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Investigating the Optimal Concentration of *Trichosanthes dioica* Roxb. Extract on Paraquat Induced Oxidative Stress in *Drosophila Melanogaster*

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ABSTRACT

Oxidative stress is associated with the pathogenesis of a variety of chronic diseases such as cancer, diabetes, Parkinson's, and Alzheimer's disease. *Drosophila melanogaster* is a commonly used model for oxidative stress as supplementation of the compound paraquat produces the respective model, which can be quantified through the use of a mortality assay. Intake of paraquat increased *D. melanogaster* mortality to 59.94% after two days of a 10 mM diet. When the extract derived from *Trichosanthes dioica* fruit, or the bitter gourd, was utilized as a potential antioxidant, oxidative stress-related mortality improved in a dose-dependent manner with treatment. The optimal concentration of the extract was found to be 1.0 mg/mL as it lowered mortality to 36.63%. The change in mortality between an oxidative stress model and one with treatment had statistically significant p-values when the dosage of *T. dioica* was greater than 0.04 mg/mL up to a dosage of 2100.0 mg/mL. The study not only underscores *T. dioica* fruit's antioxidant properties *in vivo* and establishes an ideal concentration for future studies involving the model *D. melanogaster* but also elucidates *T. dioica* as a potential candidate for treating a variety of oxidative stress-associated conditions.

Keywords: *Trichosanthes dioica,* Pointed gourd, Paraquat, Antioxidant, Oxidative Stress.

INTRODUCTION

1.1 Oxidative Stress

Oxidative stress is a biological phenomenon that occurs when there is an imbalance between the production of harmful reactive oxygen species (ROS) and the body's ability to detoxify or repair the resulting damage. ROS are chemically reactive molecules that contain oxygen and include free radicals such as superoxide anion radicals ($O_2^{\cdot -}$), hydroxyl radicals (•OH), and non-radical hydrogen peroxide (H_2O_2). While these molecules serve important roles in various cellular processes, excessive ROS

production can lead to cellular damage and dysfunction. The body constantly generates ROS as byproducts of essential metabolic processes, including respiration and energy production within mitochondria. In healthy individuals, the body has a system of antioxidants that regulate ROS levels. Under stress conditions, the balance between reactive species production and antioxidant defense is disturbed and the result is the development of oxidative stress.

Oxidative stress is linked to a range of health issues, including aging due to the accumulation of oxidative damage, neurodegenerative diseases like Alzheimer's and Parkinson's, and cardiovascular diseases through the oxidation of blood vessel components. It's also implicated in cancer by causing DNA mutations, exacerbates inflammation in chronic diseases, contributes to diabetes complications, and plays a potential role in autoimmune disease development and progression.

Under oxidative stress, antioxidants from foods, plants, or pharmaceutical supplements may enhance the antioxidant defense and help reduce the condition of stress. Antioxidants scavenge and neutralize free radicals or strengthen the innate antioxidative ability of cells acting on existing antioxidant protection systems. Plants have emerged as accessible and cost-effective treatments to alleviate oxidative stress, and specifically, gourd vegetables have shown promise. Gourds have been found to contain a statistically significantly higher quantity of phenols, flavonoids, tannins, and carotenoids as well as potent antioxidative effects (Yadav et al., 2016).

1.2 *Trichosanthes dioica*

Plants are sources of many natural bioactive compounds, which are of increasing interest for their pharmacological potential including antioxidant, antitumor, anti-inflammatory, and antimicrobial activities. Several roots and vegetables have attracted much attention in recent years due to their nutritional and pharmacological properties.

Trichosanthes dioica Roxb. (Cucurbitaceae), called pointed gourd in English, is a dioecious climber found in the wild throughout the plains of North and North-East India from Punjab to Assam and Tripura states of India. It is also cultivated in India for its fruits, a common culinary vegetable in India. In India, all parts of this plant have been traditionally used for various medicinal purposes. (Bhattacharya et al., 2011). According to Ayurveda, the traditional system of Indian medicine, its root is a drastic purgative. The root has been traditionally used in India as a purgative and as a tonic, febrifuge, in the treatment of jaundice, anasarca, and ascites. The leaves and tender shoots are also used medicinally and as culinary vegetables in West Bengal and Assam, called as Palta in Bengali. Previous works have reported different phytochemical and pharmacological studies on *T. dioica* root, stem, and seeds in experimental animal models. However, limited research has been done on the fruit of *T. dioica* (Bhattacharya et al., 2011).

