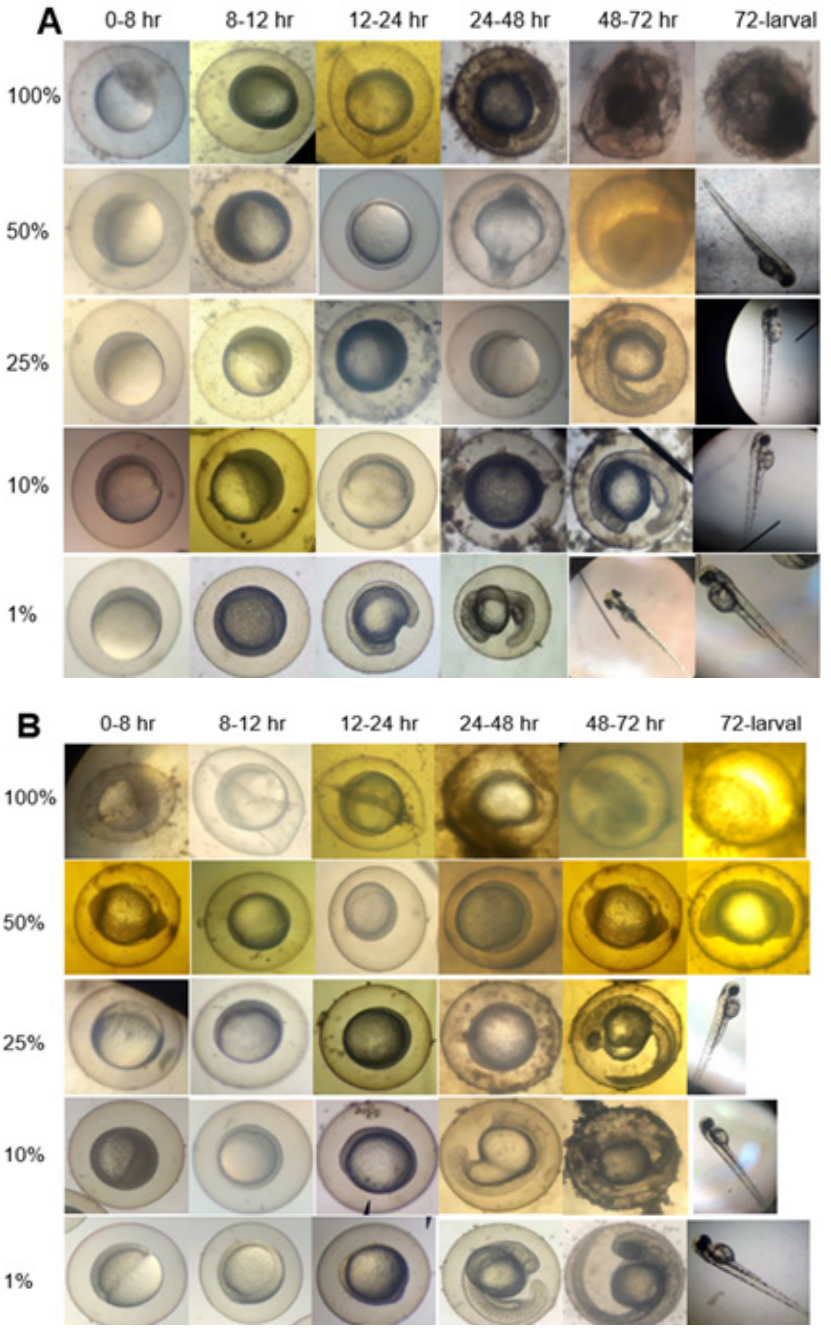
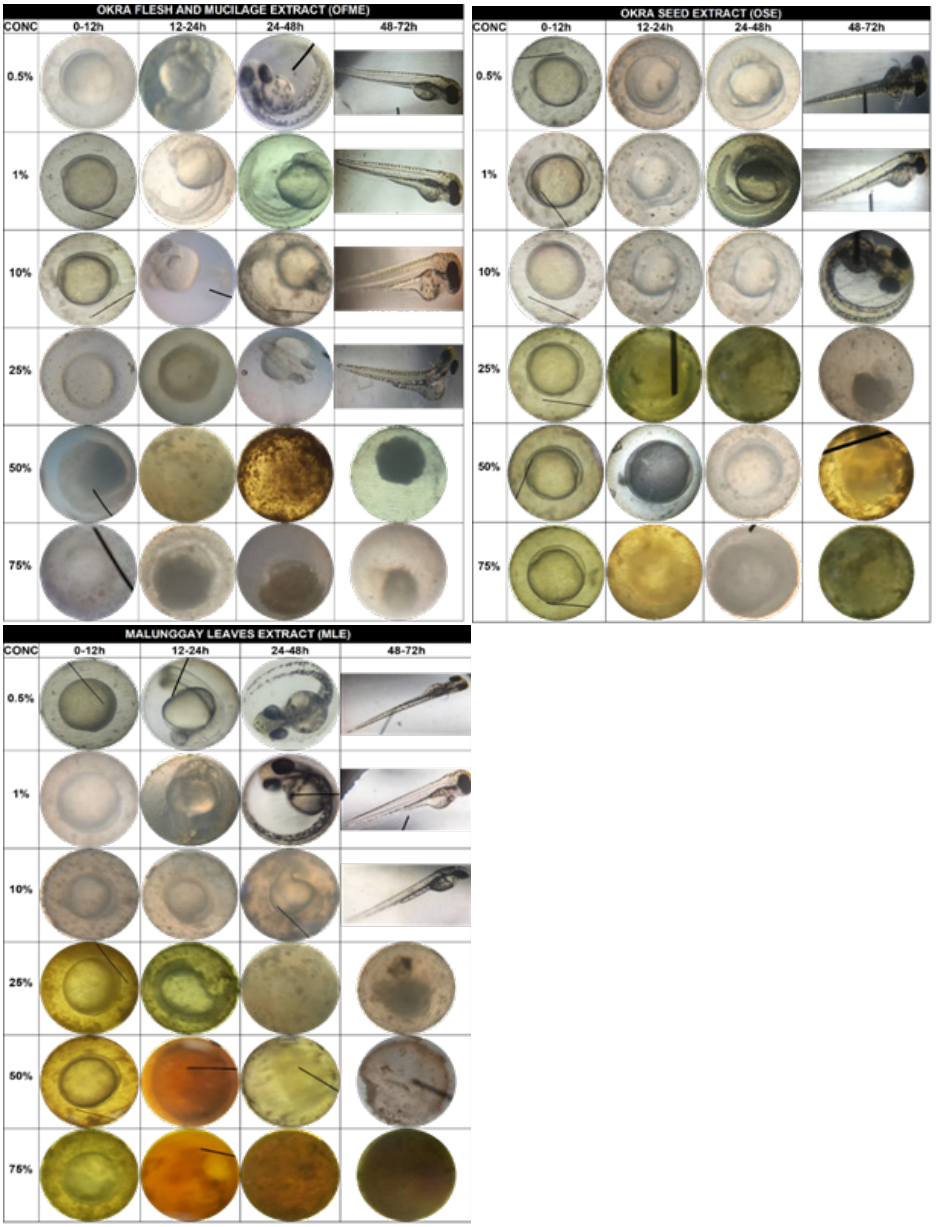
Supplementary Material



