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Prolonged Effect of Tea and Coffee Consumption on Danio Rerio Behaviour and Bone Density

Mahathir MohdUzid[1], Muhammad DanialCheRamli[2] ,NurSyahirah Zaharuddin[3], Anita Zara Weinheimer[4], JunedahSanusi[5]

[1,2,3,4]Department of Diagnostic and Allied Health Science, Faculty of Health and Life Sciences, Management and Science University, Seksyen 13, 40100, Shah Alam, Selangor, Malaysia.

[1,2,4] Center of Excellent, Research Management Centre, Management and Science University, Seksyen 13, 40100, Shah Alam, Selangor, Malaysia.

[5]Department of Anatomy, Faculty of Medicine, University Malaya, Kuala Lumpur, Malaysia

ABSTRACT

World consumption of coffee among individuals has steadily increased by 1.3% per year, next to tea with an annual growth rate of 2%. Both appear to have some effect on the women's bone density, which could lead to osteoporosis. This research evaluates the tea and coffee cytotoxicity toward bone density using zebrafish. Adult zebrafish were treated with 500mg/ml, 200mg/ml, 100mg/ml, 50mg/ml and 25mg/ml concentrations to determine the tea and coffee efficacy by visualizing bone anatomy and physiological changes. Zebrafish embryos were treated with 75mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml concentrations for cytotoxicity effects including the abnormal heartbeat, high mortality rate, pericardial oedema, somite and tail deformation. Histological findings do not interpret any significant changes in bone after prolonged consumption of tea and coffee. Behaviour pattern, yet reveals the zebrafish's anxious response. Overall, the prolonged effect of tea and coffee have shown ambiguous results that may contribute to early osteoporosis development.

Keywords
Coffeaarabica; Coffearobusta; Camellia sinensis; Daniorerio; Osteoporosis

Introduction

Tea (*Camellia sinensis*) and coffee (*Coffeaarabica* and *Coffearobusta*) are the most commonly consumed refreshment in daily life, although its beneficial or adverse effects on human health have not been fully understood. The consumption of either product has its effect on bone mineral density and investigation of the effect has been studied since the 1900s (Zhang et al., 2017). Black tea consists of almost 80% of all tea consumed being one of the world's majority choices beverages (Nie, Dong, Bai, & Xia, 2014). However, there have been suggestions that organic compounds in tea and coffee such as caffeine, fluoride and many others could affect bone mass density.

Apart from that, black tea was able to dominate unanimous approval due to its hygienic packaging in tea bags. Nevertheless, it has been reported that these tea bags have a high fluoride content of 1.15 to 6.01mg/l (Cao et al., 2006). According to American Dietary Reference Intake (RDA) in 1989, the healthy maximum limit for daily fluoride intake is 2.5mg for children and 1.5 to 4.0mg for adults per day (Jain, Manghani, Kohli, Nigam, & Rani, 2013). If each bag is expected to contain 2g of tea, approximately 1.5 to 3.0mg/l fluoride concentration (Gupta &Sandesh, 2012), 4 cups of tea are considered healthy. Excessive fluoride levels could lead to skeletal fluorosis and fractures of the bone (Jain et al., 2013). The strong antioxidant strength of black tea suggests that it could play a role in helping consumers reduce their risk of coronary heart disease and cancer. Even so, the media also claim that tea harms cognitive function, iron

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levels, dental health, bone health and fluid balance often correlated with its caffeine content (Gardner, Ruxton, & Leeds, 2007).

On the other hand, heavy coffee drinkers have been linked with bone loss, reduced bone density or fractures and increased blood pressure (de Mejia & Ramirez-Mares, 2014). Although more than 80 coffee species have been distinguished worldwide, only two types of coffee are economically important. *Coffeaarabica*, also known as Arabica coffee, is responsible for roughly 70% of the global coffee market and *Coffearobusta* or Robusta coffee accounts for the rest (Farah, 2012). The non-volatile fraction of coffee is composed primarily of carbohydrate and fibre, water, lipids, proteins and free amino acids, minerals, chlorogenic acids, organic acids, trigonelline and caffeine (Farah, 2012). In a way, caffeine can inhibit the activity of phosphodiesterase and transformed into agonist of adenosine cyclase and finally take effect on several tissues (Sun et al., 2017), especially bone.

Caffeine is the tea and coffee's predominant methylxanthine (alkaloids); where the ratio of other alkaloids to caffeine varies widely but is usually higher than 1 (Franco, Oñatibia-Astibia, &Martínez-Pinilla, 2013). Moderate methylxanthine intake from the food sources is healthy, but high doses (for example, caffeine pills) may cause increase heartrate, anxiety and gastric acid secretion (Franco et al., 2013). Depending on weight, sex, age and differences in vulnerability, the amount of caffeine needed to produce negative effects varies from person to person. Most end-users experience and enjoy increased vigilance, improved mood and focus, and the ability to stay awake. Caffeine may have unpleasant symptoms for the others; some individuals metabolize caffeine more slowly than others because of variation in the metabolizing CYP1A2 enzyme activity. Caffeine also has addictive properties with a strong tendency to ingest foods or drinks containing caffeine and signs of withdrawal when the intake of caffeine is abruptly stopped (de Mejia & Ramirez-Mares, 2014).

Amid controversy about whether caffeine is good or bad for individuals, evidence has suggested that caffeine consumption has some adverse effects on anatomy and physiological state in human osteoporosis despite being a stable compound (Hallström, 2013; Heaney, 2002; Sun et al., 2017). According to Harris and Dawson-Hughes (1994) prospective study, a woman with low calcium intake than 744mg/day but consumed high caffeine more 450mg/day had a notable high bone loss than women consuming less caffeine. It is hypothesized that daily caffeine consumption greater than that obtained from about 2-3 servings of brewed coffee will intensify osteoporosis in women with insufficient intake of calcium (Choi & Kim, 2016). While the magnitude of this impact in relation to the development of osteoporosis may be challenged in individuals with adequate calcium intake, it may be more obvious when there is a higher intake of caffeine and low calcium consumption and absorption.

