Current Topics in Toxicology
Vol. 15, 2019

Short Communication

Toxicity evaluation of instant coffee *via* zebrafish (*Danio rerio*) embryo acute toxicity test

Chamundieswary Sellathory[1], Kokila Vani Perumal[1,2], Siroshini Thiagarajan[2], Azrina Zainal Abidin[1], Santhra Segaran Balan[1] and Hasnah Bahari[2,*]

[1]Department of Diagnostic and Allied Health Sciences, Faculty of Health and Life Sciences, Management and Science University, 40100 Shah Alam; [2]Department of Human Anatomy, Faculty Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

ABSTRACT

Coffee is one of the most popular beverages in the world. However, the safety level of some instant pre-mixed coffees available in Malaysia is questionable as there have been reports of adverse effects and psychoactive chemical contents. This study aims to evaluate the toxicity level of instant coffee sold around Selangor, Malaysia. A total of 13 types of instant coffees were collected and the toxicity assessed through zebrafish (*Danio rerio*) embryo acute toxicity test (ZFET). The survival rate, hatching rate, heartbeat rate, and scoliosis were observed. Data were analyzed using linear regression and one-way ANOVA. The LC_{50} was calculated and was compared with the positive control and LC_{50} of caffeine. Four coffee samples did not exhibit an effect on zebrafish survival rate; however the rest of the coffee samples caused death in zebrafish. Three coffee samples namely samples 11, 12 and 13 caused a very low hatching rate. A normal heartbeat rate for zebrafish is between 120 to 170 beat per minute which was similar in zebrafish of both the control group and those exposed to coffee samples 4 and 10. The rest of the coffee samples caused an abnormally low range of heartbeat per minute. There was no scoliosis observed in this study. In a nutshell, this study suggests that some of the pre-mixed coffee has the potential to cause health problems due to the toxic reaction of an anonymous compound that could be toxic to humans.

KEYWORDS: *Danio rerio* embryo, heartbeat per minute, instant coffee, toxicity activity.

INTRODUCTION

Worldwide, people consume around 3 to 5 glasses of coffee daily. In Malaysia, the pre-mixed coffee products are usually packed in a sachet with a blend of coffee and herbs. The coffee itself is a mixture of various kinds of complex chemicals such as carbohydrates, lipids, nitrogenous compounds, vitamins, minerals, alkaloids and phenolic compounds [1]. So the uniqueness of Malaysian coffee is that it is mixed with various types of herbal or local fruits such as Tongkat Ali, durian, ginseng and many more. However, the safety of this pre-mixed coffee is dubious. Therefore, conducting a toxicity assessment for such products are essential, especially in their development phase. Preclinical toxicity testing of compounds will explain the species-, organ-, and dose-specific toxicology effects of an investigational product [2].

MATERIALS AND METHODS

Sample collection

Thirteen instant coffee sachets were randomly purchased in Selangor, Malaysia and labeled as samples 1 to 13 (Table 1).

*Corresponding author: haba@upm.edu.my

Table 1. The contents of 13 coffee samples collected at Selangor, Malaysia.

Coffee Samples	Ingredients listed on food label
1	Instant Coffee
2	Coffee Arabica Extract White Ginger Extract Red Ginger Extract Coal Ginger Extract Garcinia Cambogia Extract Cassia Augustilofia
3	Durian Musang King White Coffee
4	Coffee Arabica Extract Coal Ginger
5	Bentong Ginger Red sugar Licorice Honey
6	Durian flavour
7	Durian Coffee
8	Coffee Arabica Pomegranate Extract
9	Red dates Longan
10	Seven selected herbs but did not mentioned the herbs
11	Instant coffee Instant Tongkat Ali Instant Ginseng Extract Maldoxterin
12	Instant coffee Maldoxterin Smilax myosotifiora Eurycoma longifolia Ganoderma
13	Tongkat ali Guarana Maca Instant coffee

Sample preparation

The instant coffee samples were collected in the powder form and weighed. The samples were prepared in the following series of concentrations: 750 µg/ml, 375 µg/ml, 187 µg/ml, 93.75 µg/ml, 46.88 µg/ml, 23.44 µg/ml, 11.72 µg/ml and 5.86 µg/ml. Paracetamol was used as a positive control in this study.