Although there are no *in vivo* studies on *T. dioica* fruit, *in vivo* studies have been conducted using the root of the plant. The study consisted of a mice liver cell model and found that *T. dioica* root was effective in its role as an antitumor and antioxidant in low dosages (Bhattacharya et al., 2011). A similar study, however, found that *T. dioica* root in a higher dosage can also aggravate oxidative stress and promote the proliferation of tumor cells in an *in vivo* model with mice (Bhattacharya & Haldar, 2011). Both studies in conjunction display the dose-dependent nature of *T. dioica* root in its respective effects and the balance needed between a low and high concentration. The seeds of *T. dioica* were also studied *in vivo* and found to possess antiglycemic effects (Prashant et al., 2008). The stem extract of *T. dioica* was found to contain antioxidant and antidiarrheal effects and the leaf extract was also found to be antioxidative (Aktar et al., 2011; Sharma et al., 2009).

1.5 Novelty & Significance

While various parts of the plant *T. dioica* have been studied for their antioxidant and other beneficial effects, the fruit has not been tested in an *in vivo* model, nor has optimal dosage for the fruit been found. The fruit itself has been found to have potential antioxidative. anti-inflammatory, and antipyretic effects due to its high flavanol count and its overall phenolic makeup (Alam et al., 2011). In addition, the *T. dioica* fruit is commonly consumed while the stem, seeds, and leaves are less prominent in cultural diets, emphasizing the need for research on the fruit.

Furthermore, *T. dioica*'s dose-dependent nature underscores the importance of determining the optimal concentration of a fruit extract to be utilized in future research. *T. dioica* have shown negative effects

at a high dosage but positive effects at a low dosage so it is essential to find the optimal concentration for supplementation. With an optimal dosage, *T. dioica*'s efficacy as a treatment can be investigated in conditions that are commonly associated with oxidative stress, such as cancer, diabetes, Parkinson's, and Alzheimer's disease.

1.6 Model Organism

Drosophila melanogaster is commonly utilized in a large variety of studies due to its homologous genetic structure to humans and its ability to model many human diseases and conditions. Similarly, *D. melanogaster* is commonly utilized to study oxidative stress (Vitorović et al., 2021). By studying the effects of *T. dioica* on a *D. melanogaster* with oxidative stress, conclusions on the fruit's potential oxidative properties and its optimal dosage can be determined in an *in vivo* model. The results of this study can then be utilized in future research to research *T. dioica* treatments for other conditions in *D. melanogaster*.

1.7 Inducing & Measuring Oxidative Stress

The compound paraquat is traditionally utilized in *D. melanogaster* models to induce oxidative stress with new variations of administration being researched. Paraquat is a toxic herbicide and weedkiller and is toxic to both humans and animals, especially if inhaled or ingested. However, paraquat, in conjunction with a sucrose solution, has traditionally been used to produce an oxidative stress *in vivo* model. One study found that 10 mM and 20 mM concentrations of paraquat in the traditional yeast/cornmeal diet for flies yields increased oxidative stress and oxidative stress related mortality (Rzezniczak et al., 2011).

Oxidative stress has also been associated with drastic variations in lifespan and thus a measuring lifespan is commonly conducted methodology with *D. melanogaster* to study variations in oxidative stress (Rzezniczak et al., 2011). To measure lifespan, mortality is commonly utilized. Mortality refers to the percentage of deceased subjects compared to the total number of subjects provided a certain treatment over a set period of time. Thus measuring mortality would yield insight into variations in oxidative stress levels.

1.8 Purpose

The purpose of this study is to determine if there are any potential antioxidative properties from *T. dioica* fruit extract and then to investigate the ideal concentrations of the extract that would most effectively reduce oxidative stress in a *D. melanogaster* model.

MATERIALS AND METHODS

2.1 Stock Maintenance

D. melanogaster utilized in the research were wild-type flies (w[1118]) obtained from Bloomington Drosophila stock center (#27898). Flies were maintained at 22°C on a standard yeast/cornmeal diet detailed in 2.2 Fly Food Preparation. To expand the stocks, flies were tapped into new food vials every four days and to maintain the stocks, the flies were tapped into new food vials every three weeks. To dispose of old fly vials, vials were placed in a -20°C freezer for one hour.