Meanwhile, osteoporosis is a metabolic bone disease that featured by an enhanced state of bone reabsorption accompanied with diminished bone formation, leading to a reduction of bone mass density, degradation of bone quality and increasing the risk of developing fractures (Sun et al., 2017). Bone density is the major indicator for fracture risk that allows biological effects to be explored in randomised controlled trials of modest size (Tai, Leung, Grey, Reid, &Bolland, 2015). In this context, it is also possible that caffeine metabolites may be essential. This could be seen in a recent study that has stated coffee would cause high mortality, low heart rate and low LC_{50} value in zebrafish embryo prior to caffeine consumption (Sellathory et al., 2019). Also

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according to Santhos, Ruiz-Oliveira, Silva &Luchiari (2017), high doses of coffee are harmful to the individual due to disappearing stimulating effect but caffeine anxiogenic effect, which conclude through temporal analysis of zebrafish behaviour.

MATERIALS AND METHODS

Materials

Black tea *(Camellia sinenis)* was obtained from sachets originated in Sri Lanka. The Malaysia grown coffee beans were acquired before ground following the standard preparation process. Two coffee types were used in this study which are*Coffeaarabica* and *Coffearobusta*.

Breeding of Zebrafish

The breeding method was adapted from Avdesh et al. (2012), wherefertilized eggs of zebrafish can be obtained through mating in pair. The zebrafish mating tank was set up in the afternoon after being fed. Two female and one male were put between separator to opposite sides of the breeding tank. The separator was removed the next morning shortly after the onset of light. The mating occurred for 1 hour undisturbed. After that, fish were returned to their tanks. The collected eggs can be observed under a microscope. Zebrafish were fed with commercial food (pellets) or fresh food (brine shrimps) three times per day.

Cold brew experiments

At room temperature, 100g of grounded coffee samples were added into 500ml of distilled water. The samples were contained in filter paper and placed in a conical flask. The mixture was stirred and left for 1 hour. Samples collected after the first hour were evaporated using the rotary evaporator.

Embryo Zebrafish Cytotoxicity

The semi-solid extraction were taken to make different concentration of dilution (75mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25 mg/ml and 3.125mg/ml) to evaluate the parameters of viability, heart rate, tail, somite and oedema formation. Each dilution were pipetted into 96 well-plate and the results are recorded for 5 days. Data provided are the mean of triplicate studies performed in duplicates.

Adult Zebrafish Cytotoxicity

The remaining semi-solid extraction were used for 500mg/ml, 200mg/ml, 100mg/ml, 50mg/ml and 25mg/ml to investigate the prolonged effect of tea and coffee exposure toward bone density. Each dilutions were measured (500ml) and aliquot into containers before left them for 1 month. Data provided are the mean of triplicate studies performed in duplicates.

Bone Histology

After 1 month of adult female zebrafish immersion under tea and coffee treatment, the zebrafish proceeded into tissue processing. The zebrafish is thoroughly infiltrated by paraffin wax, which eventually formed into a “block”. The section of the tissue is cut using a microtome. The tissue was attached on a slide and left dried before staining with Haematoxylin and Eosin for observation under the forensic microscope.

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Behaviour Examination

Behaviour study wasadapted from Pathan, Ansari, Kanase&Mateen (2017) using Examination tank that filled with different concentration of tea and coffee as mentioned in adult cytotoxicity earlier. It consisted of a 1.5L tank with vertical and horizontal lines drawn on the middle of the tank, which divides the water into four quarters portion. The line was used to calculate the time spent in the top and bottom of the tank by fish in 2 minutes.

Ethical Consideration

The procedures were proposed from previously published studies with optimisation and performed in accordance with the ethics code MSU-RMC-02/FR01/11/L3/002. The experiments in this paper will be performed with a clinical application in mind and use carefully matched data, so the distribution is not represented as a whole. Thus, ethical form is required for animal handling.

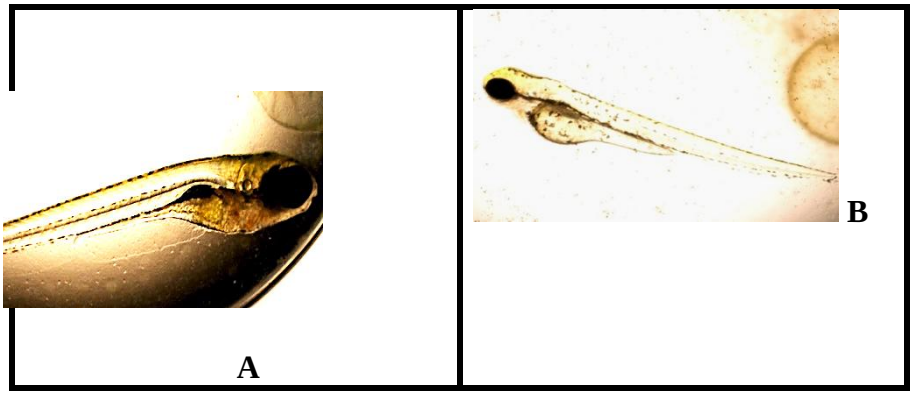
Statistical analysis

All data analysis was conducted with GraphPad Prism version 8. The results were expressed with the graph before statistics. Two subgroups model are used (adult and embryo zebrafish) when there was significant homogeneity between-studies; otherwise, the fixed-effects model was employed. Significant differences between the mean values were analysed using ordinary one-way ANOVA analysis of variance (Turkey and Bonferroni's multiple comparison test). The differences were considered to be statistically insignificant if the *P*-values were more than 0.05.

