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The effect of *Bacopa monnieri* on locomotion in a *D. melanogaster* Amyotrophic Lateral Sclerosis (ALS)

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SUMMARY

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that impacts nerve cells in the brain and spine. Specifically, it causes motor neurons to degenerate and die, stopping the transfer of neurological transmissions between the brain and muscles, eventually causing muscle weakness, locomotor deficits, and atrophy. The objective of this study is to investigate the effect of *Bacopa monnieri* on the locomotor function of *Drosophila melanogaster* with ALS. It was hypothesized that the oral administration of Bacopa monnieri to Drosophila melanogaster with ALS would suppress locomotor deficits and improve climbing ability compared to the untreated ALS group. Two hundred male flies consumed food with $4.62 \cdot 10^{-3}$ g/mL of *Bacopa monnieri* extract for seven days before undergoing a climbing assay to determine how many flies could climb eight cm in ten seconds. These results were compared to ALS flies that did not receive the extract, wild-type (WT) flies that did not receive extract, and WT flies that did receive extract. The Mann-Whitney statistical tests provided further support for the claim that ALS flies had improved climbing ability after consuming Bacopa monnieri. Based on the ALS+regular food vs. ALS+supplemented food test, it can be concluded that the extract had a significant impact on locomotive ability since the p-value was less than 0.05. From these results, it may be possible to use *Bacopa monnieri* or its extracts in ALS treatments or medications to help suppress locomotor impairments; however, more research should be conducted before doing so.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS), otherwise known as Lou Gehrig's Disease, is a progressive neurodegenerative disease that impacts nerve cells in the brain and spine. Specifically, it causes motor neurons in the spinal cord, motor cortex, and brain stem to degenerate and die, stopping the transfer of neurological transmissions between the brain and muscles, eventually causing muscle weakness, locomotor deficits, and atrophy (1). Although previous data shows that ALS is 20% more common in men than women, the presence of ALS has since become more equally distributed between the two groups (2). Approximately 90% of ALS cases have no known genetic cause; however, the remaining 10% typically inherit it via an autosomal dominant mutation from their parents (2).

The following genetic mutations are associated with ALS in humans: superoxide dismutase-1 (SOD1), fused in sarcoma (FUS), chromosome 9 open reading frame 72 (C9orf72), and TAR DNA-binding protein (TARDBP, encoding TDP-43). Specifically, the SOD1 gene is responsible for coding a Copper/Zinc superoxide dismutase, which catalyzes the conversion of Copper/Zinc superoxide ion, a toxic reactive oxygen species (ROS) produced when cellular respiration is taking place (3). Typically, in a SOD1 mutation, the proteins that are coded from the gene aggregate in the motor neurons, which results in the loss of the Copper/Zinc superoxide dismutase enzymatic activity and an increase in oxidative stress (4).

Inherently, the increase in oxidative stress due to ROS decreases the number of motor neurons and results in mitochondrial dysfunction. The mitochondrial dysfunction leads to the neurodegeneration in ALS patients, mainly affecting the brain stem and spinal cord (5). Due to this, typical symptoms of ALS include muscle weakness, speech difficulties, fatigue, and muscle twitches and cramps. Currently, there is no known cure for ALS, and most people live about two to five years after diagnosis (2).

In order to potentially alleviate a few symptoms of ALS, *Bacopa monnieri* will be tested in this study. *Bacopa monnieri*, otherwise known as “Brahmi,” “Aindri,” “water hyssop,” and the “herb of grace,” is a plant that has been used in Ayurvedic medicine due to its antioxidant, antiapoptotic, and anti-inflammatory effects. *Bacopa monnieri* is said to have a high bacosides, such as bacoside-A (6), an amphiphilic compound that contains sterol and sugar moieties, which is extremely effective in the central nervous system due to the decrease in the free radical aggregation in the brain (7). In a previous study, *Bacopa monnieri* has been tested on the symptoms of Parkinson’s disease, and the treatment has raised motor outcomes and improved emotional function amongst the test subjects (8). Additionally, in another study, *Bacopa monnieri* has been shown to regulate neuroinflammation and ROS generation in a Parkinson’s disease model. This research study concluded that the extract is a promising source of treatment for most central nervous system disorders (9).

