



UNIVERSITI PUTRA MALAYSIA

***TOXICOLOGICAL EVALUATION OF COOKED KOREAN INSTANT
NOODLES***

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BY

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**A project submitted as a partial fulfilment of the requirement for the degree of
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Abstract

Toxicological Evaluation of Cooked Korean instant noodles

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The proportional mortality in Malaysia from non-communicable disease (NCDs) keep increasing and it was estimated to account for 74% of all death reported by Department of Statistic of Malaysia in 2017. There were many risk factors of NCDs, and one main driving force to the these risk factor could be unhealthy dietary choices, for examples, high and prolong consumption of fast food and processed food like instant noodles that keeping famous and trending in our community. The forecasting report on instant noodles market in Malaysia prove that it witnessed a growth of 7.2% from 2010 to 2017, and surely, this product getting high demand because of the purchasing power by our younger generations that mostly influence by the South countries like South Korean. Prolong and high consumption of Korean instant noodles might lead to certain level where the level might beyond the normal concentration that can lead to toxicity. This study conducted in order to determine and compare the toxicity in raw and cooked Korean instant noodles. The samples were basically prepared by hot aqueous extraction with the ratio of 1:10 and the samples involved raw noodles, raw seasoning powders and cooked Korean instant noodles while Paracetamol acts as positive control. These samples were prepared by serial dilution into 5 different concentration which are 62.5, 125, 250, 500, and 1000µg/mL and the treatment administration was 100µg/mL for each embryo. The parameters observations were conducted every 24 hours for 48 hpf, 72 hpf and 96 hpf. Lethal concentration further calculated by using Linear Regression Formula and it revealed that raw seasoning powders classified as in the slightly toxic category while for the cooked Korean instant noodles classified as nontoxic. However, when compared with the amount that we consume which is per one packaging, it is much more higher that the LC_{50} value, and the higher the LC_{50} value mean higher concentration of toxin/compound is required to kill the same population, and thus it is less toxic. In conclusion, raw seasoning powders is considered as slightly toxic compared to raw noodles and cooked Korean instant noodles, and becoming the major contribution for toxicity level in cooked Korean instant noodles as if it is combination of seasoning powder and noodles. Further study need to be conducted in order to study the factor that might influence the toxicity level of this sample.

Abstrak

Penilaian Toksikologi keatas Mee Segera Korea Yang Telah Masak

Muhammad Azmi Bin Mohamad A'sri

Kadar kematian di Malaysia dari penyakit tidak berjangkit (NCDs) terus meningkat dan dianggarkan menyumbang 74% daripada semua kematian yang dilaporkan oleh Jabatan Statistik Malaysia pada tahun 2017. Terdapat banyak faktor risiko NCD, dan salah satu pendorong utama untuk faktor risiko ini adalah lebih kepada pilihan makanan yang tidak sihat, contohnya, memakan makanan segera dan makanan yang diproses dalam kuantiti yang banyak dan dalam jangka waktu yang panjang seperti mi segera yang kian menjadi terkenal dan trend dalam komuniti masyarakat. Laporan ramalan mengenai pasaran mi segera di Malaysia merekodkan bahawa pasaran mee segera mengalami pertumbuhan 7.2% dari 2010 hingga 2017, dan sesungguhnya, produk ini mendapat permintaan tinggi kerana kuasa beli oleh generasi muda yang kebanyakannya dipengaruhi oleh negara-negara Selatan seperti Korea Selatan. Memakan makanan segera dan makanan yang diproses dalam kuantiti yang banyak dan dalam jangka waktu yang panjang berkemungkinan boleh membawa kepada tahap tertentu di mana tahap tersebut mungkin melebihi kepekatan normal yang boleh menyebabkan keracunan. Kajian ini dijalankan untuk menentukan dan membandingkan ketoksikan dalam mi segera Korea yang mentah dan dimasak. Sampel tersebut pada dasarnya disediakan oleh pengestrakan berair panas dengan nisbah 1:10 dan sampel yang melibatkan mi mentah, serbuk perasa mentah dan mi segera Korea yang dimasak sementara Paracetamol bertindak sebagai kawalan positif. Sampel ini disediakan oleh pencairan bersiri ke 5 kepekatan yang berbeza iaitu 62.5, 125, 250, 500, dan 1000 $\mu\text{g} / \text{mL}$ dan rawatan diperkenalkan pada isipadu 100 $\mu\text{g} / \text{mL}$ untuk setiap embrio. Pemerhatian kepada parameter dijalankan setiap 24 jam untuk 48 hpf, 72 hpf dan 96 hpf. Kepekatan maut selanjutnya dikira dengan menggunakan Formula Regresi Linear dan ia mendedahkan bahawa serbuk perasa mentah diklasifikasikan dalam kategori toksik sedikit manakala untuk mi segera Korea yang dimasak tidak toksik. Walau bagaimanapun, jika dibandingkan dengan jumlah yang kita makan yang setiap satu pembungkusan, lebih tinggi nilai LC50 dan jika nilai LC50 lebih tinggi bermakna konsentrasi toksin / kompaun yang lebih tinggi diperlukan untuk membunuh komuniti/penduduk yang sama, dan dengan itu ia diklasifikasikan kurang toksik. Kesimpulannya, serbuk perasa mentah dianggap sebagai sedikit toksik berbanding mi mentah dan mi segera Korea yang dimasak, dan menjadi sumbangan utama untuk tahap ketoksikan dalam mi segera Korea yang dimasak kerana ia adalah kombinasi serbuk dan mi rempah. Kajian lanjut perlu dilakukan untuk mengkaji faktor yang mungkin mempengaruhi tahap ketoksikan sampel ini.