RESULTS

Embryo development

The most changes in abnormality of physical structure in *Daniorerio* embryo were seen in coffee compared to tea within low concentration from 12.5mg/ml, 6.25mg/ml and 3.125mg/ml. A comparison between normal and abnormal morphology were recorded in Figure 1.The cumulative mortality or lethal concentration (LC_{50}) of an embryo after tea and coffee exposure are shown in Figure 2 (D). Mortality rate comparison of *Camellia sinensis*(59mg/ml), *Coffeaarabica*(77mg/ml) and *Coffearobusta*(36mg/ml) have shown that *Coffearobusta* causes the highest death followed by *Coffeaarabica* and *Camellia sinensis*.



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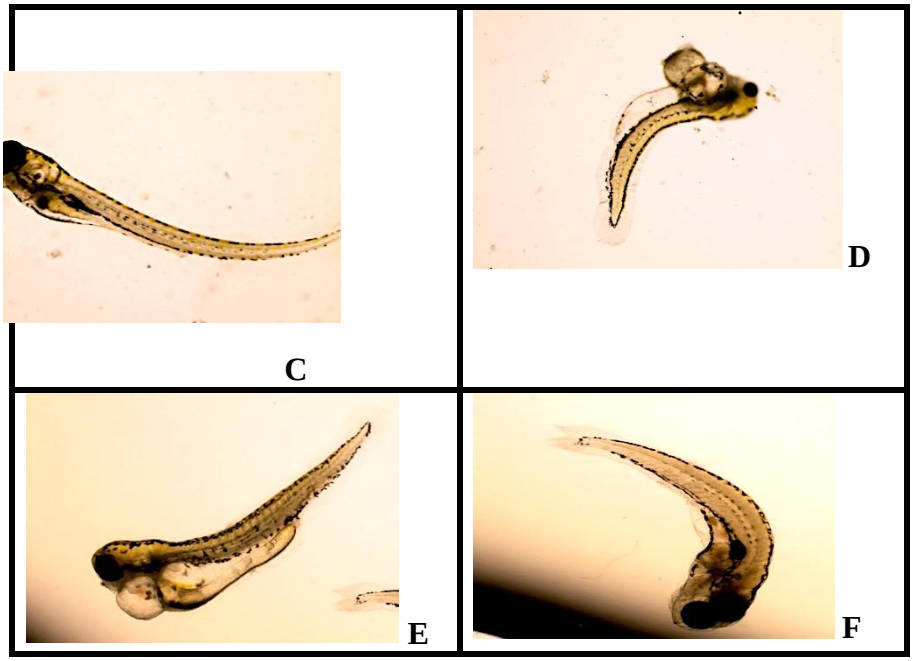
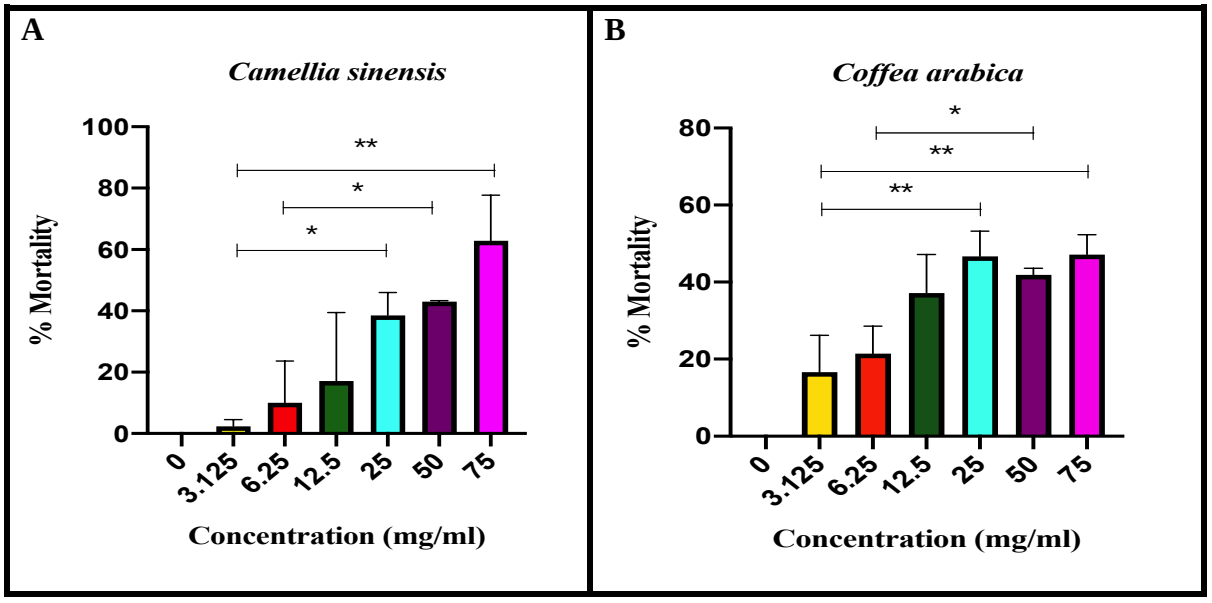


Figure 1: Observation of zebrafish larvae after 5 days of treatment using *Coffeaarabica* and *Coffearobusta*. **(A,B)** Normal larvae of zebrafish could be seen with straight body and tail shape (anterior view). Abnormal physical structure in the larvae demonstrated by anatomy changes in C, D, E, F. **(C)** Curvature of larvae physical structure was seen. **(D,E)** Larvae develop pericardial oedema. **(F)** Abnormal tail formation and curve shape was observed.