Drosophila melanogaster will be used as a model to represent ALS in humans due to the homologous nature of the genes between the two. The genome of *Drosophila melanogaster* is 60% homologous to that of humans, and 75% of these genes are responsible for human diseases in flies (10). The SOD1 gene in humans is homologous to the *dSod1* gene in *Drosophila melanogaster*, mimicking ALS type 1 in humans to ALS type 1 in flies. Consequently, the strain of *Drosophila melanogaster* that represents ALS in this study is Sod1[X-39] due to its repeated use in prior research studies (11). In past research, the antiaging and antioxidant elevating activity of different plant extracts, like blueberries, in Drosophila was at least partially associated with its interaction with genes SOD (6).

The objective of this study is to investigate the effect of *Bacopa monnieri* on the locomotor function of *Drosophila melanogaster* with ALS. The significance of this study is to alleviate the symptoms of ALS, specifically the loss of climbing ability in flies. If *Bacopa monnieri* were to improve locomotor function, it could potentially be a supplement that can help with locomotor deficits for those diagnosed with ALS. However, if no correlation between the intake of *Bacopa monnieri* and the locomotor

function of *Drosophila* is found, it would be known that the extract does not help with this specific symptom of ALS.

It was hypothesized that the oral administration of *Bacopa monnieri* to *Drosophila melanogaster* with ALS would suppress locomotor deficits and improve climbing ability compared to the untreated ALS group. In the past, it has been noted that *Bacopa monnieri* decreases the amount of ROS present, and ultimately decreases oxidative stress (7). Since the lack of antioxidant defense is a leading pathological cause of ALS, the decrease in ROS due to *Bacopa monnieri* may alleviate common symptoms of ALS such as locomotor deficits. Additionally, *Bacopa monnieri* is said to have anti-Parkinsonian, another central nervous system disease, effects when it comes to locomotor function (9).

The independent variables of this study are the *Bacopa monnieri* extract and the presence of ALS in *Drosophila melanogaster*. This is because the extract will be used to test if there was a change in the locomotor function of the flies, and the locomotor function of ALS flies will be compared to those of the wild-type flies. The dependent variable of this study is the climbing ability of flies in order to determine their locomotor function. To measure the locomotor function of *Drosophila melanogaster*, a negative geotaxis assay will be used, which is quantified by the number of flies that cross an eight centimeter mark in ten seconds. Constants in this experiment include lab equipment used, the location of the experiment (Dr. Tomlinson's lab), the temperature of the flies and fly food (approximately 22°C), and the time of day the experiment is conducted (between 11:20 AM and 1:00 PM EST). Additionally, there are three control groups. The negative control will contain wild-type that did not consume *Bacopa monnieri* to determine the standard expected result of the climbing assay for healthy flies. The second group is the toxicity control, which contains the wild-type flies that consumed *Bacopa monnieri*. The purpose of this group is to determine what effect the extract has on normal flies. The genetic control group is ALS flies that did not consume saffron. The purpose of this group is to determine the poor locomotion flies with ALS

have before treatment. Lastly, the experimental group, or the group being tested, is the ALS flies that consumed the *Bacopa monnieri* extract.

A negative geotaxis assay was performed in order to test the locomotor function. For each of the ten trials for the three control and one experimental group, ten flies were tested. All flies were aged for ten days before being transferred to an empty vial marked eight centimeters from the bottom. An iPhone camera was used to record the movement of the flies. After the flies were given 30 seconds to acclimate to their new environment, the vial was harshly tapped, so that all the flies fell to the bottom, and a ten-second timer was started. After ten seconds, the recording was stopped and the number of flies that crossed the eight-centimeter mark was recorded in a data table. The median and average of each group's dataset was recorded and used to perform Mann-Whitney tests, comparing the negative control group with the toxicity control group, the negative control group with the genetic control group, and the genetic control group with the experimental group. The statistical values obtained from the tests were used to analyze data and draw conclusions to either support or reject the hypothesis that the oral administration of *Bacopa monnieri* to *Drosophila melanogaster* with ALS would help suppress locomotor deficits and improve climbing ability.