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List of Abbreviation

Abbreviations	Page
NCDs : non-communicable disease	1
TBHQ : tertiary butyl hydroquinone	3, 11
LC₅₀ : lethal concentration	18, 20, 21, 23, 26, 28
EDTA : Ethylenediaminetetraacetic acid	11
ppm : part per millions	11
DNA : Deoxyribonucleic acid	11
Na : sodium	11
mg : miligram	11
OR : odd ratio	12
BPA : bisphenol A	12
MSG : monosodium glutamate	12
OECD : Organization for Economic Cooperation and Development	16
SEM : Standard Error of Means	19
RN : raw noodles	20, 21, 23,26, 28
RSP : raw seasoning powder	20, 21, 23,26, 28
CKIN : cooked Korean instant noodles	20, 21, 23,26, 28
HPf : hour post fertilization	20, 21, 23,26, 28
µg/mL : microgram per mililitre	20, 21, 23,26, 27, 28

Chapter 1 Introduction

1.1 Background of the study.

According to the World Health Organization (2013), the non-communicable diseases (NCDs) are growing health problem throughout the world and nowadays, the leading causes of death were mainly from cardiovascular disease, cancer and diabetes. In Malaysia, the proportional mortality from NCDs was estimated to account for 74% of all deaths (WHO, 2013).

According to the Department of Statistics Malaysia (2017), the principal cause of death in 2016 was ischaemic heart disease with rate of 13.2 per cent. Ischaemic heart disease also known as coronary heart disease or coronary artery disease which is the complication involved of the narrowing of heart arteries, this further causing less oxygenated blood delivered to some particulars organ in the body and can lead to heart attack in a long term effect with some particular symptoms of risk factors like hypertension, high cholesterol level and so on (Department of Statistic Malaysia, 2017).

This silent killer has becoming serious problem in our Malaysian community not only just causing of the increased total mortality but also give burden to the community by increased the health care cost. There are many risk factors of NCDs and one of main driving forces to the these risk factor could be unhealthy dietary choices, including increased consumption of fast food especially Korean instant noodles that getting famous in our community.

As economies of Malaysia keep into improvement, lifestyle of the citizen also keep changing which is everybody want to be quick in whatever matters. A busy career causing time constraint among the Malaysian community inducing the demand and consumption processed and fast food especially instant noodles which easy and quick to be prepared (Farzana et al., 2011)

Most of Asian country, noodles have becoming one of main food consumption and nowadays, the production of instant noodles have increase causing it's globally well-known recognized globally due to higher global intake (Sikander et al., 2017). Korean instant noodles consumption in Malaysia nowadays is getting increased and this product have given a big shots to the community social media atmosphere where most of the people are making the Korean instant noodles consumption challenge mostly known as Spicy Ramen Challenge (YouTube, Facebook, Instagram).

Previous study reported that the dietary pattern among population of Korean men that high intake of instant noodles, hamburger and pizza have the association to increase the prevalence if cardiometabolic risk factors (Lee et al., 2011).

1.2 Problem statement

Malaysia as a developing country have taken many initiative in economic strategy and the number of employment increased due to the expansion of industrialization in Malaysia while involved the employment from diverse socio-economic and culture. Generally, as our Malaysian keep growing their careers in the particulars industry or even pursue their education, their work schedule keep getting restrictive and these further influences on their dietary choices based on their daily life activities especially during working hours or peak hours, may not particularly healthy. For instance, this time constraint factor further cause them to have a mentality of thinking of take less time and more convenient by purchase processed food especially instant noodles which is currently well-known in our community with Korean instant noodles.

The prolong consumption of instant noodles may cause a various adverse health effect because of the concentration of dose of certain hazards related in instant noodles taken increase such as tertiary butylhydroquinone (TBHQ), sodium (Na), bisphenol A (BPA), monosodium glutamate (MSG), and propylene glycol. Various hazards related in instant noodles have been identified and the safety of this hazards is depends on the concentration of dose have been consume (refers to the Chapter 2). For example, consumption of high dose of tertiary butylhydroquinone (TBHQ) in the form butane in chemical preservative are related with sickness, incoherence, fall, tinnitus and retching (Sikander et al., 2017).

There are several studies have been done on the consumption of instant noodles such as instant noodles consumption with some parameters adverse health effect (Sil Huh et al., 2017; Park et al., 2016; Shin et al., 2014). However,

there is a limited studies have addressed the toxicological evaluation of Korean instant noodles. Although the study on the consumption of this product have been conducted, some of researchers have stated that it is also necessary to conduct a further study that focussed on the specific food item especially to know the concentration of the hazards substances (refers to the Chapter 2) as it might contribute to the toxicity reactions as if it exceed the limit of the normal concentration and this can further cause an affected biological system (Hamidi et al., 2014). Although there is study has been done on the toxicological evaluation of a novel umami flavour compound (Karenewsky et al., 2016) and the flavour ingredients (Karenewky et al., 2017), however, this study has not been in the case of Korean instant noodles and there are limited scientific evidence on the toxicity effect of Korean instant noodles and still at its infancy.

Therefore, more research on the toxicological evaluation of Korean instant noodles are needed because Korean instant noodles are getting popular instant noodles in our community that highly consumed by local people. However, there are lacking information on the toxicity of Korean instant noodles in which if the high and prolong consumption might have a possibilities could lead to health problems in a long term effects.

Although, there are several studies have been conducted to study the effect of consumption of instant noodles association with distinct cardio metabolic risk factors (Sil Huh et al., 2017; Shin et al., 2014) and also the study of association between instant noodles, processed food intake and dietary pattern with atopic dermatitis in an adult population (Park et al., 2016). But, those previous study were a cross-sectional study in which the information and

evidences on the toxicity data of the Korean instant noodles consumption are limited.

Therefore, the purpose of this study is to conduct in vivo study on the toxicological evaluation of Korean instant noodles by using Zebrafish method (*Danio rerio*) in order to give superior insights and provide information to the following research question:

1. Is there any significant difference of toxicity in sample of noodles only, flavour only, and mixture of noodles and flavour?
2. What is the dose of Korean instant noodles samples (noodles only, flavours only, mixture noodles and flavour) that could cause toxic effect?