2.2 Fly Food Preparation

Fly food preparation begins by mixing 6.75 grams of yeast, 3.90 grams of soy flour, 28.50 grams of yellow cornmeal, and 2.25 grams of agar in a beaker by using an electronic balance, a weigh boat, and a scoopula. The wet ingredients were added to a separate beaker in the following quantities: 30 mL of light corn syrup, and 390 mL of deionized water. The wet ingredients and dry ingredients were mixed separately at first, then the dry mixture was poured into the beaker with the wet ingredients and was mixed until all clumps were removed. The beaker was then microwaved in intervals of 45 seconds and the solution was mixed in between. The process was repeated until the mixture began boiling, at which point it was removed using heat-resistant gloves, and a cheesecloth was placed over the mixture until it stopped steaming and the components were mixed in intervals to prevent hardening. Then 1.88 mL of 10% propionic acid was micropipetted into the beaker and mixed thoroughly to act as an antifungal. The mixture was then evenly poured into 50 vials, ensuring that approximately ⅕ of the vial was filled with food.

The process for preparing fly food was adjusted based on how many vials of food were required. The adjustments were made by maintaining the same ratios of ingredients. For example, if ten vials of food were required a ⅕th batch was created by dividing all the ingredient quantities by five.

2.3 Inducing Oxidative Stress

Paraquat (GlpBio, #GB60361) was utilized to induce oxidative stress and was administered to *D. melanogaster* by producing a ⅕th batch of standard yeast/cornmeal diet. After following Procedure 2.2 Fly Food Preparation, 10 mM paraquat was measured and poured into the food mixture right before 10% propionic acid was added. The mass of paraquat utilized was prepared using 421.88 mL solvent and 257.16 g/mol molar mass for paraquat. For example, if ⅕th batch was created, the total volume of the batch was 84.38 mL and 0.217 grams of paraquat was added. Age-matched flies were then transferred to the vial with paraquat.

2.4 Preparation of *T. dioica* Extract *(Adapted from Bhattacharya et al.'s 2011 study)*

T. dioica fruit was cut into quarter-inch slices and placed in a Ninja Foodi Digital Air Fry Oven with the dehydrate option selected. After the dehydrating process was completed the *T. dioica* was ground into a clumpy powder-like texture and macerated in a 20% ethanol solution for four days and the solution was gently shaken on the fourth day and allowed to re-macerate in the same solution for another three days. The solution was then sieved using a #40 mesh sieve to remove the ethanol, and *T. dioica* was then transferred back into the Air Fry Oven and the extract was dehydrated again. The final product was then ground into a fine powder.

2.5 Application of *T. dioica* Extract

The extract from 2.4 Preparation of Extract was administered to *D. melanogaster* through the standard yeast/cornmeal diet. The food was prepared in ⅕th batches of food. Each batch of food was created by adding 3.36 mg, 16.8 mg, 84.0 mg, 420.0 mg, and 2100.0 mg to produce concentrations of 0.04 mg/mL, 0.2 mg/mL, 1.0 mg/mL, 5.0 mg/mL, and 25.0 mg/mL, respectively. The extract was added and mixed alongside the paraquat from 2.3 Inducing Oxidative Stress.

2.5 Age Matching *D. melanogaster*

The study consisted of the use of *D. melanogaster* aged 1-3 days. This was accomplished by tapping *D. melanogaster* into a new food vial. Once pupae were observed on the edges of the vial, the parent flies were tapped out of the vial and disposed of according to 2.1 Stock Maintenance. After three days of the first fly eclosing, the flies in the vial were tapped into one of the respective experimental vials.

2.6 Conducting Mortality Assay

To quantify mortality rates in each of the experimental groups, the age-matched flies from 2.5 Age Matching were transferred into one of the experimental vials (which either contained normal food for control or supplemental food with paraquat, *T. dioica* extract, or both). After two days, the living flies were tapped into an empty vial and euthanized by placing the vial in a -20°C freezer for one hour. The number in both the experimental and empty vials was totaled and the number of deceased flies in the experimental vial was then divided by the total number of flies in both vials to produce a mortality rate.

