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EVALUATION OF ACUTE TOXICITY OF AN AQUEOUS EXTRACT OF IRRADIATED *LABISIA PUMILA* ON ZEBRAFISH EMBRYO (*DANIO RERIO)*

Santhra Segaran Balan [1], Punithaamalar Manokaram [1], Norshafarina Shari [1], Mahathir Mohd Uzid [1], Hasnah Bahari [2], Muhammad Danial Che Ramli [1], Azrina Zainal Abidin [1], Mahardian Rahmadi [3], Arapoc Daryl Jesus [4]*

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Abstract

This research aimed to compare the toxicity effect of non-irradiated and irradiated Labisia pumila at a different dosage of 3, 6, 9 and 12 kilogray (kGy). Different irradiated dosages of L.pumila were prepared using Cobalt-60 gamma irradiation and the acute toxicity were assessed through zebrafish (Danio rerio) embryo. The survival rate, hatching rate, heartbeat rate and scoliosis were observed. Data were analyzed using SPSS 25.0 windows. The lethal dose (LC_{50}) value was calculated. The LC_{50} value of non- irradiated extract L. pumila is 125 µg/ml compared to irradiated extract is 62.5 µg/ml respectively. Hatchability of zebrafish of L.pumila extract reduce in the higher concentration for non-irradiated sample at 250 µg/ml and for irradiated sample at 125 µg/ml. Presence of scoliosis not observed in all concentration for irradiated and non-irradiated sample.

The heartbeat of zebrafish embryo treated with irradiated L. pumila extract (0- 62.5 µg/ml) was within the normal range (120-180 bpm for all doses), but at higher concentrations (125 µg/ml) the heartbeat differs from normal ranges for all the doses. From this time forward, irradiated and non-irradiated of this plant was safe to be consumed due to its pharmaceutical effect but it still exhibited mild toxicity effect on zebrafish embryo. The diverse irradiated doses show a change of toxicity level of this plant which higher doses show mild toxicity to the zebrafish embryo compared to low doses exposure.

Keywords: *Labisia pumila, irradiation, zebrafish, heartbeat, hatching*

Introduction

Herbal medicine is known as a complementary medicine which has high opportunity as a medicinal drug that's broadly used in many develop countries worldwide.

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Use of herbal medicine became high demand among people because human beings agree that herbal medicine has a useful compound which is help on cure many disease with less side effect compared to modern medicine. The main difference between modern medicine and herbal medicine is that modern medicine is chemically created and develop and has been scientifically proven the effectiveness by research trial. However, herbal medicine doesn't have prominent research evidence on the effectiveness in human and the side effect as well [1, 2]. This is because of the harmful compound that may be typically produced from seeds, plants, and micro-organisms in plant/herbs. This compound can be as a radioactive agent which will converts the poisonous substance widely, especially through direct contact or through various forms of transmission. Toxicology study are very important to identify the adverse effect and side effect of this herbs to human. Study on toxicity such as tissue culture and zebrafish can prove the safety usage of herbal medicine [1].

In Malaysia, *Labisia pumila* (Myrsinaceae) is referred as Kacip Fatimah. It could be found widely throughout Indochina's rainforest, especially in Malaysia. It develops rapidly *L. pumila var. alata*. Different species are distinguished by its characteristics of the petiole and the leaf *L. pumila var.alata*. It has a winged petiole with red veins, while *var.pumila* has a marginate petiole with ovate leaf blade shape. However, the var.lanceolata has a long and non-winged petiole. The *var. alata* is more frequently used in traditional medicine preparations [3].

Usage of herbal medicine become high and due to the availability on the markets in the form of tablets or drinks. This high demand on herbal medicine due to negative perception of modern medication among people. Therefore, *L. pumila* is functioning, in particular to detect bioactive phytochemicals that contribute to the pharmacological properties. A few phytochemicals from the *L. pumila* extract have been described. Most are phenolic compounds, including flavonoids and phenolic acids in this plant believe will help in preventing the cancer [3].

