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The Effects of Pomegranate Juice on *C. elegans* under Thermal Stress

Abstract:

This experiment analyzed the effects of pomegranate juice on *C. elegans* under thermal stress. *C. elegans*, a nematode with similar genes to humans, is an effective model for studying human diseases. Thermal stress occurs when an organism is exposed to high temperatures, which results in the release of heat shock proteins. Heat shock proteins act as molecular chaperones that help restore protein homeostasis following heat stress. Despite their presence, thermal stress can still weaken antioxidant defense in organisms. However, pomegranate juice is rich in antioxidants and polyphenols, which can help strengthen antioxidant defense. In this study, pomegranate extract was dissolved in water and mixed with agar powder. Using this pomegranate mixed agar, *E. coli* was cultured for 24 hours. The results showed that the 5 mg/ml concentration increased survival rates of *C. elegans.* However, the 10 and 20 mg/ml concentrations lowered survival rates, which can be attributed to dose-dependent toxicity. Interestingly, the higher concentrations of pomegranate extract significantly increased *C. elegans* reproductive rates. This research expands on previous studies that examined the effect of pomegranate juice on *C. elegans* and distinctly focuses on its role under thermal stress. This distinction helps gain a deeper insight into pomegranate juice's effect on oxidative stress.

Introduction:

Heat stress

Heat is a natural hazard that is studied by many researchers to understand how organisms act under certain temperatures (Kovats & Hajat, 2008). This natural hazard can be caused by many factors like elevated outdoor temperatures, and the body's natural increase in temperature to combat infection. Regardless of the method, when the body is exposed to severe heat, it will

attempt to decrease its temperature by radiative, convective, and evaporative means, such as vasodilation and perspiration (Kovats & Hajat, 2008). However, if the body is exposed to high enough temperatures that the previously stated methods cannot handle, it can lead to heat stroke, heat exhaustion, heat syncope, and heat cramps (Kovats & Hajat, 2008). These results of heat stress have a high case-mortality ratio leading to death within hours or, if not fatal, causing permanently damaged organs. Exposure to such high levels of temperatures will also start producing misfolded proteins and protein aggregates (Jovic *et al.,* 2017). These accumulated misfolded proteins and protein aggregates are signs and causes of aging and age-related diseases, like Alzheimer's and Parkinson's disease (Jovic *et al.,* 2017). Because of the effects extreme temperatures can cause, many organisms evolved to develop heat-shock responses.

When the body temperature exceeds the temperature tolerable for an organism, the heat-shock response system is activated (Crombie *et al*., 2016). The heat-shock response is the body's stress reaction to heat, which is utilized to preserve proteostasis (Jovic *et al.,* 2017). The response system protects and realigns the proteins by introducing molecular chaperones called heat shock proteins (Crombie *et al.,* 2016). These heat shock proteins when introduced would detect, refold, or degrade any unfolded and misfolded proteins, which prevents their accumulation (Xu *et al.,* 2023). Small heat shock proteins, a subclass of heat shock proteins, exhibit ATP-independent activity that delay the formation of harmful protein aggregates (Janowska *et al.,* 2019). This subclass of proteins is also involved with muscle protection and has treatment resistance in cancer. Their expressions are also associated with poor prognosis and play essential roles in easing Parkinson's and Alzheimer's disease (Janowska *et al.,* 2019). Mutations in small heat shock proteins are associated with several disorders, such as neuropathies and early-onset cataract formation (Janowska *et al.,* 2019). This demonstrates the significant impact

that a malfunction in this protein can have. Heat-shock responses, as well as heat shock proteins, are not limited to only animals and may occur in all plant, bacterial, and archaean species (Pires *et al.,* 2023).

Previous research on the heat shock response systems and their proteins primarily aimed to understand the response system, the heat shock proteins, and their effects when paired with other abnormalities. Small heat shock proteins are protein chaperones that received relatively little attention in research despite being recognized for a long time (Janowska *et al.,* 2019). They are defined by their α-crystallin domain, which is named after the numerous small heat shock proteins found in the eye lens, specifically αA-crystallin and αB-crystallin (Janowska *et al.,* 2019). Researchers also investigated how heat stress interacts with other abnormalities such as oxidative stress; this specific example shows a synergistic effect that reduces an organism's survival (Crombie *et al.,* 2016). When both abnormalities were tested on *C. elegans,* the response systems did not function as efficiently as they would when only one abnormality was induced (Crombie *et al.,* 2016).