2.7 Experimental Groups

The experiment consisted of four main groups, the negative control, the condition control, the toxicity control, and the experimental. The negative control consisted of *D. melanogaster* on a standard yeast/cornmeal diet and was utilized to establish mortality rates for healthy flies. The condition control consisted of *D. melanogaster* on yeast/cornmeal diets with paraquat using 2.3 Inducing Oxidative Stress. The condition control group was utilized to establish the oxidative stress-related mortality rate. The toxicity control consisted of *D. melanogaster* on yeast/cornmeal diets with 25.0 mg/mL *T. dioica* extract using 2.5 Application of *T. dioica* Extract. The toxicity control was created to ensure the concentrations of *T. dioica* extract did not adversely impact mortality rates by proving toxic. The experimental control consisted of *D. melanogaster* on diets of yeast/cornmeal diets with both paraquat and all the varying concentrations of *T. dioica* extract detailed in 2.5 Application of *T. dioica* Extract. The experimental control was utilized to depict the ideal concentration of *T. dioica* extract which effectively lowered the oxidative-stress-related mortality in *D. melanogaster*. Each of the four groups consisted of ten trials with each trial consisting of *D. melanogaster* from one vial (n=10). The purpose of this study is to determine if there are any potential antioxidative properties from *T. dioica* fruit extract and then to investigate the ideal concentrations of the extract that would most effectively reduce oxidative stress in a *D. melanogaster* model.

RESULTS

3.1 Data

Table 1. Average Oxidative Stress Related Mortality for Control Groups

	Average Mortality	Standard Error	Average Number of *D. melanogaster*	Average Deaths
Negative Control	0.39%	0.39%	49.1	0.2
Toxicity Control (25 mg/mL)	3.03%	0.64%	53.25	9
Condition Control	59.94%	3.02%	61.4	37.8

Table 2. Average Oxidative Stress Related Mortality for Experimental Groups with Differing Concentrations of *T. dioica*

	Average Mortality	Standard Error	Average Number of *D. melanogaster*	Average Deaths
0.04 mg/mL	54.79%	3.97%	67.9	36.2
0.2 mg/mL	46.40%	3.08%	58.9	27.3
1.0 mg/mL	36.63%	4.03%	48.1	17.8
5.0 mg/mL	41.74%	3.38%	56.5	26.2
25 mg/mL	45.17%	3.05%	64.1	28.2

3.2. Graph of Average Mortality per Group

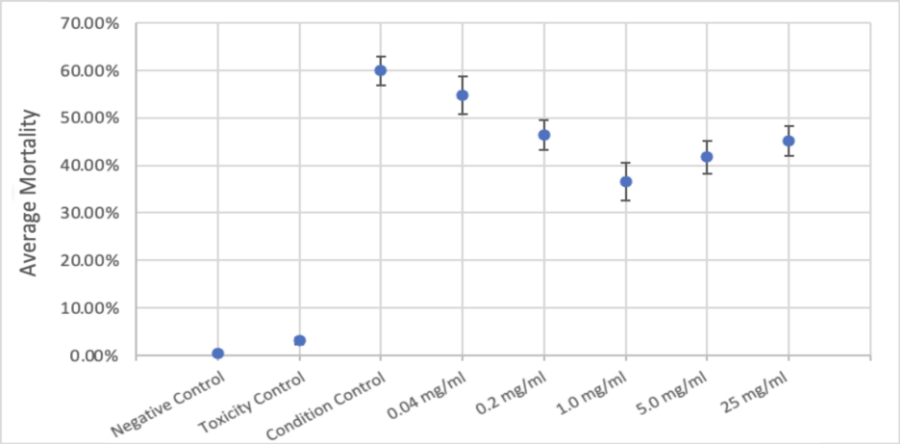

Figure 1. *T. dioica* improves oxidative stress-related mortality, the graph represents the combined data from *Table 1* and *Table 2*. The graph visualizes 1 SE.