Gamma radiation is the physically actuated pressure found to be the rapid and effective technique for improving the quality and quantity of plant attributes and has been frequently used in an elicitor in plant science as impacts from low portion incitement to high portion

disability. Bioactive mixtures, for example, maybe triggered under a sufficient portion of radiation under phenolic and flavonoids. Gamma illumination applies its mechanisms by inducing oxidative stress in the cells which increases the phenolic due to the introduction of phenolic mixes [4]. The purpose of this study is to assess the toxic effects of aqueous extract of non-irradiated and irradiated *L. pumila* in zebrafish

Methodology

Sample Preparation

Gamma irradiation is carried out at room temperature in a cobalt- 60 irradiator available at Malaysia Nuclear Agency (Science, 2008). The powder material of *L. pumila* were irradiated with different doses of 3, 6, 9 and 12 kilogray (kGy). The non-radiated plant was used as control for the experiment [5]. For extraction method, *L. pumila* extraction was prepared using a hot aqueous method. 50g of *L. pumila* was put into a Scott bottle containing about 500 ml of distilled water and well mix. The diluted *L. pumila* in the conical flask. Following 24 hours, the mixture was separated utilizing Whatman filter paper No 1 and the filtered sample was transferred into falcon tube before freeze drying. The collected aqueous extract powder of *L.pumila* was stored in the fridge at -40^{o}C [6].

Serial Dilution

The serial dilution of the hot aqueous extract was prepared by mixing the 0.1 g of *L. pumila* hot aqueous extract with 1000 µl of distilled water in a conical flask. From the 100% solution mixture, six successive series of concentration were produced which were 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml and 15.625 µg/ml. As for the control treatment, distilled water used as negative control and positive control is paracetamol [1, 2].

Zebrafish Embryo Acute Toxicity Assay

The healthy embryo was transferred into each of the 96 well plates using a pasteur pipette at 24 hours post-fertilization. The treatment was given by pipetting 200ul of a diluted sample into 96-well plates based on different series concentration [1]. The assurance of the endpoint of toxicity was through the assessment of mortality, hatching rates, heartbeat rates, and scoliosis. The rates of mortality, hatching and heartbeat as well as scoliosis were observed and recorded beginning following 24 hours of treatment till 96 hours after treatment since the embryo will start to hatch around 48 hours post-fertilization. Heartbeat rate of developing embryo/larvae was determined and recorded beginning from 48 hours of treatment as long as 96 hours after treatment. An inverted microscope was utilized to view and tally the heartbeat. The heartbeat was recorded as bpm - beat every moment. Scoliosis was seen under an inverted microscope and recorded [1, 2].

Data Analysis

Data were analyzed using SPSS 25.0 version. Data were represented as mean± SD. One way ANOVA and linear regression was used. All the data were interpreted with $p < 0.05$ indicating the result is significant. The LC_{50} value is the concentration of the sample that cause 50% death or mortality in zebrafish. This value was determined using the mortality value. LC_{50} value was obtained with the regression method using the mortality value against the log concentration.

Results

Effect of *L. pumila* extract on the survival rate

Based on the result, the survival rate of zebrafish embryo increases as the concentration of the extract decreases. At concentration of 15.63 µg/ml the maximum survival rate was seen compare at concentration 250 µg/ml for non-irradiated extract. No survival of zebrafish seen in 500 µg/ml concentration. For a dose of 3, 6, 9 and 12 kGy irradiated of aqueous *L. pumila* extract a higher survival rate of zebrafish was seen in concentration 15.63 µg/ml whereas the lowest survival rate was seen in 125 µg/ml concentration. This show that the survival rate of zebrafish embryo decreased as the concentration of *L.pumila* increase. The result of irradiated of aqueous *L. pumila* extract was shown constant results at 24, 48, 72 and 96 hours post- fertilization. For positive control the survival rate less at lower concentration as well (Figure 1).