Punica granatum

Punica granatum (pomegranate) is a drought-tolerant, long-living plant (Zarfeshany, 2014). This plant helps prevent or treat various disease factors including, but not limited to, high blood pressure, high cholesterol, oxidative stress, hyperglycemia, and inflammatory activities (Zarfeshany, 2014). Pomegranates prevent the growth and start of some inflammatory markers as their production is blocked by ellagitannins (Zarfeshany, 2014). Ellagitannins are a large group of bioactive compounds that are found in plants, which exhibit antioxidant, antimicrobial, anti-inflammatory, and anticancer properties (Lipińska, 2014). Foods that are found rich in

ellagitannins, like pomegranates, can improve health and prevent chronic conditions such as cardiovascular diseases, neurodegenerative diseases, and cancer (Lipińska, 2014). Pomegranate components have beneficial effects in animal models for respiratory diseases, RA, neurodegenerative disease, and hyperlipidemia due to its medical properties (Daniel, 2017).

Pomegranate juice has strong antioxidant potential and is rich in polyphenols (Basu, 2009). Polyphenols such as Kaempferol, Quercetin, and Resveratrol regulate proteins, which may reduce the impact of thermal stress on protein synthesis (Iqbal, 2023). Due to its antioxidant and anticarcinogenic qualities, pomegranate juice has several components, such as lipid peroxidation, free radicals, and the reduction of macrophage oxidative stress (Zarfeshany, 2014). Pomegranates are a highly rich source of ellagitannins with anti-inflammatory effects that can be utilized as an effective treatment for inflammatory bowel disease (Scaioli, 2019). Pomegranate components were employed as an anti-inflammatory medication to treat chronic inflammatory disease (Daniel, 2017).

Previous studies mainly focused on pomegranate juice's effect on prostate cancer, diabetes, and atherosclerosis (Danesi, 2017). Studies also stated that polyphenolic compounds may have robust and reproducible effects during aging (Wilson, 2006). Many disease risk factors, including oxidative stress, hyperglycemia, high blood pressure, high cholesterol, and inflammatory processes, could be treated or prevented using *Punica granatum* (Zarfeshany, 2014). Some experimental studies that involved pomegranate juice have found that it reduced atherosclerotic lesion areas in immune-deficient mice (Basu, 2009). In addition, several human clinical trials found that pomegranate juice improves blood flow and keeps arteries from becoming stiff. Additionally, pomegranate juice reduces intestinal fat in *C. elegans* and has the potential to prevent obesity (Danesi, 2017).

C. elegans

Caenorhabditis elegans, commonly known as *C. elegans,* is a multicellular eukaryotic organism that was the primary focus of many experiments because it is cost efficient, versatile, and simple. The cellular complexity and the conservation of disease pathways between *C. elegans* with the simplicity of the cultivation, make for an effective *in vivo* model (Wu *et al.,* 2012). This soil nematode offered great potential for genetic analysis, partly because of its rapid (3-day) life cycle, small size, and ease of laboratory cultivation (Riddle *et al.,* 1997). *C. elegans* can be explored to assess the toxicities of both contaminated soil and contaminated river water or sediments (Wu *et al.,* 2012). *C. elegans* can grow thousands of organisms on a single petri dish seeded with a lawn of *Escherichia coli.* In addition, the simplicity of lab cultivation and the capacity to keep thousands in a petri dish surviving off *Escherichia coli* alone caught the appeal of many bacteriophage geneticists (Riddle *et al.,* 1997). *C. elegans* can inbreed because they are self-fertilizing hermaphrodites, which have both female and male sex organs (Riddle *et al.,* 1997). This allows the nematodes to breed by themselves, which offers conveniences previously enjoyed only in a plant genetic system (Riddle *et al.,* 1997).

When *C. elegans* are exposed to high temperatures or heat stress, it triggers the heat shock response, which can have lasting effects on their cellular functions (Xu *et al.,* 2023). Exposure to mild early-life stresses can slow down aging, with protein phosphorylation serving as an essential regulator in this process (Huang *et al.,* 2020). Exposure to severe stresses often causes tissue damage and ultimately shortens the lifespan of *C. elegans*, while mild stresses usually promote defenses against damage and therefore promote longevity (Huang *et al.,* 2020). During mild heat stress, Heat Shock proteins (HSF-1) are triggered that serve as protection towards thermal stress. HSF-1 proteins can lead to extended lifespans and improved heat

hormesis (stress resistance) since these proteins mitigate the effects of thermal stress (Xu *et al.,* 2023). Heat shock proteins shield protein substrates from conformational damage to maintain protein function, stop protein aggregation, and stop the development of harmful inclusion bodies (Tutar, 2010).

