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Inhibition of the p53 Oncogene by Extracted Curcumin through Zebrafish Embryonic Models of Cancer

Sudithi Manthati[1] *

[1]Fremont High School, Sunnyvale, CA, USA
*Corresponding Author: sudithi.manthati@gmail.com

Advisor: Amanda Day, amanda_day@fuhsd.org

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Abstract

As cancer cells grow uncontrollably and spread to other parts of the body, partly due to the mutation of the p53 proto-oncogene. The mutated p53 proto-oncogene, also known as the p53 oncogene, can no longer regulate cell cycles, allowing for cells with damaged DNA to proliferate. The p53 oncogene can be inhibited by different extracted concentrations of curcumin on zebrafish embryonic models of cancer, as observed through mutations in developing embryos. Zebrafish embryos are an excellent in-vivo modeling tool because 84 percent of the genes known to be associated with human diseases have a zebrafish counterpart. Zebrafish are useful to understand the dynamics of early-stage cancers, especially since they overexpress the p53 oncogene; when this is suppressed, gross mutations of the embryo result. Data for each concentration of curcumin was graded 72 hours post fertilization (hpf) by the severity of several phenotypes (bent or hook-like tails, spinal column curving, sac mutation (reabsorption), shorter body length, or no mutations). Results of the experiment showed that increased concentrations of curcumin, which acts as an anticancer agent, led to more severe mutations, indicating a higher absorbance of curcumin by cancer cells, thus suppressing the overexpressed p53 oncogene.

Keywords: Curcumin, Danio rerio, Cancer, p53 oncogene, p53 proto-oncogene

1. Introduction

Cancer is the second leading cause of death worldwide, as the cancer cells grow uncontrollably and spread to other parts of the body (Cabezas-Sáinz et al., 1978). As researchers continue to find ways to better understand the disease and improve the treatments, they have been working with zebrafish (*Danio rerio*) embryos, a tropical fish native to southeast Asia, as models of cancer. 84 percent of the genes known to be associated with human diseases have a zebrafish counterpart and the biology of cancer is very much the same in zebrafish and humans, based on the genome sequence (Gilbert, 2016). Zebrafish are an excellent model (Khan and Alhewairini, 2018). Due to their cost-effective maintenance, high fecundity, fast development, and the transparency of the embryos which helps visualize tumor cell growth and the dynamics at early stages of cancer. Just like humans, zebrafish have a p53 gene that regulates their cell cycle. p53 acts as a proto-oncogene, and when it mutates, it leads to the mutation of the cell and metastasis to others (Wang et al., 2021). In zebrafish embryos, the same thing occurs with cell death, and as one cell dies, others around it also do so. Research has also been done on curcumin, (diferuloylmethane), the active ingredient of turmeric, which gives turmeric its bright yellow characteristics, has shown anti-cancer effects on cancer cells. Turmeric, a plant in the ginger family, is grown throughout India, other parts of Asia, and Central America (O'Brien, 2018). The therapeutic applications of turmeric (its rhizome, the underground stem) have a long history and are used in culinary and traditional Asian medicines. The objective of this experiment was to investigate if active ingredients in natural ingredients, such as turmeric, could be used during combination therapy to treat cancer patients. Based on the results, the increased concentration of extracted curcumin has implications for significantly inhibiting the p53 oncogene, as displayed through the severe mutations in the zebrafish embryos.

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2. Materials and Methods

2.1 Curcumin Extraction:

1 g, 2.5 g, 5.0 g and 10.0 g of turmeric were weighed using a digital scale and were placed into test tubes labeled with weights to extract the concentrated turmeric. Laboratory grade acetone, bought from Carolina Biologicals (North Carolina, USA), was used as a solvent. Using a 10 mL graduated cylinder, acetone was poured into each of the four test tubes labeled with the turmeric weight and 10 mL of distilled water was poured into one test tube labeled as control. The test tubes with turmeric were stirred for about 5 minutes separately until the turmeric in the test tubes became a homogeneous slurry. The slurry was filtered out using filter paper and poured into test tubes labeled with turmeric mass. The standard curve was calculated using a spectrophotometer for the different weights of turmeric, using 6 glass cuvettes of which 4 were labeled with turmeric mass, one as control (water) and one as acetone to act as a blank. 1 mL of each filtrate from the test tubes were pipetted into the labeled cuvettes to observe the absorption of photons which was then recorded. The filtrate from the cuvettes was then placed back into the original test tubes respectively and the filtrate was then let to evaporate ensuring acetone was completely evaporated. After the filtrate is complete evaporated 1 mL of acetone was poured into each of the test tubes and the contents were stirred using a glass stirrer separately and using different pipettes, 1 mL of the mixture was placed into the weight labeled petri dishes, and 1 mL of distilled water was placed into the control petri dish. Negative absorbance defines the concept that less light can pass through the extracted substance, which correlates to a greater amount of curcumin being extracted. The sensor used was the GoDirect SpectroVis Plus Spectrophotometer with a wavelength range of 380 nm to 950 nm, a wavelength accuracy of ± 4.0 nm, and a photometric accuracy of ± 0.10 AU. The blank, which was different from the control, was used to calibrate the spectrophotometer and was not further used in data analysis. The regression line in Figure 1 implies that as the amount of turmeric used increased the concentration of curcumin increased.