Effect of *L. pumila* extract on LC_{50} values

The LC_{50} of the different irradiated samples of *L. pumila* was plotted using mortality ratio verses the log concentration. Sample of *L. pumila* which irradiated with 3, 6, 9 and 12 kGy showed a very low LC_{50} value (62.5 µg/ml) compared to LC_{50} value for a non-irradiated sample which is 125 µg/ml concentration. The highest LC_{50} was observed for positive control (paracetamol) which is 144.54 µg/ml (Table 1).

Effect of *L. pumila* extract on scoliosis

The toxic effect on scoliosis was differentiated between irradiated and non-irradiated *L. pumila* extract and was determined at 96 hours post-fertilization (hpf). No scoliosis was observed in all concentration and similar pattern was observed in negative control zebrafish.

Effect of *L.pumila* extract on heartbeat

The heartbeat of the zebrafish embryo was evaluated to study the toxic activity of irradiated and non-

irradiated aqueous *L. pumila*. There were no heartbeat seen for concentration of 1000µg/ml, 500µg/ml and 250 µg/ml for irradiated extract however for non-irradiated *L.pumila* show zebrafish death at 1000µg/ml and 500µg/ml only. Based on the result, at higher concentration (250 µg/ml) of non-irradiated sample the heartbeat counting eventually reduces. For the 3 kGy test extract the heartbeat of zebrafish was higher at concentration 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml and 15.63 µg/ml compared to the negative control. Therefore, in overall heartbeat of zebrafish embryo increases inconsistently as concentration. As for 6 and 9 kGy test extract result shows that the heartbeat increases as the concentration increases. As for 12 kGy test extract, the result shows that the heartbeat was normal. Irradiated aqueous irradiated *L. pumila* shows that the heartbeat was abnormal as the concentration of the test extract increases. Stable heartbeat counting was achieved for the positive control.

Dose of irradiated samples	LC_{50} (µg/ml)
0	125
3	62.5
6	62.5
9	62.5
12	62.5
Positive (Paracetamol)	144.54
Negative (Distilled Water)	-

Table 1: The lethal concentration 50 (LC_{50}) value of test extract and control solution examined on zebrafish embryo.

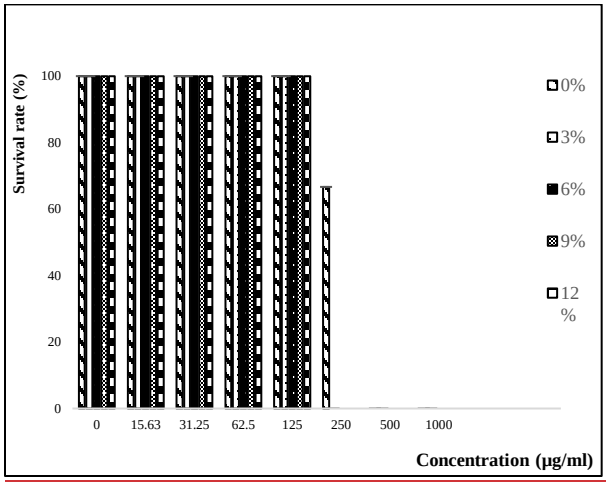

Figure 1: Effect of non-irradiated (0kGy) and irradiated (3, 6, 9 and 12) of *L.pumila* extract on survival rate of zebrafish. Data were expressed at mean ± SD.

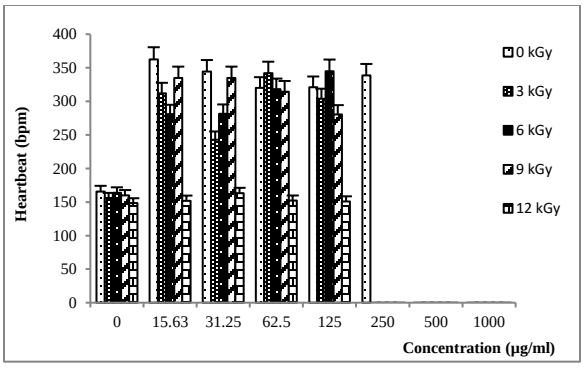

Figure 2: Effect of non-irradiated and irradiated *L.pumilla* in the heartbeat of zebrafish. Data were expressed at mean ± SD.