1.3 Significance of study

Many studies regarding the prolong consumption of processed food product are easily available nowadays, even the studies regarding toxicological evaluation of the flavour or ingredients might have a discoveries but not much existing information regarding the toxicity of well-known instant noodles in our community which is Korean instant noodles . Therefore, the findings of this current study will provide information variation and some insights on toxicity in Korean instant noodles.

Moreover, the toxicological data of Korean instant noodles are really important and useful because it could help local communities to reduce their frequency consumption of Korean instant noodles to particular levels that can prevent from any adverse health effects happen that related to the toxicity of these fast food products. This is an important issue because the Korean instant noodles are among the most popular fast food products that highly consumed by local people and its getting an attention nowadays.

Apart from that, the finding from this study will give a significant contribution to the knowledge as it's provide information variations on the toxicity problem in Korean instant noodles to other researcher. In addition, advance understanding of toxicity in Korean instant noodles can be developing through this study as if it does can be a benchmark for others study for the fast food products.

This study will be conducted in vivo study to evaluate the toxicity of Korean instant noodles by using Zebrafish embryo (*Danio rerio*) method. For instance, the result and findings of this study will provide preliminary evidences on the toxicity of Korean instant noodles and can contribute to the body of knowledge for further research area by exploring more on possibilities of present toxic product. In addition,

the results of this study also can further develop to an early awareness to the community as well as further intervention information can be developed.

1.4 Objectives

1.4.1 General Objective

To determine the toxicological evaluation of raw and cooked Korean instant noodles.

1.4.2 Specific Objectives

- 1) To determine and compare toxicity in raw and cooked Korean instant noodles (noodles and seasoning powder) using Zebrafish acute toxicity test.
- 2) To determine and compare the lethal concentration (LC₅₀) of raw and cooked Korean instant noodles (noodles and seasoning powder).
- 3) To determine the morphological changes of Zebrafish embryo after treatment with raw and cooked Korean instant noodles (noodles and seasoning powder).

1.5 Null Hypothesis

- 1) There is no significant different of toxicity in raw and cooked Korean instant noodles (noodle and seasoning powder) using Zebrafish acute toxicity test.
- 2) There is no significant different of the lethal concentration (LC₅₀) of raw and cooked Korean instant noodles (ramen and seasoning powder).
- 3) The dose that beyond a normal concentration do not cause changes in morphological of the embryo

Chapter 2 Literature Review.

2.1 Instant noodles

The consumption of instant noodles among Asian populations is relatively high and in some way of some other country influences on our Malaysia culture especially Korea, for example, causing the increment demand for Korean instant noodles among our population. Usually this product was available in dried and precooked noodles block with the seasoning powders and flakes. Generally, Kim (1996a) states that instant noodles are partially cooked by steaming and further cooked, then dehydrate from various types of component and the composition ingredients of instant noodle are mainly made from wheat flour starch water, salt or kansui (an alkaline salt mixture of sodium carbonate, potassium carbonate, and sodium phosphate), and other ingredients that improve the texture and flavor of noodles.

As the time flies, the food industry revolution has taken place, this causing the menu improvement. Taneya et al. (2014) reported that instant noodles made from combination ingredients of flour, water and eggs and then crafted from unleavened dough into long thin piece of dough that is can be prepared from various types of components and usually cooked in soup or boiling water. As for Sikander et al. (2017), the main ingredients in instant noodles are flour, water, salt, starch and additional substitute of salt (kansui) and usually produced by using a blend of wheat flour and other flour.

2.2 Demand of instant noodles worldwide and Malaysian market particularly

According to the World Instant Noodles Association, instant noodles that were eaten in 2018 were reported about 103.6 billion servings and it was account as 280 million servings are eaten per day. This association also states that nearly 80% of instant noodles were consumed were basically from 10 countries out of 15 were from Asia. However, there was limited study and information conducted on the real statistics of consumption of Korean instant noodles among Malaysian community. Instant noodles market in Malaysia witnessed a growth of 7.2% from 2010 to 2017 (Instant Noodles Industry in Malaysia: Analysis of Growth, Trends and Forecast 2017 - 2022) and this happen due to the purchasing power among younger generation in our community caused this product getting high demand in our country.

2.3 Nutrient composition in instant noodles

The primary constituents for instant noodles were basically from flour, starch, water, salt and additionally a salt substitute (Sikander et al., 2017).

2.4 Toxicity testing

Toxicity research on laboratory organisms was conducted to evaluate and determine the chemical substances such as medicines drug, industrial and agriculture chemical, and food additives in term of its potential to cause problem of adverse effect for living things. Toxicity testing will lead to improve the availability information so that it can serve as crucial part of public health actions so that further regulation or decision towards concerning of toxic chemicals can be comply. The result from animal testing of toxicity study can be used to predict health effects for human with take into account of the related assumptions and extrapolations. (Nurain, 2010).

However, there's still limited study conducted on toxicity screening for instant noodles or similar samples

2.5 Examples of other available toxicity study and methods

According to The National Academics of Sciences Engineering Medicines, there were several methods that available for toxicity testing which are developmental toxicity, reproductive toxicity, carcinogenicity, neurotoxicity and mutagenicity. This study was include in the developmental toxicity in which its end point involves the mortality, growth retardation, skeletal variations, gross external malformations, defects of soft tissue/internal organs. Examples of the developmental toxicity study are Zebrafish acute toxicity test and Brine Shrimp acute toxicity test (just to name a few).

2.5.1 Zebrafish (*Danio rerio*)

Zebrafish (*Danio rerio*), are used widely as model organisms for biomedical research and also known as a small freshwater fish. These type of fish have high degree of genetic conservative with human which is 70 per cent of genetic structure in Zebrafish similar with human genetic structure and geneticist found that about 84 per cent of Zebrafish genes counterpart known to be associated with human disease. The Zebrafish embryo development and viability enhances a good and easier examination of the internal structures to conduct because of its transparency characteristic so that this make it more ideal model organism for studying early development. The Zebrafish are fast in growing due to its life cycle and the maintenance cost is cheap. (JoVE Science Education Database, 2018).