Several other studies were conducted that were like this experiment that revolved around the use of *C. elegans* and thermal stress. The novelty of this experiment is that we used pomegranate juice with *C. elegans* under thermal stress. A previous study shows the gene expression of *C. elegans* under thermal stress. It was found that gene expression significantly changes due to protein aggregates and misfolding (Jovic *et al.,* 2017). To avoid the detrimental effects of cytotoxic misfolded protein species and protein aggregates, multiple stress response systems have evolved as a first line of defense to maintain proteostasis, with the highly-conserved heat-shock response (HSR) pathway being prominent (Jovic *et al.,* 2017). Another study shows the Stress-Induced Alterations in the *C. elegans* phosphoproteome. In this study, it states that the essential regulators of the insulin-signaling pathway, AKT-2, DAF-18, and DAO-5, can regulate the aging process by reducing the effects of thermal stress. Furthermore, it was reported that proteins that respond to temperature changes, such as LMD-3, can regulate the lifespan of *C. elegans* (Huang, 2020). These results offer phosphoproteome-wide datasets that can deepen our understanding of early thermal processes in *C. elegans* (Huang, 2020).

Another closely related article shows the effects of thermal stress on longevity and regulation of *C. elegans.* It was shown that there was a high correlation between stress resistance and longevity, which suggested that the same molecular activities that defend the cell from stress can defend the cell from the damage caused by aging (Crombie *et al.,* 2016). Additionally, overexpressing the heat shock protein HSP70F and the old-1 gene improves thermotolerance and

longevity. This study focused on using pomegranate juice for its distinctive cooling properties on the thermal stress of *C. elegans* unlike other researchers who mainly studied pomegranate juice's effect on prostate cancer, diabetes, and atherosclerosis.

Materials and Methods:

Agar and pomegranate extract preparation

Agar solution was prepared by adding 4.6 grams of agar powder to 200 ml of water. The pomegranate juice was prepared using a stock solution of 200 mg of pomegranate juice and 10 mL of agar. This created a stock solution of 20 mg/mL concentration. Serial dilution was performed by setting up 5 test tubes. The stock solution created was poured into the first test tube. 5 mL of agar was poured into each test tube (excluding first) and labeled accordingly. Using a micropipette, 5 mL stock solution from the first test tube was poured into the second test tube to create 10 mg/ml concentration. 5 mL of solution was transferred and mixed from the previous beaker to the next beaker. The process continued until all beakers were diluted. The remaining concentrations were 20 mg/ml, 10 mg/ml, 5mg/ml, and 2.5 mg/ml. The agar was left to cool for a few minutes before adding to the petri dishes.

E. coli Cultures

After an hour, the incubated agar plates solidified. The inoculation loop was sterilized using a Bunsen burner and used to transfer small amounts of *E. coli* into each of the plates. *E. coli* plates with pomegranate extract were cultured and placed into an incubator.

M9 buffer preparation

The M9 buffer was prepared by measuring 1 L of distilled water in a graduated cylinder. 1 L of distilled water was poured into a flask. 3g of potassium phosphate (KH_2PO_4), 6g of sodium phosphate dibasic (Na_2HPO_4), 0.5g of sodium chloride (NaCl), and 1g of ammonium

chloride using a microspatula (NH_4Cl) was measured and mixed together. This mixture was placed into the flask with 1 L of water. A glass stirring rod was used to mix the solution completely until the solids were completely dissolved. The solution was transferred into a small glass mottle and loosely covered with a bottle cap. This small glass was placed into the autoclave at 120^o kpa. After autoclaving for 20 min, the bottle was sealed and placed in the lab at room temperature.

Culture of C. elegans

Small chunks of N2 *C. elegans* were placed into each petri dish. Petri dishes with *C. elegans* were sealed with a small opening (to stop condensation) using parafilm. Parafilm was stretched to match the circumference of a petri dish. Sealed petri dishes were placed into an incubator at 20 ^{o}C. To ensure that the incubator was not opened, a sign indicating not to touch the incubator was placed.