3.3 Statistical Analysis

Table 3. Tukey Honest Significant Difference Post-Hoc Test P-values

	p-value
Negative Control vs Toxicity Control	0.99610
Negative Control vs Condition Control	**0.00001**
Condition Control vs 0.04 mg/mL	0.94500
Condition Control vs 0.2 mg/mL	**0.03890**
Condition Control vs 1.0 mg/mL	**0.00003**
Condition Control vs 5.0 mg/mL	**0.00120**
Condition Control vs 25.0 mg/mL	**0.01590**

*Bold values represent significant p-value for $p < 0.05$

DISCUSSION

4.1 Data Analysis

The mean mortality of the negative control of 0.39% was notably lower than the condition control's mean mortality of 59.94%. Additionally, the p-value comparing the mortality between the negative control and the condition control at 0.00001. This result suggests the increase in mortality is significant and verifies that paraquat increased oxidative stress mortality.

Additionally, the mean mortality for the negative control of 0.39% was similar to the mean mortality of the toxicity control of 3.03%. The p-value between the groups was 0.9961 which suggests that the difference in mortality rates is statistically insignificant. The results suggest that the dosages of *T. dioica* extract tested were non-toxic to *D. melanogaster* and did not cause any adverse effects.

For the experimental groups, the mean mortality of 54.79%, 46.40%, 36.63%, 41.74%, 45.17% for the concentrations of 0.04 mg/mL, 0.2 mg/mL, 1.0 mg/mL, 5.0 mg/mL, 25 mg/mL respectively were all lower than the mean mortality of the condition control of 59.94%. Furthermore, the p-values for the experimental groups all had a significant p-value ($p < 0.05$) except for the concentration of 0.04 mg/mL. This supports the hypothesis that *T. dioica* fruits possess antioxidant properties *in vivo*.

Looking at the most optimal concentration of *T. dioica*, a concentration of 1.0 mg/mL has the lowest average mortality rate at 36.63%. This concentration had a p-value of 0.00003 when compared to the condition control indicating a very strong decrease in oxidative stress-related mortality. The results suggest that there is a high probability that the concentration of 1.0 mg/mL is the most optimal concentration for alleviating oxidative stress from the concentrations tested in the study.

4.2 Implications of the Study

The study corroborates the results determined by Bhattacharya et al.'s 2011 study that *T. dioica* is dose-dependent, even with an extract derived from the fruit instead of the root. The study further underscores *T. dioica* fruit's antioxidant properties and establishes an ideal concentration to be utilized going forward in future *in vivo* studies regarding the model *D. melanogaster*. The study also elucidates *T. dioica* as a potential candidate for treatment in a variety of oxidative stress-associated conditions.

4.3 Limitations & Errors

While the study conducted research on the extract's antioxidant properties, the study did not account for the potential anti-inflammatory and antipyretic properties that were suggested by Alam et al., in their 2010 study. The study also did not specifically test the levels of reactive oxygen species present after each treatment, thus failing to conclude on region-specific variations in oxidative stress. The study also did not investigate potential tumor propagating or antitumor effects of *T. dioica* fruit extract as this effect was detailed in the root (Bhattacharya & Haldar, 2011).

4.4 Future Work

Possible future work for this study includes finding the optimal concentration of *T. dioica* fruits in a mammalian model. This would allow better conversion of the ideal concentration to a human. Furthermore, the exact mechanism of *T. dioica* which limits oxidative stress could be studied and isolated to create a more potent treatment for reducing oxidative stress.

Oxidative stress is also prevalent in a variety of debilitating neurodegenerative diseases such as Alzheimer's and Parkinson's disease and *T. dioica*'s ability to reduce oxidative stress in a Parkinson's disease model could also be researched.

T. dioica fruit has also been found to have potential as an anti-inflammatory and antipyretic agent, both of which were not studied in this study and could be pursued in future research.

CONCLUSION

Based on the results, when *D. melanogaster* was given *T. dioica* extract at various concentrations after being induced with oxidative stress through the dietary supplementation of paraquat, the concentration of 1.0 mg/mL proved to be the most effective at reducing oxidative stress-related mortality. As a result, the scope of the project has been achieved. This study allows for future research to study the effects of oxidative stress through the lens of *T. dioica* extract in vivo and with the usage of *D. melanogaster*. Furthermore, the study streamlines the process for

future research to utilize *T. dioica* as a potential treatment for various diseases and conditions that have elevated oxidative stress.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

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