2.6 Hazards related with prolong consumption of instant noodles.

2.6.1 Tertiary butylhydroquinone (TBHQ)

According to Sikander et al. (2017), TBHQ used widely in food industry as the chemical preservative for the food products and butane form is the usually form that being used. Its usage in the food product can deferment the rancidity onset and longer the food's shelf life. The amount of TBHQ that permitted by FDA to be in food is up to 0.02% of the total oils. High dosages consumption of TBHQ in the proximity around of 1g and 4 g can cause retching, tinnitus (ringing in the ears), incoherence, fall and sickness. In research facility creatures, it showed that high dosages of TBHQ in a long period of time have tendency to induce malignant antecedents in the stomach and further DNA damage (Choy et al., 2012). The mixture of TBHQ (200ppm) and disodium EDTA (500 ppm) in the frying oil quintupled the usability time span. The usage of the inside surface of TBHQ (200ppm) prolonged the usability time span two times of an similar proximate TBHQ measure in frying oil (Choy et al., 2010).

2.6.2 Sodium (Na)

Sikander et al. (2017) stated the range of 821 mg of sodium substance was observed for each 100 g in which, it was above points of confinement set by the British sustenance wellbeing organization and about 30 percent of the aggregate sodium intake contributed by instant noodles utilization. Chang (2006) revealed that the sodium levels in instant noodles need to decrease as it contains 20% of sodium content which was about 350 mg. According to The Toyama Birth Cohort Study in Japan, the habitually consume instant noodles among middle school understudies which was more than 3 days per week showed a increase hazard for a lower personal satisfaction from the age of 3 (OR: 1.49; P=0.007) (Wang et al., 2008). Park et al.

(2011) also stated that the inclination of salty sustenance among youngsters leads to fortify and sustain this inclination in their future and programs that related to suitable sodium utilization must be conducted.

2.6.3 Bisphenol A (BPA)

The Journal of Nutrition have reported a review according to information from the Korean National Health and Nutrition Examination Survey from 2007-2009 regarding the inspection of the in Styrofoam compartment used of a few brands of instant noodles that they found compound of bisphenol A (BPA) and Yang et al. (2003) reported that body's hormones especially the female sex hormone estrogen interfere by BPA.

2.6.4 Monosodium Glutamate

Sikander et al. (2017) stated that the issue of contains high measures of monosodium glutamate (MSG) was commonly known related with instant noodles and its seasoning powder and an unfavourably susceptible response can be trigger by MSG in about 1 to 2 per cent of the populace. Consuming sensations, cerebral pains, trunk and facial torment are the common symptoms for the people who are oversensitive when consume high of MSG (Liu et al., 2008).

2.6.5 Propylene Glycol

A liquid catalyst property known as Propylene Glycol recognized to be available in instant noodles which is act as to hold the dampness of the instant noodles in order to prevents it's from drying but our body effortlessly consume this compound because it will amasses in the kidneys, heart and liver (Sikander et al., 2017). Consistent finding found by Kim et al. (2016) that stated that this compound can differ the standard of norm in our body and further harm the indicated ranges.

2.6.6 Adverse health effect that associated with prolongs and over consumption.

Park et al. (2016) found that the consumption of instant noodles and meat and processed foods was associated with increased prevalence of atopic dermatitis in adults. Consistent finding of adverse health effect found by Sil Huh et al. (2017) reported that college student who consumed instant noodles frequently were more likely to have multiple cardiometabolic risk factors ($P < 0.0001$) and Shin et al. (2014) stated that the consumption of instant noodles more than 2 times/week was associated with a higher prevalence of metabolic syndrome in women but not in men.

Chapter 3 Methodology

3.1 Reagents and chemicals

This study do not involves any harmful reagents and chemicals apart from distilled water as the medium for dissolving the samples for preparation of the concentration. This factor can enhance the validity of the result as if no other reagents or chemical that can influence the toxicity effect on the embryos.

3.2 Equipment and apparatus

Cooking set (including stove and cooking temperature), plastic wrap, freeze dried vacuum pump, grinder, test tube, Falcon tube, well plates, conical flask, magnetic stirrer, centrifuge machine, Whatman filter paper no. 1, weighing balance, dropper, beaker, tissue paper, gloves and black sheet.

3.3 Sample collection

The latest arrived stock of Korean instant noodles was obtained from local market at Selangor. The non-probability convenient sampling will be conducted in determine the local market and the types of flavour for purchasing the samples.

3.4 Sample Preparation

In this study, it's required three different samples which were raw instant noodles, raw seasoning powder, and cooked Korean instant noodles. Cooked Korean instant noodles basically prepared according to the cooking method that have been set by the manufacturer on the packaging. For the raw samples (noodles and seasoning powder), it proceed with freeze dried process directly same goes with cooked Korean instant noodles. All samples were started freeze dried at the same time for at least 3 days in order to get a constant period of time. Then, the sample

will be cut into small pieces then further with grinding using heavy duty grinder into much more smaller pieces so that it can increase the surface area of the samples until fine powder obtained.

3.5 Preparation of Hot Aqueous Extraction

After the samples were freeze dried, the preparations of hot aqueous extraction according to the sample were proceed. The hot aqueous extractions of the samples were prepared by decoction. The method of extraction was modified according to Cheurfa & Allem, 2015, Meneguetti et al., 2014 and Tan et al., 2014.

The extract of the samples were prepared by dissolved 5 g of each sample into 50 ml of deionized water (distilled water). The ratio concentration of this extraction was 1:10. In order to achieve an optimal extraction, the extractions incubate at 80°C for 10 minutes (Meneguetti et al., 2014). Then, the extraction will be filtered in order to remove or separate the supernatant from the insoluble sediment by using Whatman filter paper No. 1. The filtrate proceed with freeze dried process at range temperature of -80°C to -20°C for certain particular period so that the powdered extract obtained (Azwanida, 2015), and further can be stored at 4°C in the chiller. As for the further analysis, the specific dose for administration in the study was prepared by dissolved the specific amount of prepared extract in distilled water.