Survival assay

All 4 plates were placed excluding Control-1 into the incubator. They were incubated for 12 hours at 34 ^{o}C. Control-1 was left in an incubator at 20 ^{o}C. After 12 hours, the incubator was set back to 20 ^{o}C for all plates. Using the gentle touch assay, we observed the behavior of the *C. elegans* and noted how many comprehended to the assay. To calculate the survival rate, divide the amount of C. elegans alive over the sample size and multiply by 100 to gain your answer.

Gentle Touch Assay

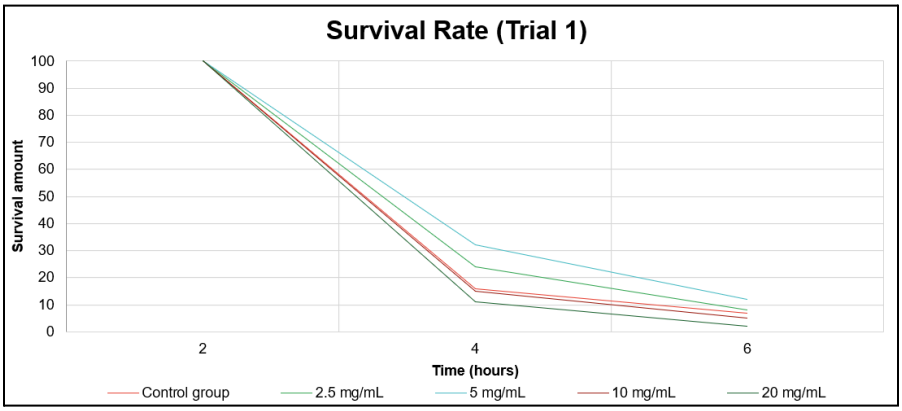
Eyelash hair was removed and sanitized with 70% isopropyl alcohol. A toothpick was obtained and sanitized using sanitizing wipes and alcohol. The sanitized eyelash hair was attached to the end of a toothpick. The *C. elegans* were gently stroked 5 times with the toothpick. Any movements made by the *C. elegans* were recorded and observed in 30 sec increments. This

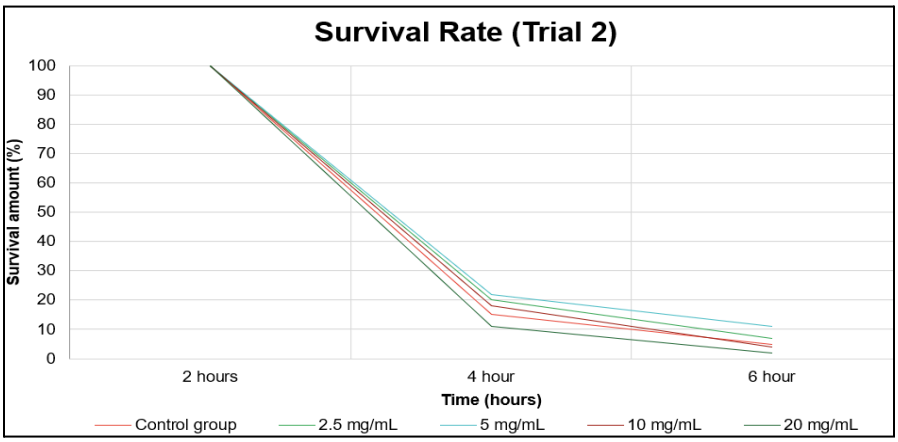
process was repeated 3 times to observe any backward locomotion or shallow turns. The toothpick was thoroughly disinfected with sanitizing wipes and alcohol.

Data analysis plan

Data were collected from the analysis of the *C. elegans* behavior/movement, survival rate. Data was stored on Google Sheets in CSV format and Excel. The data was obtained from several different graphs, tables, diagrams, and photographs. Standard deviation was calculated, and error bars were added using Excel. The data's significance was calculated using ANOVA followed by Tukey HSD. P-values below 0.05 were considered significant. The graphs that are needed include line graphs and bar graphs. The bar graphs would show the average lifespan of each group and reproduction rates. The line graphs would show the change in survival rate of each group every hour. The data tables needed for this experiment was recording the average lifespan of each group and the survival rate of each group. Reproduction rates were calculated using ImageJ.