Currently, it is known that *Bacopa monnieri* has antioxidant effects in the hippocampus, frontal cortex, and striatum brain region. Additionally, *Bacopa monnieri* has been recently tested on mice, and it was determined that the free radical scavenging attribute of the extract might be a herbal remedy for stress-induced activities in humans. Another study was done on a *C. elegans* model of Parkinson's disease, and it was determined that the extract decreased aggregation of α-synuclein and neurodegeneration, providing a promising Parksinon's treatment (7). On the other hand, other extracts such as Spirulina have been tested on symptoms of ALS. From an experiment conducted on mice models of ALS, it was determined that the antioxidant properties of Spirulina has slowed the progression of the disease compared to untreated mice (12). Despite the past and current research done on *Bacopa monnieri* and ALS, there has been no correlation between the two yet. Additionally, the locomotor deficits caused by ALS have

rarely been tested in the past. Through results collected in this study, it is hoped to find a correlation between the consumption of *Bacopa monnieri* and locomotor deficits in the *Drosophila* model of ALS. If promising results are presented, the extract could be tested on other cognitive impairments such as decision-making in the future.

RESULTS

It has been noted that *Bacopa monnieri* decreases the amount of ROS present in neurons and ultimately decreases oxidative stress (7), but it is still unknown whether *Bacopa monnieri* affects locomotion in *Drosophila melanogaster* with amyotrophic lateral sclerosis (ALS). It is hypothesized that if flies with ALS are fed *Bacopa*, then they will be able to climb a great distance compared to flies with ALS that are not fed *Bacopa*. Locomotion was quantified using a climbing assay in which ten male flies were placed in a vial and given 10 seconds to climb eight centimeters. The number of flies that successfully climbed or flew past the eight centimeter mark "passed," and these values were converted into pass rate values and recorded (Table 1). After the pass rates were compiled, the mean pass rate was calculated. Additionally, the median pass rates were recorded using the "=MEDIAN()" command, which would be used during the Mann-Whitney U-tests.

Based on the raw data, it is apparent that the wild-type (WT) flies that did not consume *Bacopa* possessed the best climbing ability, followed shortly by WT flies that consumed *Bacopa*. When compared to the ALS flies that did not consume *Bacopa*, a significantly greater amount of ALS flies that consumed *Bacopa* seemed to have passed the climbing assay. Therefore, based on the raw data alone, it can be assumed that the *Bacopa monnieri* administration effectively improved the climbing ability of the ALS flies.

The Mann-Whitney statistical tests provided further support for the claim that ALS flies had improved climbing ability after consuming *Bacopa monnieri*. Based on the WT+regular food vs. WT+supplemented food, it was concluded that the supplement had no effect on the healthy flies since the p-value was greater than 0.05. Similarly, the

WT+regular food vs. ALS+regular food test demonstrated that the ALS phenotype had a significant impact on locomotive ability since the p-value was less than 0.05. Based on the ALS+regular food vs. ALS+supplemented food test, it can be concluded that the extract had a significant impact on locomotive ability since the p-value was less than 0.05. Finally, the WT+regular food vs. ALS+supplemented food test showed that the locomotive ability that was restored to the ALS flies by the extract is similar to that of the healthy flies.