3.6 Experimental Animals (Zebrafish Embryo)

3.6.1 Zebrafish Embryo Acute Toxicity Test.

The zebrafish embryo acute toxicity test was conducted and adopted according to the guidelines from Fish Embryo Acute Toxicity (FET) Test No. 236 from Organization for Economic Cooperation and Development (OECD 203) with some modifications. The species embryo of zebrafish that used in this study was

Danio rerio. The zebrafish embryo maintained in aquatic condition according to the guidelines with water temperature of $26.0^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ and good water quality in terms of oxygen saturations, total organic carbon, residual chlorine, water purification and so on.

The zebrafish embryo acute toxicity tests conducted to explain and describe the adverse effects of the sample extraction that arises from a single exposure or from multiple exposures within a short period of time further give the determination value of Lethal concentration (LC_{50}). The newly fertilised embryonic zebrafish eggs introduced with the different concentrations of the sample extraction for 96 hours and four observations recorded in every 24 hours.

3.7 Embryo handling and allocation

The 40 eggs were transferred to the test solutions at latest 60 minutes after light had been turned on to initiate spawning. Fertilized eggs were separated from the non-fertilized eggs with a pipette using a stereo microscope and allocated in the 24-well-plates (1 embryo per 1 well plate). 20 fertilized eggs were allocated individually into 20 well plates that contain 2 ml of the test solutions to exclude mutual influences. The remaining four wells of each plate were used as internal control filled with distilled water and allocated one fertilized egg in each well. The plates then were sealed and the 24-well-plates were covered with a self-adhesive foil and incubated at $26^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

By using a dissecting microscope, lethal, sublethal and teratogenic endpoints were recorded within 48h. If 90% of the embryos in the control treatments showed neither sublethal nor lethal effects, a test was classified as valid.

3.8 Observation on the parameters

3.8.1 Morphological Observation

There were three observation conducted in this study. First was scoliosis observation which is based on the coagulation of fertilised eggs, failure of somite formation and non-detachment if the tail-bud from the yolk sac. Second observation was by recorded the Zebrafish heart rate by using a microscope. Lastly, the lethal concentration (LC_{50}) calculated based on mortality of the half of the Zebrafish population used in the study.

Observations conducted every 24 hours until it reaches 96 hours of exposure and the result was recorded. The number of coagulated embryos will be determined after 48, 72 and 96 hours of exposure. Meanwhile, non-somite formation will be detected using microscope at latest after 48 hours of exposure. At 24, 48, 72, and 96 hours of exposure, the non-detachment of the tail will be recorded. Last but not least, the heartbeat will visible after 48 hours of exposure. Therefore, the absence of heartbeat will be recorded after 48, 72 and 96 hours of exposure (Busquet et al., 2014).

3.8.2 Determination of lethal concentration (LC_{50})

Determination of lethality was according to the Linear Reggression Formula and Probit Analysis by using Microsft Excel 2010. To determine the lethal concentration the percentage of lethality for every concentration was used to get the probit value and the concentration value was change to $\log_{10}$ concentration. Graph of sampes concentration versus percentage of lethality was plotted for the Linear Regression Formula, while for the Probit Analysis, the $\log_{10}$ concentration and probit value for each concentration was used in order to get the regression result.

3.9 Statistical Analysis

In this study, IBM SPSS software version 22 will be used to perform all the statistical analysis. The results obtain from the analysis will be expressed in the form mean $\pm$ standard deviation. One way ANOVA (Tukey test) was applied to determine the significance effect of the sample dose on the parameters. Results were considered significance if $p < 0.05$.



Chapter 4 Results and Discussion

The parameter data that were observed every 24 hours for 48 hpf, 72 hpf and 96 hpf were analysed and standard error of means (SEM) reported as below. Briefly, this study conducted by introduced the different concentration of samples treatment to the Zebrafish embryos. The treatment administrations were introduced after 24 hpf of 100µL per embryo for each well plates and the parameters were observed every 24 hours after.

As for the first objective which is to determine and compare toxicity in raw and cooked Korean instant noodles (noodles and seasoning powder) using Zebrafish acute toxicity test, the result discussed in term of percentage of lethality, number of heartbeat and percentage of hatching rate.

Lethality is measured by a lack of living process (Plant Growth and Development, 2002) and in this study, the percentage of lethality was observed by the coagulation of the embryos. The coagulation was observed the dark and rupture of the embryos organ under the microscope observation.

4.1 Percentage of Lethality of the Zebrafish embryos

Table 1 below shows the percentage of lethality for different samples at 1000µg/mL.

Table 1: Percentage of lethality for different types of samples at 1000µg/mL.

Sample	Lethality (%)		
	48hpf	72hpf	96hpf
RN	16.67±9.62 ^a	16.67±9.62 ^{ab}	16.67±9.62 ^a
RSP	0.00±0.00 ^a	33.33±19.25 ^{ab}	66.67±0.00 ^b
CKIN	33.33±19.25 ^a	50.00±9.62 ^b	50.00±9.62 ^b
Paracetamol	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a

Result was expressed as mean±SEM (n=4). Mean with different letter between samples are significant at p<0.05. Paracetamol was used as positive control. Noted that RN, raw noodles; RSP, raw seasoning powder; CKIN, cooked Korean instant noodles; hpf, hour post fertilization.