Results:





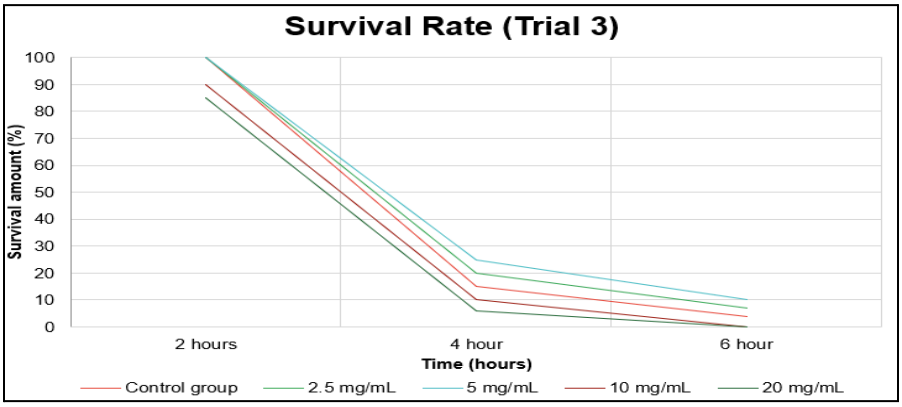


Figure 1. Survival rate of *C. elegans* under thermal stress.

Each color represents either the control group or a different concentration of pomegranate juice. Survival rate of the *C. elegans* were measured using a gentle touch assay and survival assay. The *C. elegans* were measured over a timespan of 6 hours in 2-hour intervals. The incubators were

set to 32°C to induce heat stress. The data was stored in Excel and the percentage of *C. elegans* alive was calculated per interval. It is shown that the 5 mg/mL group had the highest survival percentage throughout the three trials.