Zebrafish embryo toxicity test (ZFET)

Zebrafish embryos were purchased from Danio Assay Laboratories Sdn. Bhd. The zebrafish embryo toxicity test was carried out according to Zhu *et al.* [3]. Only healthy embryos were selected and were transferred into a 96-well plate. The plate was incubated for 24 hours at 28 ± 2 °C. The embryos were left for 24 hours post

fertilization. Next, the embryo media were replaced with 100 µl of diluted coffee samples. The determination of endpoint of toxicity was through the evaluation of mortality, hatching rates, heartbeat rates and scoliosis. The rates of mortality, hatching and heartbeat as well as scoliosis were observed and recorded starting after 24 hours of treatment till 72 hours after treatment since the embryo will start to hatch around 48 hours post fertilization [4]. Heartbeat rate of embryo/larvae was calculated and recorded starting from 24 hours of treatment up to 72 hours after treatment. An inverted microscope was used to view and count the heartbeat. The heartbeat was recorded as bpm - beat per minute. Scoliosis was observed under an inverted microscope and recorded.

Data analysis

Data were analyzed as mean ± SEM relative to negative control. One-way analysis of variance (ANOVA) by SPSS window version 23 was used. All the data were interpreted with the p-value of <0.05 indicating the result to be statistically significant. The LC_{50} value represents the concentration of sample that causes 50% mortality in the zebrafish embryo. Probit value was determined using mortality value. To get LC_{50} value regression method was applied using probit value and log concentration.

RESULTS

Effect of 13 coffee samples on LC_{50} of zebrafish embryo

The LC_{50} of 13 coffee samples were plotted using mortality ratio (Table 2). Samples 4, 5 and 10 caused no death and hence there is no LC_{50} value. Samples 3 and 9 showed a very low LC_{50} value because the zebrafish died at the lowest concentration and only 1 died. The highest LC_{50} value was observed for paracetamol, which is 2398.83, followed by coffee samples 7 and 8. Paracetamol acted as a positive control in this study. The lowest LC_{50} value was observed for coffee samples 11, 12 and 13. This is because a lot of zebrafish embryos died at most of the tested concentrations.

Effect of 13 coffee samples on hatching rate of zebrafish larvae

The hatching rate of zebrafish larvae exposed to thirteen coffee samples with different concentrations was observed at 48 hpf (hours post-fertilization) until 96 hpf (Figure 1). Overall hatching rate was more than 70% in both control group and in larvae exposed to all coffee samples at 72 hpf except for coffee samples 11, 12 and 13. However, at higher concentration, there was a delay in hatching rate which was observed for paracetamol-administered group and treatment groups 1, 2, 3, 4, 5, 6 and 7.

Table 2. LC_{50} values of 13 coffee samples collected at Selangor, Malaysia.

Samples	LC_{50} (Mortality in probit value)
1	831.76
2	146.01
6	231.74
7	1703.01
8	1335.21
11	83.07
12	98.77
13	58.88

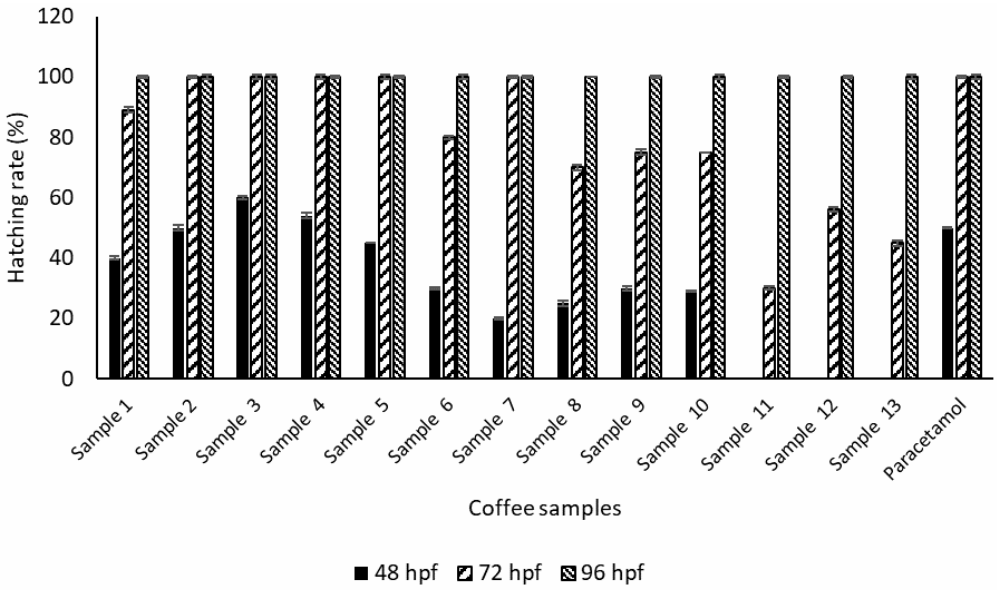

Figure 1. Effect of 13 coffee samples on hatching rate of zebrafish larvae. Frequency of hatching rate of thirteen coffee samples at 48 hpf, 72 hpf and 96 hpf. Data are expressed as mean ± SEM and analyzed using one-way ANOVA. The values with $p < 0.05$ are significantly different.