Table 1: Absorbance Values Based on Mass of Turmeric Used for Curcumin Filtrate.

Substance (mg)	Absorbance (AU)
0.0	0.00887
1.0	0.00166
2.5	-0.00101
5.0	-0.00605
10.0	-0.01

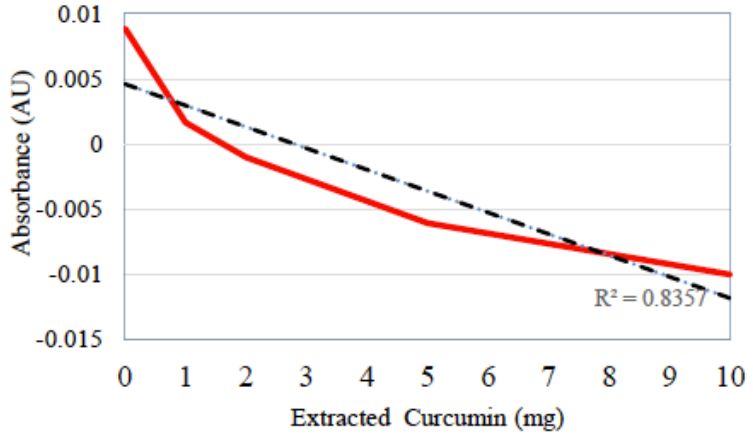

Figure 1: Standard Curve Absorbance Slope Based on Extracted Curcumin from Different Masses of Turmeric

2.2 Zebrafish Breeding and Eggs:

The male and female zebrafish were bought from Carolina Biologicals and were placed into two separate tanks. The tanks were set up with 30 liters of water and included a filter system and automatic food feeder, and the water temperature was maintained at 22 ^{0}C with automatic lights turned off at night. The fish were let to get acclimated to their environment for 3 days. On the 4th day, a breeding tank bought from Carolina Biologicals was used to breed the fish. Water from both the male and female fish tanks was used; two male fish and one female fish were placed into the breeding tank with the divider for the fish to get acclimated. After one and half hours the divider was removed to let the fish mate naturally. After 2.5 hours later when the eggs were collected at the bottom of the breeding tank, the male and female fishes were placed back into their respective tanks. Water from the breeding tank was placed in the

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petri dishes. Five eggs were carefully pipetted one at time into the turmeric weight labeled petri dishes and into the control petri dish with water. The petri dishes were then placed in a warm area and let the eggs develop.

2.3 Data Recording and Disposal:

At 24 hours post fertilization (hpf), curcumin was introduced to the embryos. The mutations (Wu et al., 2007) were observed from 48- 72 hpf and the phenotype data was recorded. Severity score defines the qualitative observation of how much the extracted curcumin at differing concentrations has affected zebrafish development by inhibiting the p53 oncogene. Mild indicates that the zebrafish embryo is following normal development markers, while moderate refers to more obvious mutations and severe defines embryos that have been mutated to the point that development has stopped. Within 168 hours of post fertilization, all embryos except in the distilled water control group were euthanized by the supervisor in a two-step process involving freezing and bleach. Embryos in the distilled water control group (with no abnormalities during development) were placed into a fish tank to develop into adults. The above process breeding and observing mutation was conducted for two more trials.

3. Results

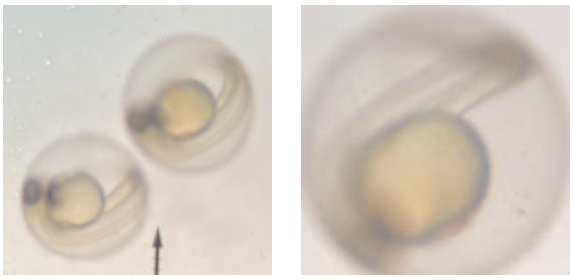

Figure 2: Control group embryonic development (exposed to only distilled water) at 72 hpf was observed to have normal expected zebrafish development.

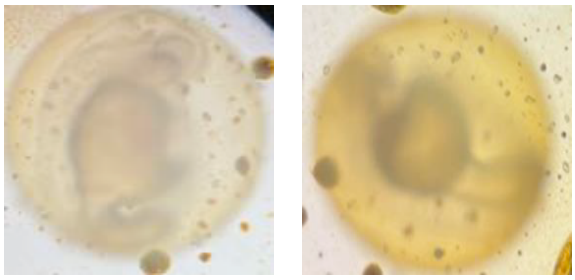

Figure 3: The 1 g group's embryonic development at 72 hpf showed slight mutations in tail development, spinal column curving, body length, and sac reabsorption. Phenotypes were comparable to the control group.