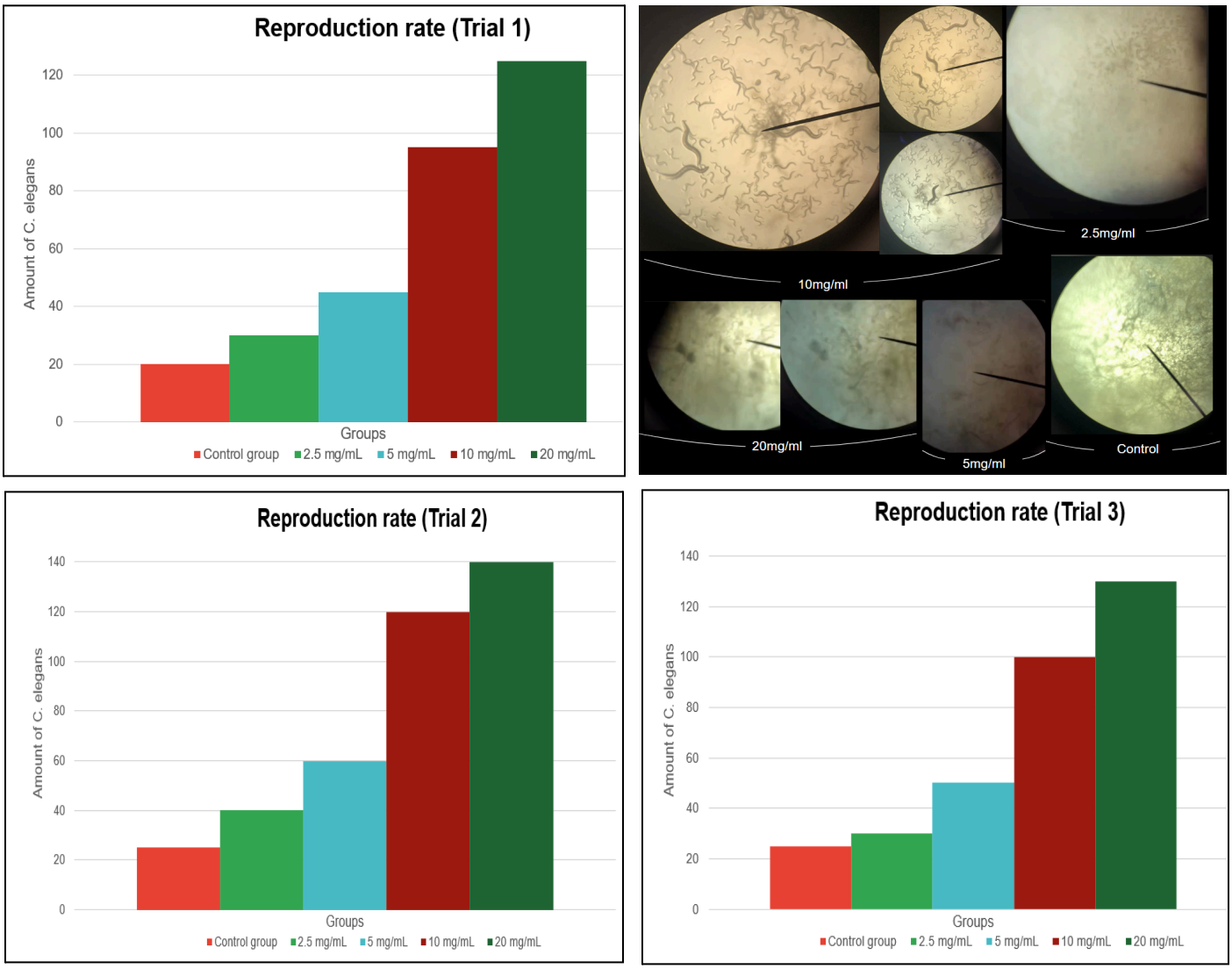


Figure 2. Shows the reproduction rate of the different groups.

Each color represents either the control group or a different concentration of pomegranate juice. The reproduction rate was analyzed using ImageJ. The *C. elegans* were cultured using *E. coli* in

20°C incubators. The *C. elegans* were allowed to reproduce and grow for two days. The data was stored in Excel and pictures were taken after the two days to analyze in ImageJ. As the amount of pomegranate extract increased, the reproduction rate also increased.

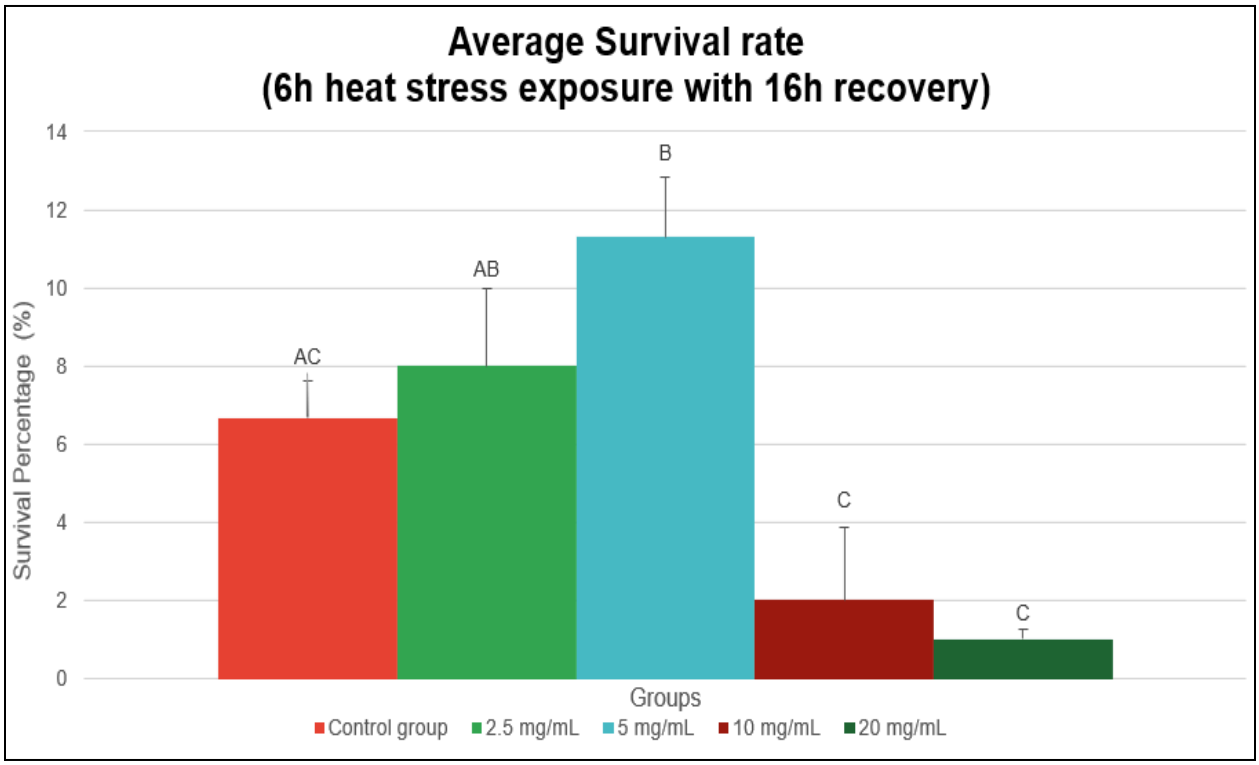


Figure 3. Shows the average survival rate of the *C.elegans* in the different groups after 6 hours of heat stress exposure and 16 hours of recovery.