Discussion

Effect of survival rate of *L. pumila* extract

Endurance rate refers to the capacity of the living being to adjust in a specific situation as indicated by its ideal condition necessity. A part of the ideal condition which can impact the continuance pace of zebrafish undeveloped organism incorporates the pH, temperature, water compund and hardness, just as nitrogenous waste factor. Higher continuance shows that the zebrafish creating life can withstand the specific condition where low perseverance rate exhibits that the early life form can't drive forward through the incorporating air. As revealed, the ordinary criteria of zebrafish developing life endurance rate are therefore expected to decrease as the concentration of test solution [7]. At higher concentrations, the sample has a high content of bioactive compounds [8]. A low dose of gamma irradiation stimulates the growth by changing the hormonal influence on the plant cell. Previous research show that the carbohydrate and protein content will decrease with increased of gamma-irradiation which this caused the increase the metabolic and hydrolyzing in plant [9, 10, 18].

In view of the outcome, the survival rate of the zebrafish embryo of irradiated and non-irradiated *L.pumila* increase as the concentration of test extract decline. The survival rate of embryo tested on non-irradiated *L.pumila* extract shows that increased in low concentration of 250 µg/ml whereby the survival rate of embryo tested on irradiated *L.pumila* extract shows that increased in low concentration at 125µg/ml. The summary of results reflect that the non-irradiated *L.pumila* extract has a higher survival rate in comparison irradiated sample. Other than that, a slide difference can be seen on the survival rate between these two types. This is because all types of the irradiated dose of *L.pumila* extract have the same component but a low dose of gamma irradiation induces the breakdown of glycosides and releases phenolic compound

which increases the antioxidant activity. Whereas higher dose of gamma irradiation generates a flux of free radicals which causes more deleterious effects and decrease antioxidant activity this may be one of the factor which contributes to the higher survival rate of non-irradiated aqueous *L.pumila* compare irradiated extract [9,10].

Exposes to the negative control portion not impact when contrasted with all the test separate seen on the survival rate of zebrafish developing life. Finally, the structure is one of the key parts that should be underlined as it can modify the psychology research of zebrafish incipient organisms affecting the survival rate. Based on all the five types of the irradiated dose of *L.pumila* extract it is visible that non-irradiated aqueous *L.pumila* extract is the best option whereas it exhibits a lower toxicity effect as compared with irradiated aqueous *L.pumila* extract.

Effect of *L. pumila* extract on LC_{50}

The LC_{50} estimations of all types of the irradiated and non-irradiated samples of *L.pumila* and control tests are organized. In general, the higher LC_{50} values indicates that the toxicity level is low because higher concentration is needed to result in 50% of mortality rate of an organism, whereas a lower LC_{50} points out that the toxicity of the sample is higher which means harmful because in higher concentration itself there is greater death of organism [12].

Based on the result, there is a wide variability of LC_{50} value which comparable between the five types of the irradiated and non-irradiated dose of aqueous *L. pumila*. All this five types of dose of aqueous *L.pumila* was compared with control positive that is the paracetamol. The LC_{50} of non-irradiated aqueous *L.pumila* considered safe to be consumed below 250 µg/ml of zebrafish and for irradiated aqueous *L.pumila* is safe to be consumed below 125 µg/ml of zebrafish. It shows that the non-irradiated aqueous *L.pumila* has higher LC_{50} value in comparison with the other types of irradiated aqueous. Thus, it can be sum up that the non-irradiated aqueous *L. pumila* reflects a higher LC_{50} value which indicated that it is safe to be consumed than irradiated aqueous *L.pumila* as it ends in a lower toxicity level observed on the zebrafish embryo. In general irradiated aqueous *L.pumila* has the lower content of total flavonoid and phenolic in root and stem extracts might be the reason of lower antioxidant activity [13,14]. This factor can eventually affect the mortality rate of zebrafish embryo, causing the irradiated aqueous *L.pumila* to result in toxic effect.