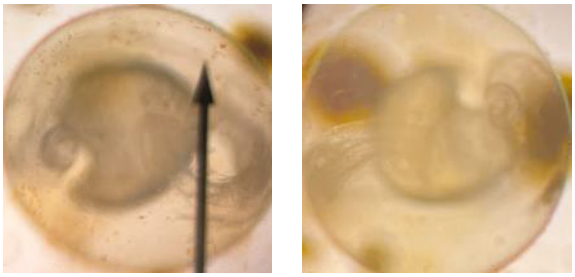

Figure 4: The 2.5 g group's embryonic development at 72 hpf showed some mutations in tail development, spinal column curving, body length, and sac reabsorption. Phenotypes were comparable to 1 g embryos and the control group.

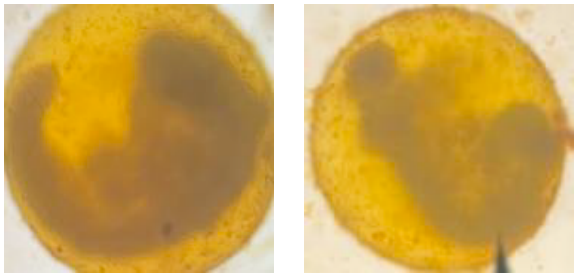

Figure 5: The 5 g group's embryonic development at 72 hpf showed moderate to severe mutations in tail development, spinal column curving, body length, and sac reabsorption.

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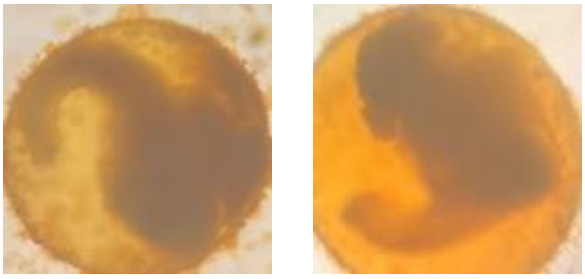

Figure 6. The 10 g group's embryonic development at 72 hpf showed extremely severe mutations in tail development, spinal column curving, body length, and sac reabsorption.

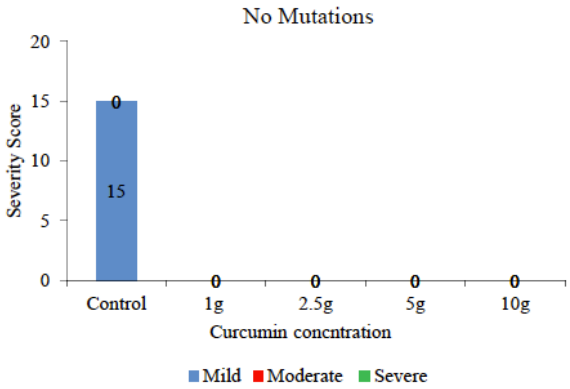

Figure 7. Graph of groups that received no mutations (only control).

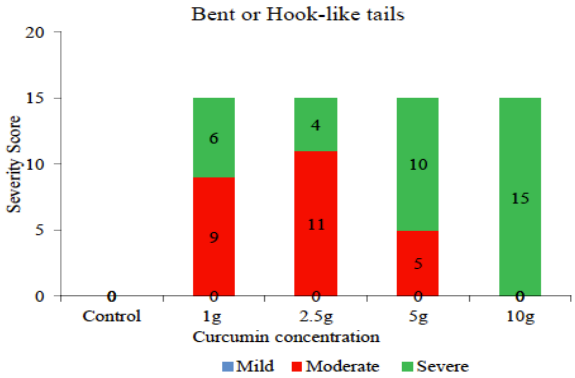

Figure 8. Severity (on a scale of mild to moderate to severe) of bent or hook-like tail mutations.

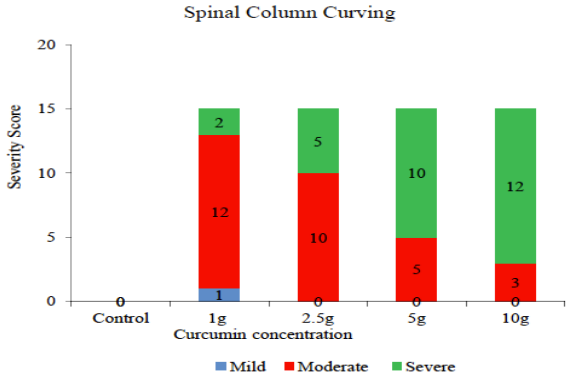

Figure 9. Severity (on a scale of mild to moderate to severe) of spinal column curving.