Supplementary Figure 1. Morphological changes of *D. rerio* embryos exposed to different concentrations of A. okra flesh extract B. okra seed extract.



Supplementary Figure 2. Morphological changes of *D. rerio* embryos at different time points as exposed to different concentrations of A. *A. esculentus* okra flesh extract B. okra seed extract and C. *M. oleifera* (malunggay) extract. Magnification = 40x.

Table 1. Forward and reverse primer sequence of col9a2, mcf2la, and housekeeping genes, β-Actin and EF1α.

Gene	Forward	Reverse	Reference
col9a2	5'-GCAGGACAAAAC-GGAAGACC-3'	5'-CACCCTTAAC-CCCCACTTCA-3'	[33]
mcf2la	5'-GAGAAAGC-CCCGTCATACAG-3'	5'AATTAACCCTCACTAAAG-GGAGTTTCTTCCCTCCCT-CATCCT-3'GAGTTTCTTC-CCTCCCTCATCCT-3'	[9]
β-Actin	5'-CCAGCTGTCTTC-CCATCCA-3'	5'TCACCAACGTAGCT-GTCTTTCTG-3'	[33]
EF1α	5'-CTGGAGGC-CAGCTCAAACAT-3'	5'ATCAAGAAGAGTAGTAC-CGCTAGCATTAC-3'	[33]
col1a1a	5'-GCTTTTGGCAA-GAGGACAAG-'3	5'-TGTCTTCGCAGAT-CACTTCG-'3	
Bglap	5'CTGCCTGATGACT-GTGTGTGTGAG-CG-3'	5'-GGCGGCATGATTC-CAGACG-3'	
PTPN11a	5'- ATGTGCCCAAG ACTATCCAGATG -3'	5'- CCCACGTTCTCA TAGACTCGAGA -3'	[15]
PTPN22	5'- GCACCAGATG GGATACAGCA -3'	5' GGAGCCGTTT TTGGCTTCTG -3'	[14]