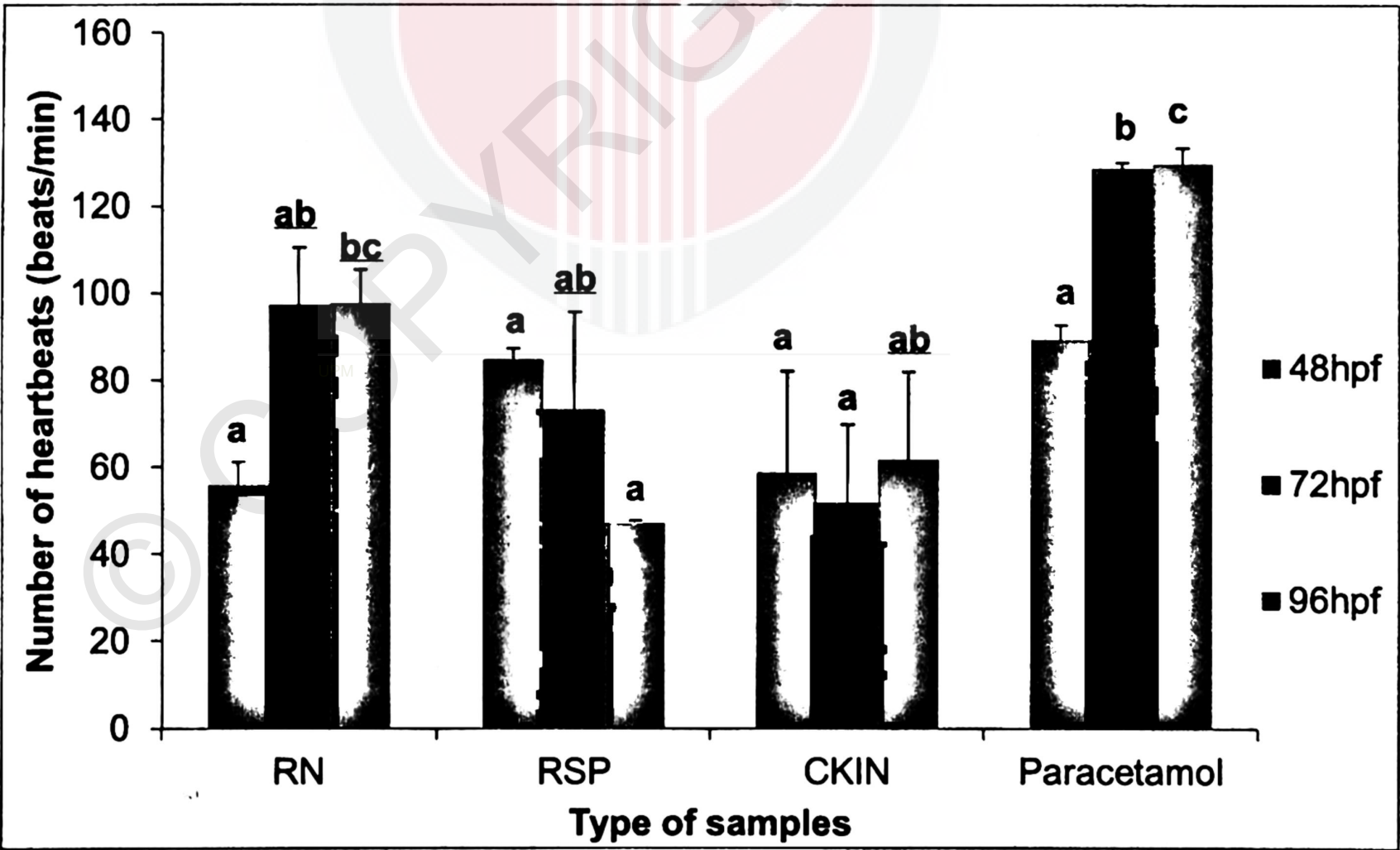
As shown in the Table 1, cooked Korean instant noodles recorded the highest percentage of lethality for 48 hour post fertilization (hpf), and 72 hpf, however, the result was only significant for 72 hpf while for the 96 hpf, raw seasoning powder reported significantly higher compared to other samples. The lethality effect caused the coagulation to the embryo indicates that the samples have the possibilities cause of toxicity effect to the embryos. Based on this result, all the samples still considered as toxic because of the purpose of its administration still cause a lethality effect on the embryo.

4.2 Number of heartbeats of the Zebrafish embryos

Next parameter observed was number of heartbeats of the embryos. The heartbeats of the embryos was observed as indications for the toxicity because the heartbeats shows the breathing capability of the embryos, which is in other words, if the embryos have the lower heartbeats meaning that the embryos have the breathing difficulty and it was further discussed that it does happen because of unsuitable environment for survival of the embryo that lead to the coagulation of the embryos. This also consider as toxic condition.

Figure 1 below show number of heartbeats of the Zebrafish embryo at 1000µg/mL of different samples treatment.

Figure 1: Number of heartbeats of the Zebrafish embryo at 1000µg/mL of different types of samples at 1000µg/mL



Result are expressed as mean±SEM (n=4). Bar with different letter between samples are significant at p<0.05. The number of heartbeats were calculated within 15 second. Paracetamol was used as positive control. Noted that RN, raw noodles; RSP,

raw seasoning powder; CKIN, cooked Korean instant noodles; hpf, hour post fertilization.

From the Figure 1, there was no significant result observed for raw noodles for 48 hpf, 72 hpf, and 96 hpf, but for the raw seasoning powder, there was significantly different between the numbers of heartbeats for 96 hpf compared to other samples, and the number of heartbeats from 48 hpf to 96 hpf for raw seasoning powder recorded a decreasing pattern. For the cooked Korean instant noodles, there were significant different between the number of heartbeats for 72 hpf and 96 hpf compared to other samples, and the heartbeats pattern for cooked Korean instant noodles was quite stagnant. The normal range for Zebrafish embryo heart rate was at 120–180 beats per minute, much closer to that of humans (Luca et al., 2014 & Baker et al., 1997) and this heartbeats range were observed in the control group that treated with Paracetamol as positive control. From this result, the raw seasoning powders and cooked Korean instant noodles can be considers as toxic because the numbers of heartbeats recorded in that particular samples were significantly low compared to raw noodles (not significant) and control.

4.3 Hatching Rate of the Zebrafish embryo.

Apart from that, this study also observed on hatching rate of the embryo in which to observed the development and viability of the embryo. In the embryology terms, hatching refers to the separation process of blastocyst (embryo) from the zona pellucida or known as embryo sacs (Medical Dictionary, 2009). The higher the hatching rate observed indicates that the condition was good for the development and viability of the embryos meaning that the embryo was alive. For instance, the lower heartbeat indicates low development and viability of the embryo, so, this condition consider as toxic for the embryo.