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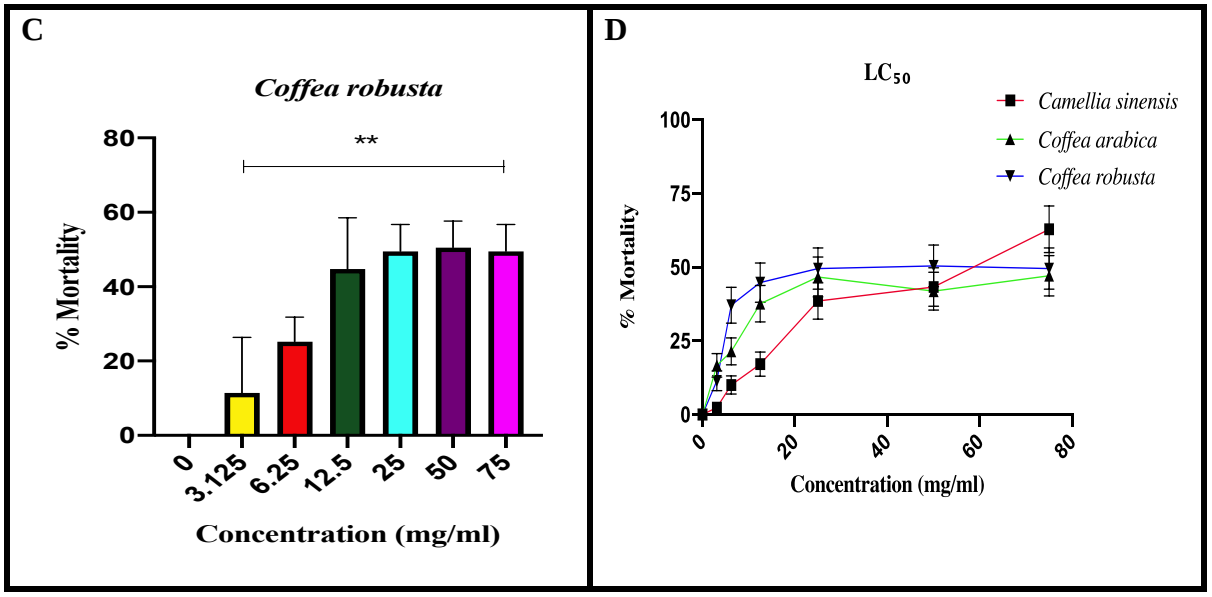

Figure 2: The mortality rates of **(A)***Camellia sinensis*, **(B)***Coffeaarabica* and **(C)***Coffearobusta***(D)** The lethal concentration (LC_{50}) of following aqueous exposure in *Camellia sinensis*, *Coffeaarabica* and *Coffearobusta*. Error bars indicate SD; *p<0.05, **p<0.001 by one-way ANOVA; n=30 for each group.

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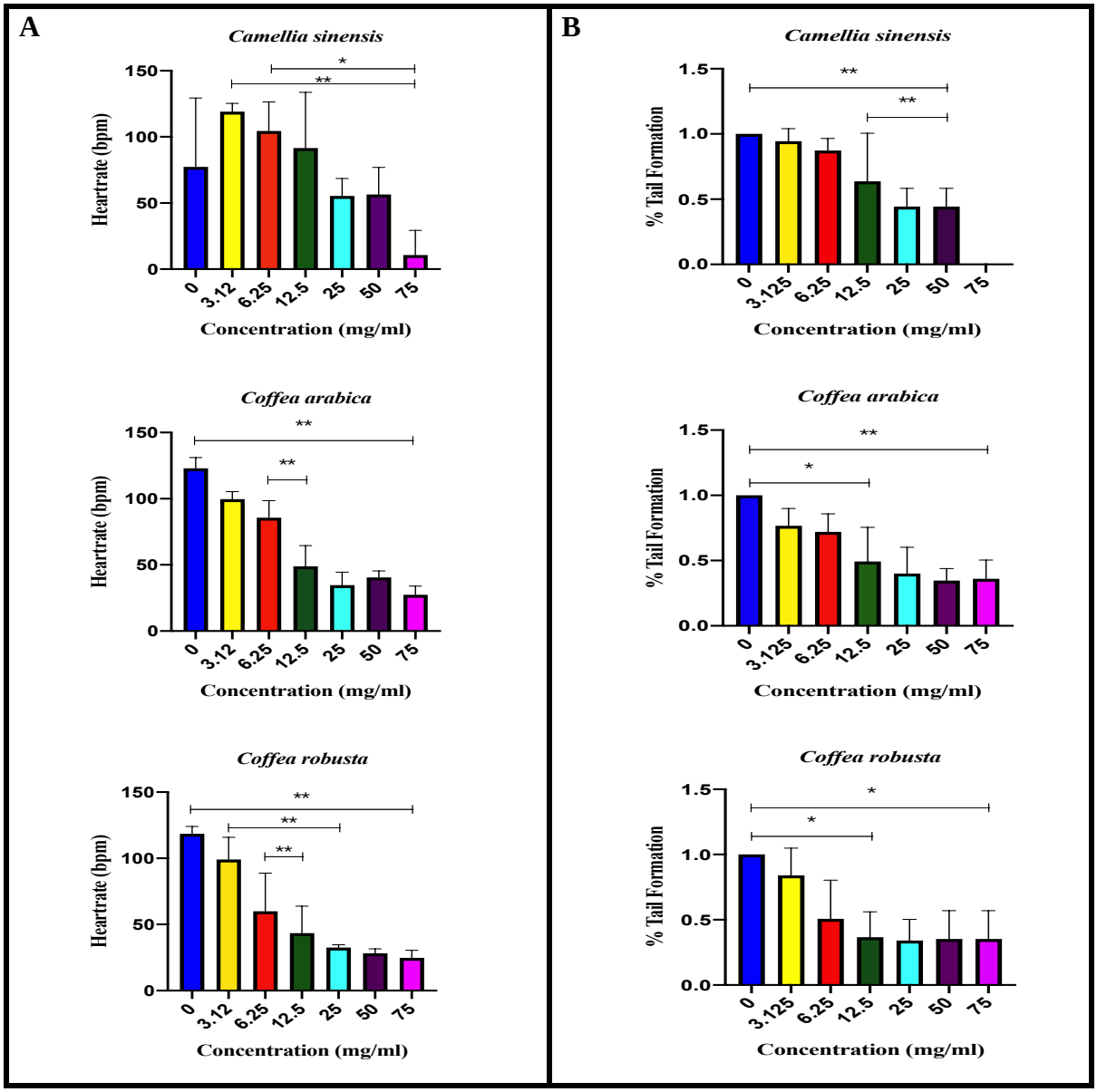

Figure 3: The heartrate and tail formation after aqueous exposure to tea and coffee. **(A)** The decreased heart rate of *Daniorerio* embryo were noted on tea and coffee with the formation of oedema. **(B)** The decreased tail formation interrelated with viability of the embryo in *Camellia sinensis*, *Coffeaarabica* and *Coffearobusta*. Error bars indicate SD; *p<0.05, **p<0.001 by one-way ANOVA; n=30 for each group.