From the data recorded, four Mann-Whitney tests were conducted using INSTAT, and the p-values and significance for each were recorded (Table 2). The first Mann-Whitney test was conducted to determine if there is a statistically significant difference between the median pass rates of wild-type flies that fed on regular food and wild-type flies that fed on $4.62 \cdot 10^{-3}$ g/mL extract-treated food. This Mann-Whitney test was conducted to check if the *Bacopa monnieri* powder treatment also improves the climbing ability of regular wild-type flies in addition to the ALS flies. From this test, a p-value of 0.7045 was obtained, which is greater than the significance level of 0.05 (Table 2).

The second Mann-Whitney test was conducted to determine if there is a statistically significant difference between the median pass rates of wild-type flies that fed on regular food and ALS flies that fed on regular food. This Mann-Whitney test was conducted to check if the ALS flies expressed poor locomotive function. From this test, a p-value of 0.0002 was obtained, which is less than the significance level of 0.05 (Table 2).

The third Mann-Whitney test was conducted between ALS flies that fed on regular food and ALS flies that fed on $4.62 \cdot 10^{-3}$ g/mL extract-treated food. This Mann-Whitney test was conducted to determine if the treatment food helped improve the climbing ability of the treated flies compared to their untreated counterparts. This test resulted in a p-value of 0.0002, which is less than the significance level of 0.05 (Table 2).

Lastly, the fourth Mann-Whitney test was conducted between wild-type flies that fed on regular food and ALS flies that fed on $4.62 \cdot 10^{-3}$ g/mL extract-treated food. This Mann-Whitney test was conducted to determine if the climbing ability that was restored by the *Bacopa monnieri* powder was similar to healthy levels. A p-value of 0.6216 was obtained from the results of this Mann-Whitney test, which is greater than the significance level of 0.05 (Table 2).

DISCUSSION

The purpose of this study was to determine if *Bacopa monnieri* powder would increase the locomotive abilities of *Drosophila melanogaster* with Amyotrophic Lateral Sclerosis caused by a SOD-1 gene mutation. It was hypothesized that the oral administration of the powder would increase climbing ability in *D. melanogaster* with ALS.

The mean pass rate for the wild-type flies that fed on regular food was 91.36%, while the mean pass rate for wild-type flies that fed on extract-treated food was 92.40% (Table 1). Even though there was a slight difference in the mean pass rates, since the p-value for the Mann-Whitney test comparing these two groups was 0.7045, which is greater than the significance level of 0.05, there is sufficient evidence from the sample data to support that there is no statistically significant difference between the median pass rates of wild-type flies that fed on regular food and wild-type flies that fed on *Bacopa monnieri*-treated food (Table 2). Therefore, it was concluded that the climbing ability of the wild-type flies that consumed the *Bacopa monneri* was not affected.

The mean pass rate of the wild-type flies that fed on regular food and ALS flies that fed on regular food, however, were significantly different. The mean pass rate for the wild-type flies that fed on regular food was 91.36% while the mean pass rates for the ALS flies that fed on regular food were 64.80% (Table 1). The difference between the two pass rates portray that, when left untreated, the ALS flies had significantly lower pass rates compared to the wild-type flies. The results of the Mann-Whitney test support this conclusion; the p-value of the test was 0.0002, which is less than the significance

level of 0.05 (Table 2). Therefore, there is sufficient evidence from the sample data to support that there is a statistically significant difference between the median climbing assay pass rates of wild-type flies that fed on regular food and ALS flies that fed on regular food. Therefore, the climbing ability of ALS flies was seen to be significantly worse than regular wild-type flies.

The mean pass rate for the wild-type flies that fed on regular food was 91.36%, while the mean pass rate for ALS flies that fed on extract-treated food was 89.84% (Table 1). Even though there was a slight difference in the mean pass rates, since the p-value for the Mann-Whitney test comparing these two groups was 0.6216, which is greater than the significance level of 0.05, there is sufficient evidence from the sample data to support that there is no statistically significant difference between the median pass rates of wild-type flies that fed on regular food and ALS flies that fed on *Bacopa monnieri*-treated food (Table 2). Therefore, it was concluded that the climbing ability restored in ALS flies by the *Bacopa monnieri* is similar to that of normal, healthy flies.