In addition, in another study show that the median lethal concentration of irradiated *L.pumila* is safe to intake by human. The study shows increasing the light intensity resulted in an enhancement in phenolics such as gallic acid and caffeic acid and also flavonoid compounds. Antioxidant activities were higher in the leaves compared to stems and roots. With increasing total flavonoid and total phenolic content, the free radical scavenging power increased in all three varieties of *L.pumila*. Antioxidant activities increased in samples from all parts of the plant with increasing light intensity [13,14].

Effect of *L. pumila* extract on scoliosis

Based on the current study, there's no scoliosis found in the higher concentration and also in a lower concentration of all test extracts irradiated or non-irradiated dose. This manifest that the control negative and test sample does not reveal any toxic effect in particular to the entire test extract.

Effect of *L.pumila* extract on heartbeat

Heartbeat estimation is significant in evaluating cardiovascular capacity in light of the fact that the variation in heart rhythm can be the reason just as an impact of covered up pathological heart condition zebrafish has risen as one of the most valuable model creatures for cardiovascular research. In fact, the zebrafish heart is effectively available for optical break down without leading intrusive methodology and demonstrates anatomical similitude to the human heart. 120 to 180 is normal range heartbeat of zebrafish per minute. Additionally, the heartbeat of zebrafish can be affected by a couple of viewpoints, for example, the compound factor, hereditary instigated adjustment temperature and numerous others [15]. This causes the heartbeat rate to change bringing about bradycardia or tachycardia relying upon the specific factor controlling the zebrafish model. .

According to the result interpreted, all the irradiated and non-irradiated aqueous *L.pumila* extract presented an abnormal heartbeat, but at higher concentration of test extract, the heartbeat counting different. The type of irradiated aqueous *L. pumila* had a different heartbeat for all series of concentration, but at higher concentration, there was no heartbeat observed because it's 100% dead. In general heartbeat rate of zebrafish embryo increases incompatibly as the concentration of test extract decreases. This can be concluded that the decrease in a heartbeat is identified with the gross malformation observed. The potential component for the expansion and afterward decreases in pulse could be clarified by homeotic. Homeotic impact related to exposure to alpha specific and microbeam photons were recently observed in zebrafish developing life. Besides, the increase in pulse shows an enduring cardiovascular effect of the advancement of ionized radiation exposure [16].

As per difference sources, it is reported that extra adverse health effects of ionized radiation were realized including changes in to the cardiovascular and nervous systems. Moreover, only less information on biological changes on embryo due to the exposure. In previous study, it revealed that radiation ionization exposure will cause the

changes in morphology and also will alter the gene expression which involving on cardiovascular and neurological development. This study furthers our understanding of the immediate ironized radiation-induced biological responses during early development at the genetic level that trigger processes leading to short term cardiovascular effects [(16)].

Conclusion

It is concluded that effect of *L.pumila* exposure on zebrafish embryo model through acute toxicity assay was evaluated in this study. A comparison of two different type of sample (irradiated and non-irradiated) show non irradiated manifested lower toxic effects compared to irradiated sample. Therefore the result show that, although this plant extract is safe to be consumed irradiated or non-irradiated due to high promising pharmaceutics, it still exhibits mild toxicity effect on higher concentration level toward to zebra fish. It is suggested that further study to be done on the phytochemical of irradiated and non-irradiated extract and screening should be conducted to compare both.