Irregular decreasing heartrate of zebrafish embryo was seen in *Camellia sinensis* for each concentration compared to the zebrafish embryo's heartrate in *Coffeaarabi*ca and *Coffearobusta*, which eventually decreased. The heartrate was noted interrelated with the oedema formation in Figure 3 (A) and 4 (B) due to prolonged exposure of tea and coffee. After 24hpf, the embryo starts to develop a tail, which gradually decrease in the line with mortality of the embryo in *Camellia sinensis*, *Coffeaarabica* and *Coffearobusta*as seen in Figure 3 (B).

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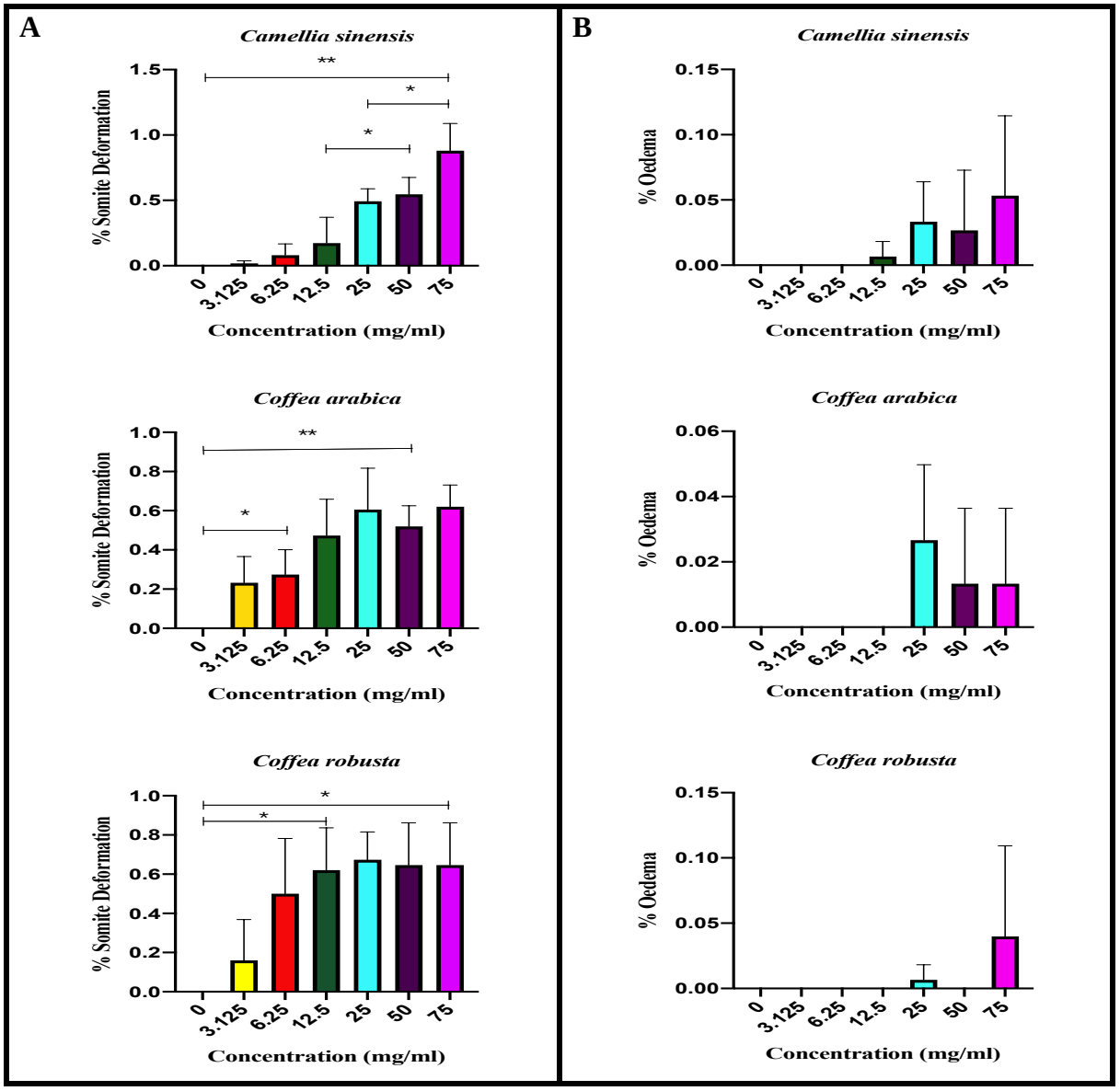

Figure 4: The somite deformation and oedema formation after prolonged exposure of tea and coffee. **(A)** The rapid increased in somite deformation was seen for each treatments. At 25mg/ml, both types of coffee show a constant results compared to tea. **(B)** The oedema formation rate in zebrafish embryo exposed to *Camellia sinensis, Coffeaarabica* and *Coffearobusta.*

The rapid increased in somite deformation was seen for *Camellia sinensis, Coffeaarabica*and*Coffearobusta*, which noted in Figure 4 (A). Starting from 25mg/ml to 75mg/ml, both types of coffee show constant results compared to tea that gives a high rate of somite deformation around 90% after 50mg/ml.Meanwhile, the oedema formation rate in zebrafish embryo shows less than 6% for overall concentrationsexposed to *Camellia sinensis, Coffeaarabica* and *Coffearobusta*thatwere considered not significant ($p > 0.05$) in Figure 4 (B).