Perhaps most importantly, the hypothesis that the climbing ability of ALS-afflicted flies will improve with the consumption of *Bacopa monnieri* powder was accepted. The pass rate of ALS flies that fed on regular food was 64.80%, and the pass rate of ALS flies that fed on extract-treated food was 89.84% (Table 1). There was a significant increase in the climbing ability based on the difference between the average pass rates alone. This was also supported by the Mann-Whitney statistical test: since the p-value of 0.0002 was less than the significance level of 0.05, there is sufficient evidence from the sample data to support that there is a statistically significant difference between the median pass rates of ALS flies that fed on regular food and ALS flies that fed on treated food. The climbing ability of ALS flies that fed on $4.62 \cdot 10^{-3}$ g/mL *Bacopa monnieri* food was significantly better than that of ALS flies that fed on regular food, and therefore, the hypothesis was accepted.

There are limitations to this experiment; however, the most evident limitation would be the amount of time we had to complete the experiment. As we only have 100 minutes

each block to complete the trials, some of the experimentation was done outside of class to collect timely data.

This study also had a few areas where errors might have occurred throughout the experiment. Some of these instances include the amount of fly food in each vial. Usually only an inch of the vial should be filled with food, but sometimes there was an excessive amount of food in the vial. Another error would be the chunks of food in some of the vials, in the extract food these chunks would result in some *Drosophila melanogaster* to have more concentration of the extract than other flies in the vial. The chunks of food were most likely caused by improper mixing while making the fly food.

Experimentation for this study can be continued by looking into the dosage of *Bacopa monnieri* administered to see if it can be altered to achieve better climbing ability in *Drosophila melanogaster* affected by ALS. Eventually, experimentation should also be done in a controlled experiment with other organisms, like mice as they have similar processes to humans in terms of aging, similar endocrine systems, and similar responses to infections and diseases, to see if results remain consistent. If so, *Bacopa monnieri* extract may be used as a supplement for humans with ALS. This research will allow further understanding of the connection between *Bacopa monnieri* extract and locomotive function in those affected with ALS.

MATERIALS AND METHODS

Variables and Constants:

The independent variables in this experiment are the presence of *Bacopa monnieri* in the fly food and the presence of ALS in the flies. This is because the extract will be used to test if there was a change in the locomotor function of the flies, and the locomotor function of ALS flies will be compared to those of the wild-type flies.

The dependent variable is the climbing ability of the flies in 10 seconds. More specifically, it quantifies the number of flies that are able to climb past an 8 cm line. This

would be the dependent variable as the locomotion of the fruit flies will be tested in order to determine if the *Bacopa monnieri* extract had affected it. This dependent variable will be measured using the Negative Geotaxis Assay, which is used to measure the climbing ability of *D. Melanogaster*.

While conducting this study, there were a few constants throughout each of the trials. Throughout the study, only male *Drosophila melanogaster* were tested. For all of the ALS trials, the Sod1[X-39] flies were used, and for all of the Wild-type trials, the w[1118] flies were used. The *Drosophila melanogaster* were tested seven days after they emerged from their larvae, where they were either feeding on regular fly food or *Bacopa*-concentrated fly food for the entire duration. Each vial of flies had 10 flies, which were maintained at a temperature of 22°C and a constant humidity. Other rearing constants include a 12 hour day and 12 hour night cycle for the flies throughout the study, and the time of day the flies were tapped and tested was between 11:20 A.M. and 1:00 P.M. For all trials, a negative geotaxis assay was performed with a constant climbing height of 8 centimeters.