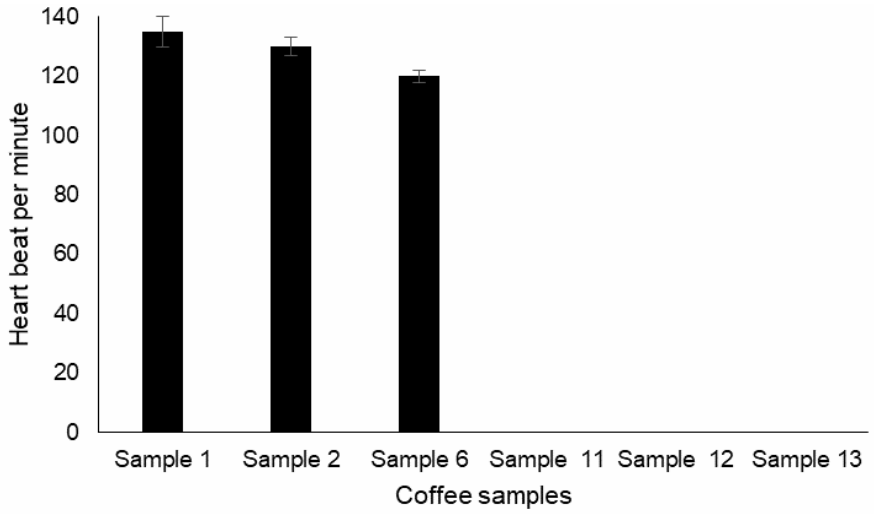

Figure 2. Effect of 13 coffee samples on heartbeat of zebrafish larvae. Heart beat frequency of coffee samples 1, 2, 6, 11, 12 and 13. Data are expressed as mean ± SEM and analyzed using one-way ANOVA. The values with $p < 0.05$ are significantly different.

Effect of 13 coffee samples on heartbeat of zebrafish larvae

The effect of coffee samples on the heartbeat rate was observed at 48, 72, and 96 hpf (hours post-fertilization) (Figure 2). Heartbeat rate measurement is essential in evaluating cardiac performance. Coffee samples 3, 4, 5, 9 and 10 showed very low LC_{50} values and hence can't be considered as baseline toxicity which has an impact on the heartbeat rate. Whereas, in the case of coffee samples 8 and 6 the LC_{50} values were too high and they are non-toxic to the zebrafish.

DISCUSSION

This study investigated the effects of 13 different types of instant coffee on zebrafish embryo at early development stages of 24 hpf, 48 hpf, and 72 hpf. Zebrafish embryos are broadly used to identify the negative effect of chemical interaction [5]. This study emphasized the effect of different instant coffee sachets on mortality, hatching rate, heartbeat rate, and scoliosis. Paracetamol was used as a positive control in this study. The LC_{50} value obtained for paracetamol was 2398.83 which is higher compared to all the thirteen samples studied. Paracetamol LC_{50} value acts as a threshold value for toxicity assessment. Coffee samples 7 and 8 have LC_{50} values closer to paracetamol LC_{50} value. From this it can be deduced that these coffee samples are mildly toxic compared to the other coffee samples. Although coffee samples 1, 2, 6, 7 and 8 showed lower LC_{50} values compared to paracetamol somehow they are higher than the LC_{50} value of caffeine proposed by Zhang *et al.* which is 108 mg/liter [6]. Caffeine is the main bioactive compound in coffee [7]. Therefore these coffee samples are not toxic since the LC_{50} values are higher compared to caffeine.

Nevertheless, coffee samples 11, 12 and 13 have lower LC_{50} values compared to caffeine. Therefore from this research, it can be deduced that these coffee samples are toxic. Coffee samples 4 and 5 did not result in any mortality at all the concentrations. Hence the LC_{50} value cannot be calculated. Besides that the mortality caused by coffee samples 3 and 9 are very low; death occurred at one of the low concentrations and thus the LC_{50} value is too low and can't be considered as baseline toxicity [8]. The zebrafish use its skin and gills to absorb low molecular weight compounds that are diluted in the maintenance solution it lives [9]. Mammals and zebrafish share a similar pathway and mechanism of apoptosis [6]. The result that was obtained from this study agrees and confirms with the outcome of results of Abdelkader [10]. Four out of 13 coffee samples showed that there is no effect of coffee on the survival rate of zebrafish. Even so, eight out of thirteen coffee samples showed decrease in survival as the concentration increases. From this it can be concluded that as the concentration increases the toxic level in the samples increases which leads to low survival rate at higher concentration.