Adult zebrafish assessment

The histology changes comparison between *Camellia sinensis*, *Coffeaarabica* and *Coffearobusta* for adult *Daniorerio* were shown in Figure 5. The normal structural layout of bone were

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classified either in spongy and compact as shown in 5 (A). Spongy tissue (arrow) is the porous with a hollow type of bone with the harder compact bone (arrowhead) surrounding it. Also, there are no obvious changes in the bone structure prior to exposure toward *Camellia sinensis*, *Coffeaarabica*and*Coffearobusta compared to normal. Osteoporosis bone is more likely characterized by structural deterioration of bone tissue (more empty spaces in the bone with less spongy bone or thinning of compact bone) or also known as porous bone. It is either when the body loses too much bone, make too little bone or both.*

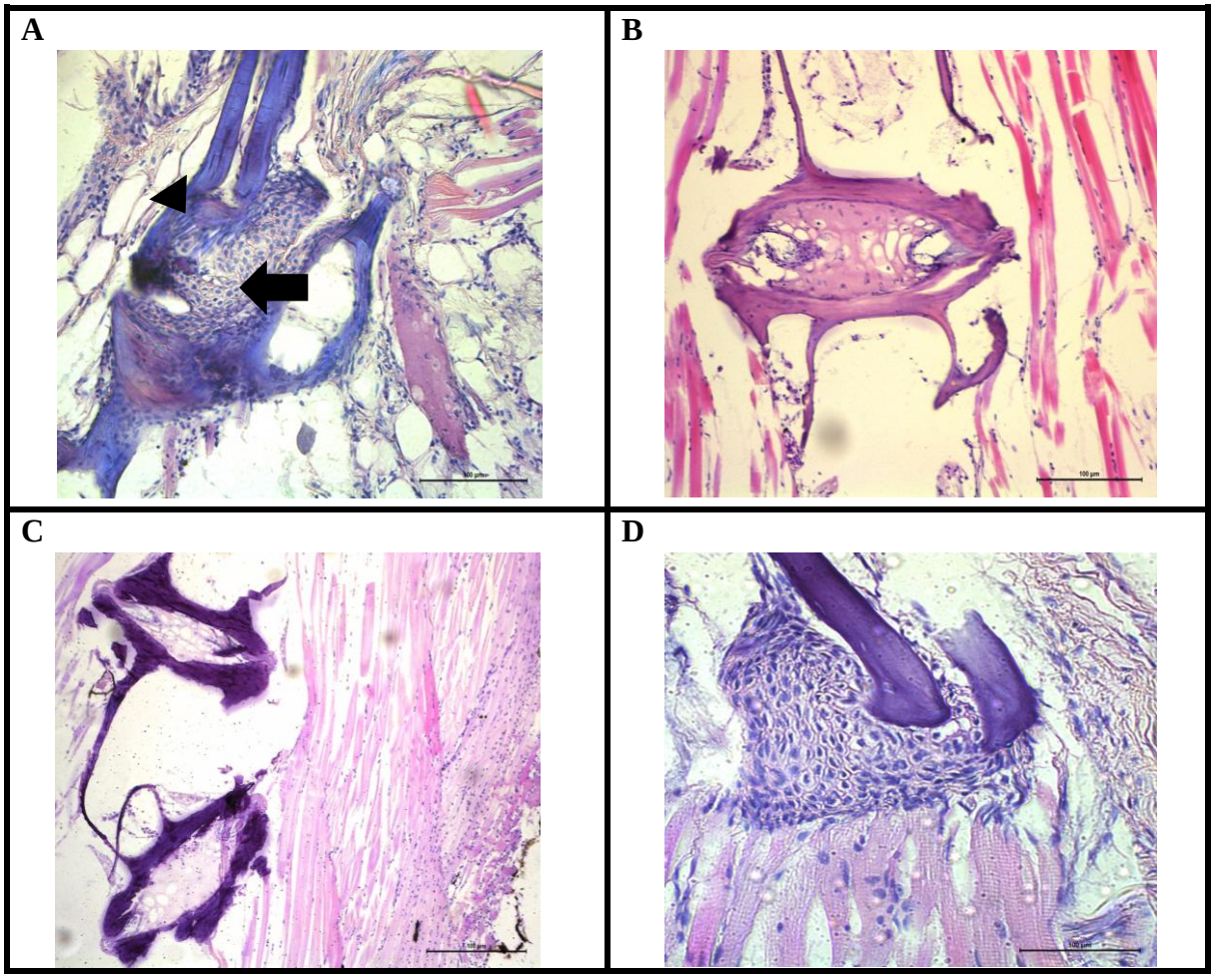

Figure 5: Histological observation of adult zebrafish bone. **(A)** Normal zebrafish bone structure. **(B,C,D)**Histopathological changes in the bone of adult zebrafish of *Camellia sinensis, Coffeaarabica*and *Coffearobusta*respectively.

On the other hand, behaviour study was evaluated with different quarters using Examination tank method according to Figure 6. Based on behavioural study analysis, the results show that zebrafish tend to spend their time in quarters C and D despite the exploratory actions that Pathan, Ansari, Kanase&Mateen (2017) well described. Compared with the control group ($p>0.05$), the zebrafish were scattered more evenly at four quarters A, B, C and D for overall observation.