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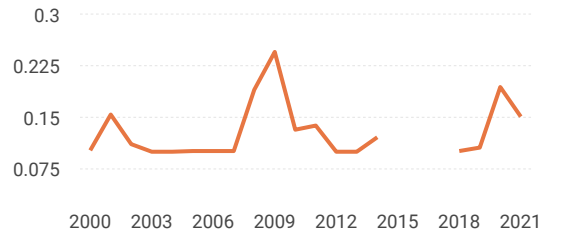

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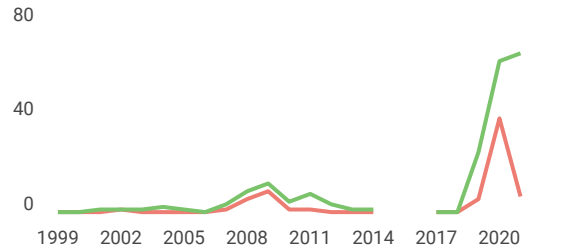

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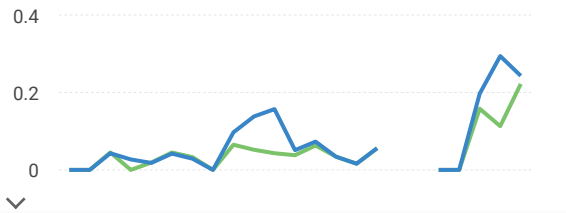

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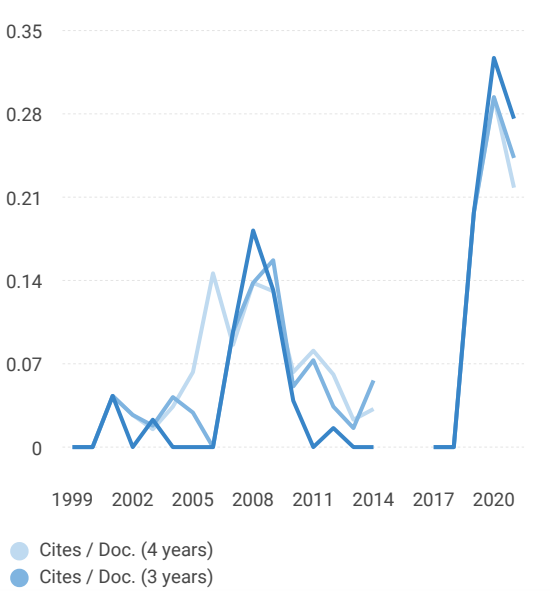

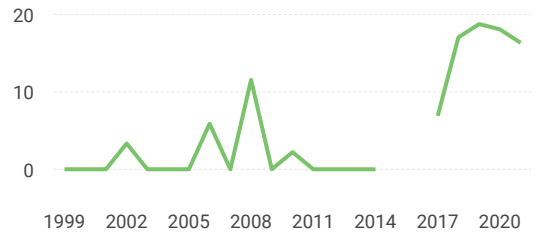

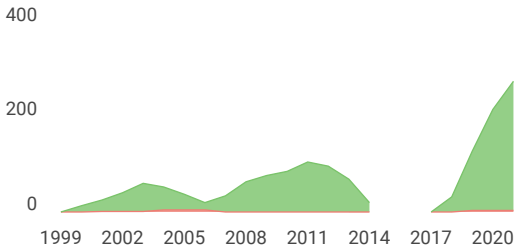

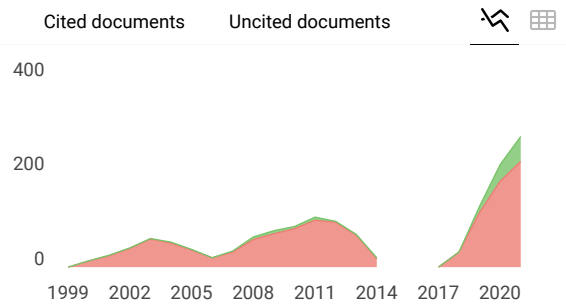

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