Table 2 below show the percentage of hatching rate of the Zebrafish embryo for different samples at 1000µg/mL.

Table 2: Percentage of hatching rate of the Zebrafish embryo for different types of samples at 1000µg/mL.

Sample	Percentage of hatched embryo (%)		
	48 hpf	72 hpf	96 hpf
RN	0.00±0.00	16.67±9.62 ^a	66.67±0.00 ^{ab}
RSP	0.00±0.00	33.33±0.00 ^a	33.33±0.00 ^a
CKIN	0.00±0.00	16.67±9.62 ^a	33.34±19.25 ^a
Paracetamol	0.00±0.00	16.67±9.62 ^a	100.00±0.00 ^b

Result was expressed as mean±SEM (n=4). Mean with different letter between samples are significant at p<0.05. Paracetamol was used as positive control. Noted that RN, raw noodles; RSP, raw seasoning powder; CKIN, cooked Korean instant noodles; hpf, hour post fertilization.

From the Table 2, there was no percentage of hatched embryo recorded for 48 hpf because of the period of exposure still early and to soon to observed the hatching rate. For the 72 hpf, raw seasoning powder recorded the high percentage of hatching rate, however there were no significantly different of the percentage of hatching rate between the samples. For the 96 hpf, raw noodles recorded the highest percentage of hatched embryo while raw seasoning powder recorded the lowest hatching rate after cooked Korean instant noodles however the result was significant for raw seasoning powder and cooked Korean instant noodles only.

From this result, raw seasoning powders and cooked Korean instant noodles considered as toxic because of the lowest hatching rate reported and this finding

consistent with previous study in which found that instant noodles do not promote growth because it has low protein and high carbohydrate content (Zailani et al., 2016).

4.4 Lethal Concentration of Raw and Cooked Korean Instant Noodles.

There were limited study reported on the specific food samples toxicity that related with instant noodles using Zebrafish acute toxicity test that can be used as the guidelines for the toxicity level for lethal concentration in this study. For instance, previous study reported on antiplasmodial activity of plant extracts that used to study drug and herbs toxicity, and it was assessed using Lactate Dehydrogenase Assay (pLDH) and the cytotoxicity estimated according to the specific organ cells, e.g.; LLC-MK2 monkey kidney epithelial cells (Zofou et al., 2011).

Table 3 below shows the toxicity classes according the value of LC_{50} as comparison with the samples used in this study.

Table 3: Toxicity classes according to the value of LC₅₀ as comparison for the consumption. (Clarkson et al., 2004)

Toxicity Classes		
LC ₅₀	>1000µg/mL	Nontoxic
	<1000µg/mL	Toxic
	500-1000µg/mL	Slightly toxic
	100-500µg/mL	Moderately toxic
	0-100µg/mL	Very toxic

According to Table 3, the toxicity category was classified according to the value of LC₅₀ of the samples. If the LC₅₀ was more than 1000µg/mL, then it was considered as nontoxic, but if the LC₅₀ value was less than 1000µg/mL, it was considered as toxic. The lowest the LC₅₀ value, it caused the increase the toxicity level. This is happen because of the smallest concentration only that required to kill half of the population that been studied.

Table 4 below show the lethal concentration, LC₅₀ for different samples considering all the concentration used in this study which were 62.5,125, 250, 500, and 1000 µg/mL by using Linear Regression Formula.

Table 4: Lethal concentration, LC₅₀ for different type of samples

Sample	Lethal Concentration, LC ₅₀ (µg/mL)			Classification category for LC ₅₀ value
	48 hpf	72 hpf	96 hpf	
RN	ND	ND	ND	
RSP	ND	ND	843.68	Slightly toxic
CKIN	ND	1094.29	1094.29	Nontoxic
Paracetamol	ND	ND	ND	

Result was expressed as mean±SEM (n=4). Paracetamol was used as positive control. Noted that RN, raw noodles; RSP, raw seasoning powder; CKIN, cooked Korean instant noodles; hpf, hour post fertilization; ND, not detected.

According to the Table 3 above, raw seasoning powder was classified as slightly toxic as the LC₅₀ value is 843.68µg/mL which was this value within slightly toxic categories while for the cooked Korean instant noodles classified as nontoxic because its LC₅₀ value more than 1000µg/mL which was 1094.29µg/mL. As for the raw noodles, the result was not detected because of the concentration that used in this study was low to cause the toxic effect. LC₅₀ value was referring to the concentrations of the chemical that kills 50% of the test animal during the observation period (Canadian Centre for Occupational Health and Safety, 2019). So, from this estimated LC₅₀ value, it can be the borderline for further safety consumption.

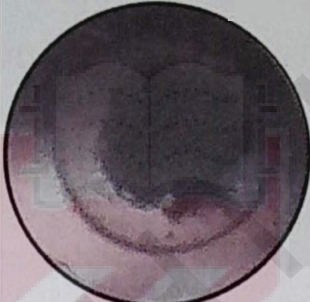
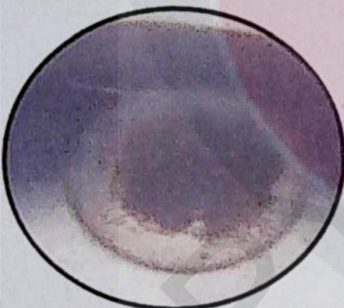
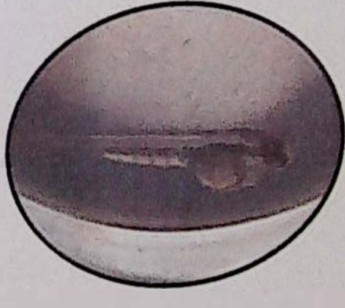
The LC_{50} value for the raw seasoning powder is $843.68\mu\text{g}$ was the weight in term of the samples from hot aqueous extraction which is this weight was the weight that undergo to time of freeze dried process that were during raw dried sample ad during hot aqueous extraction. So, if the value was change to the real food amount, it was accounted for about 3 mg. Furthermore, the higher the LC_{50} value mean higher concentration of toxin/compound is required to kill the same population, and thus it is less toxic, so when comparing with the weight for one set raw seasoning powder of the packaging which was 32.75g, meaning that we consume more than the LC_{50} value, so can consider that the amount that we consume is less toxic.