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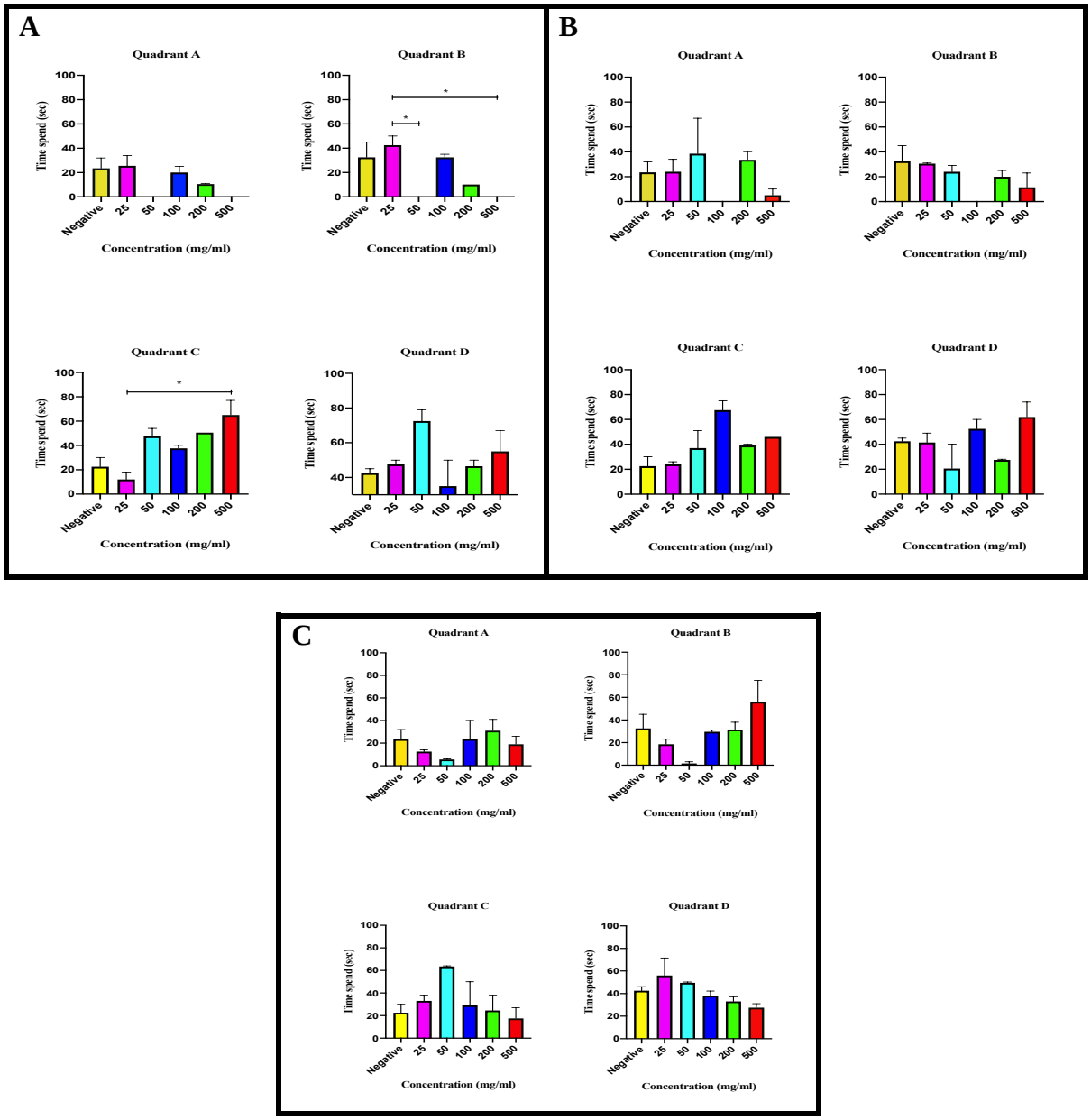

Figure 6: Behaviour study of adult female zebrafish in **(A)***Camellia sinensis***(B)***Coffeaarabica***(C)***Coffearobusta.* Error bars indicate SD; *p<0.05 by one-way ANOVA, n=30 for each group.

DISCUSSION

From the study, we discovered that tea and coffee result in developmental toxicity in zebrafish embryo, particularly morphological abnormalities and mortality. The cumulative mortality of embryo after tea and coffee exposure is shown in Figure 2. Mortality is recorded at 6, 24, 48, 72, 96 and 120 hour per fertilisation (hpf) in order of increasing concentrations. In other words, the rate of mortality increases as the concentration of tea and coffee increases, which noted by coagulation as a marker for death. Lethal concentration (LC_{50}) shows the concentration of tea and coffee that will kill 50% of zebrafish populations when administered as single

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exposure for 5 days. These values give an idea of the relative acute toxicity of consuming tea and coffee. From the graph in Figure 2 (D), the LC_{50} value of *Camellia sinensis* shows that 59mg/ml will kill 50% of populations which is higher compared to *Coffearobusta* with 36mg/ml but lower than *Coffeaarabica* with 77mg/ml. Therefore, *Coffearobusta* appears more lethal followed by *Camellia sinensis* and *Coffeaarabica*. Based on Braunbeck&Lammer's (2006) study observations, zebrafish eggs were exposed to the compounds 2,4-dinitrophenol and 3,4-dichloroaniline with different starting points from 1 to 9 h of egg age to investigate the function of exposure. For either drug, after 24 and 48 h of exposure, a strong correlation was established between the egg age at exposure onset and cumulative mortality. Mortality, however, had occurred at such a pace after 6 days of exposure that the impact could no longer be seen (Braunbeck&Lammer, 2006).

Although many researchers have investigated the biological activities of caffeine, there is limited information on its toxic effects, particularly concerning embryonic development. In this study, heartrate changes were found in a dose-dependent manner with exposure of tea and coffee and a statistically significant decrease was noted compared with the normal group. It has been observed that non-lethal malformation, pericardial oedema also led to low heartrate. Embryos were characterized by oedema with low heartrate in the phase after the coffee exposure, which is shown in Figure 3(A) and Figure 4 (B). It has been suggested that the inadequate synthesis of cardiac troponin-T leads to pericardial oedema in the zebrafish embryo (Dambal, Selvan, Lite, Barathi, &Santosh, 2017).