Hatching rate is an important parameter in the development of zebrafish [4]. The ability of the high choriolytic enzyme (HCE) and low choriolytic enzyme (LCE) to work together without any interference of any chemical to digest the chorion will help the zebrafish to hatch at a normal rate [11]. In this research, there was no effect on hatching rate between the control group and samples 8, 9, and 10 suggesting that, these coffee do not cause hatching delay which is similar to previous reports by Abdelkader *et al*. [12]. The reports stated that caffeine significantly decreased hatching ($p < 0.05$); however, the overall hatching rate at 96 hpf was 94% in control and 93% in caffeine-exposed group with no significant difference ($p > 0.05$). However, the zebrafish embryo exposed to coffee samples 1, 2, 3, 4, 5, 6, and 7 when compared to negative and positive control groups did not show a delay in hatching rate at lower concentration but at higher concentration the hatching rate was slow. This could be due to the higher concentration of coffee that can interrupt the behavioral and biochemical activity. This shows the hatching rate of zebrafish embryos was dose-dependent.

Nevertheless, there is a delay in hatching observed for coffee samples 11, 12 and 13 at lower concentration itself. For this reason, it can be concluded that the contents in coffee samples 11, 12 and 13 may interact with LCE and HCE and cause a delay in hatching. A previous report stated that a drug that causes cardiotoxicity will cause delay in hatching rate of zebrafish compared to a non-cardiotoxic drug [13]. In a nutshell, linking the previous study with the results obtained from the current study, we can conclude there is an unknown compound that causes a delay in the hatching of the zebrafish embryos. Delay in hatching implies that the samples are toxic to zebrafish embryo.

A non-invasive method was used to determine the heart contractions of zebrafish. The heartbeat rate gives an insight into the effects of chemical exposure on the cardiac regulatory mechanism [14]. Zebrafish exposed to two out of thirteen coffee samples showed heartbeat rate between

the range 120-140 per minute which is similar to control group but the heartbeat ceases as the concentration increases; this could be because increase in concentration makes the coffee sample toxic to zebrafish embryo heart. Coffee contains the main ingredient caffeine which is a white crystalline xanthine acting as a stimulant drug that directly acts on the human central nervous system by increasing the heartbeat rate. It has been proved that caffeine causes a significant increase in heartbeat rates ($p < 0.05$) which were observed at 72 and 96 hpf [12].

On a different note, three coffee samples showed inconsistent data as the concentration increases. However, seven out of thirteen samples caused a heartbeat range below the normal value. On top of that another study on five-day-old larvae exposed to cocaine obtained similar results where the five-day-old larvae exposed to cocaine at different concentrations displayed a bell-shaped dose-response curve, tachycardia at lower doses and bradycardia at higher doses, showing a similar response to cocaine as humans [15]. Milan states that drugs that produce cardiac toxicity in human by inducing repolarization abnormalities consistently produced bradycardia in zebrafish [16].

As mentioned earlier humans and zebrafish share 70% of the homologous chromosomes and have similar heartbeat rates. Higher mortality was observed with samples 11 and 12. Slow *et al*. reported that bradycardia was seen in infants receiving steroid drugs [17].This can be related to a very similar case that occurred in 2017 where United States Food and Drug Administration (FDA) recalled Kopi Jantan Traditional Natural Herbs Coffee. FDA confirmed the presence of desmethyl carbodenafil that has similar structural properties as sildenafil, the active ingredient found in Viagra. FDA confirmed that it is a drug similar to sildenafil that is used to treat erectile dysfunction. On top of that, Kopi Jantan Traditional Natural Herbs Coffee also contains undeclared milk. This undeclared milk has the ability to interact with nitrates such a nitroglycerin that usually can be found in drugs that can lower blood pressure to dangerous levels.

Scoliosis also known as curvature of the trunk normally happens due to changes in myosin levels that lead to defects in somite formation [18]. However, in this current study, there was no scoliosis observed in zebrafish due to the 13 coffee samples. There is no documented evidence showing that caffeine can cause scoliosis in zebrafish. A previous study by Marques *et al.* [19] has shown that caffeine doesn't cause scoliosis of the zebrafish embryo. This was supported by Abdelkader *et al*. who found that no morphological alterations are induced by caffeine treatment [12]. Besides, Tsika *et al*. reported that steroids have no effect on myosin change [20].