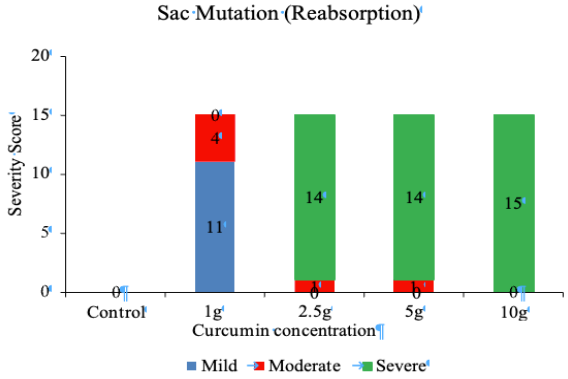

Figure 10: Severity (on a scale of mild to moderate to severe) of sac mutations (sac reabsorption)

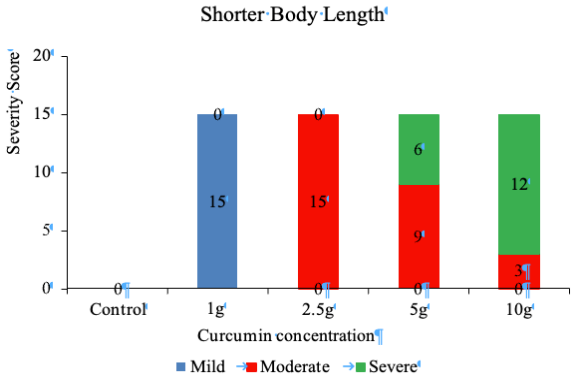

Figure 11. Severity (on a scale of mild to moderate to severe) of shorter body length mutations.

4. Discussion

A standard curve was used to determine the exact concentration of curcumin from acetone through the measure of absorbance. As a blank, acetone was used with an absorbance of 0.00887 AU. Smaller measurements were used and then scaled to fit the spectrophotometer. 1.0 mg had an absorbance of 0.00166 AU, 2.5 mg had a level of -0.00101

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AU, 5.0 mg had an absorbance level of -0.00605 AU, and 10 mg had an absorbance of -0.01 AU. The decreasing values can be seen on the standard curve. The control group, where there were just eggs with water, there were 15 eggs with no mutations. For 1 gram of concentrated curcumin, there were 9 with moderate bent or hook-like tails, and 6 with severe bent or hook-like tails. There were also 1 mild, 12 moderate, and 2 severe embryos with spinal column curving. 1 g of concentrated curcumin also had 11 mild and 4 moderate mutations of sac reabsorption. All 15 showed mild mutations of shorter body length. For 2.5 g of concentrated curcumin, 11 embryos had moderate bent or hook-like tails and 4 with severe mutations. 10 had moderate spinal column curving and 5 were severe. There was 1 moderate sac reabsorption and 14 severe mutations. All 15 showed mild shorter body length. For 5 g of concentrated curcumin, there were 5 with moderate bent or hook-like tails, and 10 with severe bent or hook-like tails. There were also 5 moderate, and 10 severe embryos with spinal column curving. 5 g also had 1 moderate and 14 severe mutations of sac reabsorption. 9 showed moderate mutations and 6 showed severe mutations of shorter body length. For 10 g of concentrated curcumin, all 15 embryos had severe bent or hook-like tail mutations. 3 had moderate spinal column curving and 12 were severe. All 15 showed severe mutations. 3 showed moderate shorter body length and 12 showed severe body length mutations.

The objective of this experiment was to observe the effect of the concentration of curcumin extracted by acetone on its anti-tumor properties, as observed through zebrafish eggs as a model of cancer. The hypothesis was that if the concentration of the curcumin extracted from turmeric is increased, then the extracted substance will have higher anti-cancer properties. The hypothesis was supported due to the increased concentration of turmeric used in the acetone extraction process, causing more curcumin to be extracted. All trials showed some signs of mutation properties compared to water. However, there were sources of error. Some of the pipetting may have not been exact, so the exact concentration of curcumin per dish may have varied slightly. Additionally, because all embryos came from the same parents, there was very little genetic variation, which must be considered as cancer affects those with certain DNA variations differently.

A future direction could be to consider sex differences into account by using full grown fish, as gender in healthcare leads to prominent reactions to the same treatment. Mutations in females could respond differently to curcumin than those in males, and the concentration of curcumin may work more efficiently on one sex than the other. Another area of improvement for the project would be to test curcumin on actual tumor cells to observe its effect on the rate of cell division and metastasis, because although zebrafish embryos are a good model of cancer in humans, cancer cells have other mutations present which may cause them to respond differently to treatment.

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