As for the cooked Korean instant noodles, the LC_{50} value was $1094.29\mu\text{g/mL}$ was considered as nontoxic and if further change the borderline value which was $1000\mu\text{g/mL}$, it might get about 20.987mg and when compared with the weight of cooked Korean instant noodles that we consume which is 266.98g for one packaging, then the amount that we consume higher than the LC_{50} value, so that it can be consider as nontoxic for consumption because the higher the LC_{50} value mean higher concentration of toxin/compound is required to kill the same population, and thus it is less toxic.

4.5 Morphological Changes of the Zebrafish embryos.

Figure 2 below show the examples of parameters of morphology changes that been observed in this study for different type of samples at $1000\mu\text{g/mL}$.

Figure 2: Parameters of morphological changes of Zebrafish embryo for each sample at 1000µg/mL

	48 hpf	72 hpf	96 hpf
RN	 Noncoagulated and not hatched embryo	 Noncoagulated and not hatched embryo	 Noncoagulated and not hatched embryo
RSP	 Noncoagulated and not hatched embryo	 Coagulated and not hatched embryo	 Coagulated and not hatched embryo
CKIN	 Coagulated and not hatched embryo	 Coagulated and not hatched embryo	 Coagulated and hatched embryo
Paracetamol	 Noncoagulated and not hatched embryo	 Noncoagulated and not hatched embryo	 Noncoagulated and hatched embryo

Paracetamol was used as positive control. Parameters were observed under inverted microscope with magnification of 10X. Noted that RN, raw noodles; RSP, raw seasoning powder; CKIN, cooked Korean instant noodles; hpf, hour post fertilization.

The morphological changes of the embryo were observed according to the noncoagulation, hatching rate and number of the heartbeats. This morphology viability of the embryo were observed basically if the embryo still clear or manage to observe the organ inside it, then it considered as have noncoagulate and have a good viability rather than if the coagulated embryo, it was observed as dark spot or ruptured of the embryo organs/morphology.

Based on the Figure 2 and other morphological changes observation, raw seasoning powder and cooked Korean instant noodles consider as toxic because of its administration mostly causing the ruptured on morphology of the embryo.

Chapter 5 Conclusion, Limitation and Future Recommendation

An experiment was done to study the toxicological evaluation of raw and cooked Korean instant noodles using Zebrafish acute toxicity test by using different concentrations (62.5, 125, 250, 500, 1000 μ g/mL) for each samples that used. The percentage of lethality revealed that every sample from Korean instant noodles still considered as toxic due to the lethality effect on the embryos. Furthermore, the number of heartbeats and percentage of hatching rate reported that only raw seasoning powders and cooked Korean instant noodles influence on providing the toxicity level because of lower numbers of heartbeats and hatched embryos.

However, for the lethal concentration, raw seasoning was the only that classified as slightly toxic compared to cooked Korean instant noodles and this further concluded that raw seasoning becoming the major contribution for toxicity level of cooked Korean instant noodles as if the cooked Korean instant noodles is a combination of cooked noodles and seasoning powders. The amounts of consumption still need to be considered as if these food products have lethality effect observed as in this study.

As for the limitation in this study, the amount of seasoning powder in cooked Korean instant noodles lesser due to some of its component loss during the mechanical process (e.g.; cooking process, transferring process, handling process) influence the toxicity level. The cooking process also takes a big role in influence the toxicity level because certain component might denatured or broken down that have the possibility to influence either to inhibit any other compound or to facilitate other compound that have the role to increase or decrease the toxicity level. The experiment also involves a movement of the well plates in which the experiment conducted in other place while the observation was conducted another place that

involves different specification of the environment, so this might give the stress effect on the embryos and this further might influence the result. Apart from that, this study dealing with an oily samples especially raw seasoning powder and cooked Korean instant noodles, so the purpose of administration of the samples treatment into the embryos have the possibility to have a layer of oil in the well plate that can prevent the embryo from getting the oxygen gases for respiration process.

As for the recommendation, further study need to be conducted by using a different range or higher concentration between the samples or by using organism testing (e.g.; rodent) in order to confirm the toxicity value that give the significant toxicity effect on a particular organ. Increasing the repetition of triplicate into five times double for each concentration and increase the range period of observation while observe on much more parameters also can improve the validity of the result

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Appendixes

Figure A: Conversion of water extracts into the real food amount for the raw seasoning powder

From hot aqueous extraction into raw dried sample	5g 843.68µg	→ 2.8212g → $\frac{843.68\mu g}{2.8212g} \times 5g = 1495.25\mu g$
From raw dried sample into real food	32.7482g 1495.25µg	→ 16.281g → $\frac{1495.25\mu g}{16.281g} \times 32.7482g = 3007.6\mu g$ or 3.0076 mg

➤ LC50 value for real food is about 3mg

The higher the LC50 value mean higher concentration of toxin/compound is required to kill the same population, and thus it is less toxic.

➤ Compared with 32.75g of raw seasoning powders that being provide in one set of packaging

Figure B: Conversion of water extract into real food amount for cooked Korean instant noodles

From hot aqueous extraction into raw dried sample	5g → 0.589g
	1000µg → $\frac{1000\mu g}{0.589g} \times 5 = 9289.389\mu g$
From raw dried sample into real food	266.983g → 118.173g
	9289.389µg → $\frac{9289.389\mu g}{118.173g} \times 266.983\ g = 20987.103\mu g$ or 20.987mg

➤ Real food amount as borderline is about 20.987mg

The higher the LC50 value mean higher concentration of toxin/compound is required to kill the same population, and thus it is less toxic.

➤ Compared with 266.98g of the cooked Korean instant per packaging