Some drugs cannot be detected in products which are sold without proper labeling of contents, such as the coffee samples used in this research. The counterfeiting of drugs is detected by the FDA when the drugs contain ingredients different from the ones present in authentic drugs. Illegal manufacturers of herbal products such as herbal coffee may have included unapproved drugs that do not meet the required FDA standards in these products. FDA has banned several drugs that are actively being used in the market that lead to heart attack, non-fatal stroke and other related events [21]. Further studies to identify the compounds in coffee sachets should be done. Zebrafish toxicity test can be done as a screening test in food industry as this test is cost saving and can be done in a shorter period compared to rat toxicity studies.

CONCLUSION

Overall the coffee samples 11, 12, and 13 caused higher mortality, lower heartbeat rate, and low LC_{50} value compared to other coffee samples. This could be due to some contents in the instant coffee sachets that lead to toxicity. A detailed research should be carried out to determine these compounds in the coffee samples.

ACKNOWLEDGEMENT

The authors would like to thank Universiti Putra Malaysia (GP-IPS/2018/9639700) and Ministry of Higher Education (MoHE) for the assistance in funding.

CONFLICT OF INTEREST STATEMENT

The authors claim no conflict of interest regarding the publication of this paper.

REFERENCES

1. Higdon, J. V. and Frei, B. 2006, Critical Reviews in Food Science and Nutrition, 46, 101-123.
2. Parasuraman, S. 2011, Journal of Pharmacology & Pharmacotherapeutics, 2, 74.
3. Zhu, X., Zhu, L., Duan, Z., Qi, R., Li, Y. and Lang, Y. 2008, Journal of Environmental Science and Health, Part A, 43, 278-284.
4. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. 1995, Developmental Dynamics, 203, 253-310.
5. Hill, A. J., Teraoka, H., Heideman, W. and Peterson, R. E. 2005, Toxicological Sciences, 86, 6-19.
6. Zhang, C., Willett, C. and Fremgen, T. 2003, Current Protocols in Toxicology, 17, 1-7.
7. Nurminen, M. L., Niittynen, L., Korpela, R. and Vapaatalo, H. 1999, European Journal of Clinical Nutrition, 53, 831.
8. Klüver, N., König, M., Ortmann, J., Massei, R., Paschke, A., Kühne, R. and Scholz, S. 2015, Environmental Science & Technology, 49, 7002-7011.
9. Langheinrich, U. 2003, Bioessays, 25, 904-912.
10. Abdelkader, T. S., Chang, S. N., Kim, T. H., Song, J., Kim, D. S. and Park, J. H. 2013, Journal of Applied Toxicology, 33, 1277-1283.
11. Fraysse, B., Mons, R. and Garric, J. 2006, Ecotoxicology and Environmental Safety, 63, 253-267.
12. Abdelkader, T. S., Seo-Na, C., Tae-Hyun, K., Juha, S., Dongso, K. and Park, J. H. 2012, African J. Biotech., 11, 10816-10823.
13. Han, Y., Zhang, J. P., Qian, J. Q. and Hu, C. Q. 2015, Journal of Applied Toxicology, 35, 241-252.
14. Sarmah, S. and Marrs, J. A. 2016, Int. J. Mol. Sci., 17, 21-23.
15. De Luca, E., Zaccaria, G. M., Hadhoud, M., Rizzo, G., Ponzini, R., Morbiducci, U. and Santoro, M. M. 2014, Scientific Reports, 4, 4898.
16. Milan, D. J., Peterson, T. A., Ruskin, J. N., Peterson, R. T. and MacRae, C. A. 2003, Circulation, 107, 1355-1358.
17. Slow, H., You, C. and Hsu, D. T. 2008, J. Ped. Hema/onco., 30, 119-20.
18. Grimes, D. T., Boswell, C. W., Morante, N. F. C., Henkelman, R. M., Burdine, R. D. and Ciruna, B. 2016, Science, 352, 1341-1344.
19. Capiotti, K. M., Menezes, F. P., Nazario, L. R., Pohlmann, J. B., de Oliveira, G. M., Fazenda, L., Bogo, M. R., Bonan, C. D. and Da Silva, R. S. 2011, Neurotoxicology and Teratology, 33, 680-685.
20. Tsika, R. W., Herrick, R. E. and Baldwin, K. M. 1987, Journal of Applied Physiology, 63, 2128-2133.
21. Maryam, A. 2016, J. Clin. Toxicol., 6, 1-2.