Early embryonic exposure to polycyclic aromatic hydrocarbon mixtures impaired cardiac conduction, accompanied by secondary effects such as pericardial oedema and disturbed cardiac morphogenesis (Incardona, Collier, &Scholz, 2004). A rise has been seen in *Camellia sinensis* for oedema formation at 12.5mg/ml with 0.006% until 25mg/ml at 0.03% before up and down rate (due to high mortality) as concentrations rise. At low concentration, there is no oedema formation for both types of coffee until 25mg/ml, where *Coffearobusta* boosts to 0.006% and *Coffeaarabica* to 0.03%. However, due to death coagulation, *Coffearobusta* shows a depleted mean reading afterwards at 50mg/ml but increases to 0.04% for a new development of oedema at 75mg/ml. The same goes for *Coffeaarabica*, a drastic increases with 0.03% at 25mg/ml before remain constant at 50mg/ml and 75mg/ml with 0.01% respectively. Oedema is characterized by an abnormal accumulation of body fluid in the interstitium, located in the cavities of the body, beneath the skin (Li et al., 2016). These results showed that tea and coffee consumption could affect the heart function and also a cause of oedema. These findings also demonstrate the risks of excessive coffee and tea consumption during pregnancy.

After 24 hpf, the embryo starts to develop their tail, which later gradually decreases as shown in Figure 3 (B). This may be hypothesized as the higher the mortality of embryo, the lower the chances of zebrafish embryo to develop a complete tail formation. But those few embryo that survived after 96 hpf were seen to form an abnormal tail. This could be seen in Figure 1 (F), where the observed ectopic tail phenotype that developed may be attributable to kinases inhibition (Gebruers et al., 2013). Previous studies have shown that tail formation is a continuation of gastrulation and varies depending on traits that are already required throughout embryogenesis (Gont, Steinbeisser, Blumberg, & De Robertis, 1993). According to Hammerschmidt et al. (1996), some of the tail's material is produced in the tailbud and later in the tail's tip, where there is a 'mini-gastrulation'. Nonetheless, ectopic tail deformation exhibit different defects during tail formation, while gastrulation and tail material formation tend to be mostly normal in the tailbud and tail tip. This indicates that the genes control biological processes in addition to the ongoing processes of gastrulation to make it possible a normal tail outgrowth

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(Hammerschmidt et al., 1996). Instead, during gastrulation, the impact of the mutations could be very slight or balanced by the gene products produced by the parent.

The paraxial mesoderm sections in metameric somite containing progenitor cells for the axial skeleton, skeletal muscle and connective tissue during chordate embryogenesis (Draga et al., 2019). Somite arise from the anterior end of the unsegmented paraxial mesoderm called the pre-somitic mesoderm or segmental plate by rhythmically budding. In this study, somite deformation starts to occur at 12.5mg/ml, apart from 6.25mg/ml and 3.125mg/ml that mainly cause mortality before they start to develop any segments during embryogenesis. The somite deformation keeps increasing until 75mg/ml for *Camellia sinensis* while *Coffeaarabica* and *Coffearobusta* shows irregular findings for each concentration. Therefore, a genetic approach may classify genes that are required for somite formation, which are new or previously unanticipated. Stickney, Baressi&Devoto (2000) stated that several other zebrafish mutations have been identified with somite structure defects, beamter (bea), fused somites (fss), mind bomb (mib) and deadly seven (des). Such abnormalities, including after eight (aei), develop a paraxial mesoderm but do not grasp the early somite boundaries. Even so, there has been some kind of segmental pre-pattern in all these defects because the zebrafish probably grow unusual segmented vertebrae and somite borders (Eeden et al., 1996).

In order to investigate the strength of bone tissue for osteoporosis evaluation by analysing the zebrafish bone fragility and morphology, haematoxylin and eosin stain were used. There were no obvious changes in bone density structure of zebrafish that were exposed long-term in *Camellia sinensis*, *Coffeaarabica* and *Coffearobusta*. The structure of zebrafish bone in the control group was normal, as described in Figure 5 (A). Bone architecture are classified with trabeculae (cancellous or spongy) and compact bone tissue. We hypothesised was that no bone loss occurred in all osteoporotic models in comparison to the control group. There are no significant histopathological changes on bone mass density prior exposure to tea and coffee as shown in Figure 5 (B, C and D). Bone strength or fracture resistance are determined by both structural and material properties of the bone (Chavassieux, Seeman, &Delmas, 2007). As the focus of this research was on structural properties, all bone mechanical properties were determined.

Based on the analysis of the behavioural study, the results show that zebrafish tend to spend their time in quarters C and D despite the exploratory actions that Pathan, Ansari, Kanase&Mateen (2017) well described. Zebrafish are considered to be surface fish, meaning they float near the water surface. Originally, they spend longer at the base of the tank when they are replaced with a new tank then after some time they swim to the surface, this is attributable to their exploratory behaviour and most often due to their fear (Pathan, Ansari, Kanase, &Mateen, 2017). The time spent on the tank below the horizontal line is measured in 120 seconds and this indicates the magnitude of zebrafish's reaction to anxiety being observed.

CONCLUSION

Caffeine is the compound in coffee and tea, which commonly consumed bioactive substances around the world. Coffee and tea are complex drinks that contain more than 1000 compounds, in addition to caffeine, responsible for its good flavour and aroma (de Mejia & Ramirez-Mares, 2014). In conclusion, different doses of prolonged tea and coffee had shown an ambiguous effect on anatomy and physiology of *Daniorerio* embryo that would lead to delay hatching period. Abnormal heartbeat, pericardial oedema, high mortality rate, tail and somite deformation were observed in coffee compared to tea on zebrafish. This shows that coffee has a higher chance of causing abnormality in comparison to tea. Histology evaluation of adult female zebrafish bone

structure shows no differences compared to normal, which concluded tea and coffee may not affect the bone density that would lead to osteoporosis. However, from the behaviour evaluation, the anxious response from zebrafish could be interpreted as tea and coffee has the potential to cause anxiety in people.

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