Each color represents either the control group or a different concentration of pomegranate juice. The *C. elegans* were cultured using *E. coli* in 20°C incubators and tested under 32°C heat stress. Afterwards, the *C.elegans* were allowed to recover in 20°C incubators where survival rate was counted. Data was analyzed in Excel and Tukey-HSD. Values represent means and ± standard deviation values with different superscripts being significantly different with a $p<0.05$. The data

shows that the 5 mg/mL group had the highest survival percentage after heat stress exposure and recovery.

Discussion and Conclusions:

Discussion

This experiment highlighted pomegranate extract as a valid treatment to heat stress. However, there are some limitations of this experiment. As pomegranate extract is insoluble in agar solutions, pomegranate extract was mixed with hot or warm water before mixing with agar powder. A more significant limitation would be that some pictures taken of *C. elegans* groups were blurry or unclear. In future trials, we will take pictures in timed intervals and ensure the quality of the pictures are clear. In addition, more trials are needed to validate the results, as significance may be difficult to determine with lower timed trials. Furthermore, due to time constraints, we were unable to include a negative control group for comparison with the experimental control. This control would have clearly demonstrated the effects of heat stress on the lifespan of *C. elegans*. Despite these limitations, this experiment highlights several benefits of pomegranate extract. For example, antioxidant and anti-inflammatory effects of pomegranate extract may reduce heat stress in N2 wild-type *C. elegans* as postulated. This could be seen in how *C. elegans* in the 5 mg/ml group had higher survival rates compared to the other groups. This group had significantly higher survival rate compared to 10 mg/ml and 20 mg/ml groups, despite the fact the latter groups received more pomegranate extract due to dose dependent toxicity. This shows that having high doses of antioxidant properties past 5 mg/ml is counterproductive to resisting heat stress. Inversely, higher amounts of pomegranate extract seem to increase the amount of reproduction rates in the *C. elegans*. Our hypothesis was valid as we

hypothesized that pomegranate extract would mitigate the effects of heat stress due to its properties.

Conclusions

The results from this experiment allow us to conclude that greater concentrations of pomegranate extract past 5 mg/ml is counterproductive to mitigating the effects of heat stress on *C. elegans* because of dose dependent toxicity. The 5 mg/ml group portrayed an 11% survival rate which was the greatest among the groups. This survival rate decreased significantly in the higher treatment groups, with the 10 mg/mL and 20 mg/mL concentrations showing survival rates of 2% and 1%. This demonstrated a statistically different result from every group besides the 2.5 mg/ml. On the other hand, the reproductive rate showed a direct relationship. As pomegranate extract concentration was increased, the number of *C. elegans* increased. This may highlight a correlation between pomegranate extract concentrations and reproductive health or fertility of *C. elegans*.

Future works can focus on improving the experiment's accuracy. There are several variables that may have not been consistent across the groups. For example, the *E. coli* counts can be altered due to pomegranate extract. To isolate *E. coli* count and pomegranate extract (or keeping *E. coli* numbers consistent), certain variables can be divided by the number of colonies forming units of *E. coli*. Moreover, changes in *C. elegans* can be monitored in smaller intervals. Measuring heat stress over 2 hour periods alone may result in inaccurate assessments of survival rates. By recording the survival ratio in hourly intervals, it may also highlight differences between length of heat stress. Future researchers could also experiment with qualities other than antioxidant properties and see if dose dependent toxicity also applies to those as well. For

example, studies testing the properties of green tea extract and coffee have shown significant results in increasing plant growth and lifespan.

Our findings build upon the existing literature on pomegranate juice with *C. elegans* by discovering the benefits of pomegranate juice on mitigating oxidative stress. This research can serve as a guideline for future research that explores the application of pomegranate juice with heat stress or oxidative stress.

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