Control and Experimental Groups:

There will be three control groups. The negative control will contain wild-type flies that are not given *Bacopa monnieri*. The purpose of this group is to determine the standard expected result of the climbing assay for healthy flies. The second group is the toxicity control; the group of wild-type flies that are given *Bacopa monnieri*. The purpose of this group is to determine what effect *Bacopa monnieri* has on healthy flies. It is expected that these flies will not be affected by the *Bacopa monnieri* and will climb similarly to the negative control flies. If the *Bacopa monnieri* affects the wild-type flies, there may be a flaw in the experimental design. The third and final genetic control group will be ALS flies that are not given *Bacopa monnieri*. This group will be used to determine how much a fly with ALS that receives no treatment should act. Since the ALS flies should display locomotor defects, it is expected of them to perform significantly worse than the negative control, toxicity control, and experimental groups. The experimental group, or the group being tested, are ALS flies who receive the *Bacopa monnieri* treatment.

Materials:

The materials used for this study were abstained through the Academies of Science unless otherwise specified. However, the w[1118] (#3605) and Sod1[X-39] (#24490) strains of *Drosophila melanogaster* were abstained from Bloomington *Drosophila* Stock Center. The *Bacopa monnieri* powder used for the treatment food was obtained from Amazon.com. Additionally, a stopwatch was used from an iPhone to time the climbing time for the climbing assay performed on the flies.

To prepare *Drosophila melanogaster* food, water, soy flour, yellow corn meal, light corn syrup, agar, yeast, 10% Propionic acid, *Bacopa monnieri* powder, a scoopula, a 500 mL beaker, a stirring rod, a weigh boat, an electronic balance, a cheesecloth, vials, and flugs were used. Additionally, a microwave (*Panasonic NN-SN936W*) was used to heat up the food during the process of combining all the ingredients. For cold sorting, a cold sorting pad (*serial no. 213200912*) was used to sort the anesthetized flies. Additionally, an ice bucket, weigh paper, feather, and magnifying glass was used to separate the male flies from female flies before transferring to vials before testing.

Lastly, safety precautions were taken during this study. To prevent damaging clothes from hot fly food, lab coats, goggles, gloves and close-toed shoes were worn. Additionally, when operating the microwave, hot hands were used to prevent skin from burning from the hot fly food.

Fly Food Preparation (13):

To prepare regular fly food, ensure goggles, lab coats, and closed toed shoes are worn at all times. Also, make sure to tie back long hair. Goggles will protect your eyes from the chemicals and flies, lab coats will protect your body and skin from the chemicals, closed toed shoes will protect your feet from glassware, and tying back hair will prevent it from getting caught in machinery or in chemicals. It is important to follow these precautions at all appropriate times. Obtain a weigh scale, weigh boat, and scoopula. Zero the scale before starting, and measure out 28.50 grams of yellow cornmeal. Add to

a 1 L beaker. Similarly, measure 3.90 grams of soy flour, 2.25 grams of agar, and 6.75 grams of yeast and add them to the 1 L beaker as well. Use a 100 mL graduated cylinder to measure 30 mL light corn syrup, and pour it into the 1 L beaker that contains the dry ingredients. Measure out 390 mL of distilled water in the same 100 mL graduated cylinder to get the corn syrup off the sides. Add the water to the 1 L beaker. Stir well until the wet and dry ingredients are well incorporated. Microwave the mixture at 30 second intervals until it starts to boil. This typically takes about 2 minutes, or four intervals. Between each interval, remove the beaker from the microwave, place it on a paper towel, stir with a glass stirring rod, and put it back in the microwave until it starts boiling. Use hot hands to take the beaker out of the microwave as the glass will be extremely hot. Once the mixture is boiling, use hot hands to remove the beaker from the microwave and allow the food to cool to room temperature. Make sure to cover the food with a cheesecloth and put a textbook on top of the beaker to prevent contamination. After it reaches room temperature, use a micropipette to add 1.88 mL of propionic acid to the food. Stir with a stirring rod until well incorporated. Pour the food into individual vials. The vials should be about one-fourth full of food. Cover the vials with cheesecloth and put a textbook on top of them. Wait until the food cools entirely before putting the foam plugs on the vials. Store the plugged vials in the refrigerator until necessary. If preparing treatment food with *Bacopa monnieri*, follow the above steps exactly, but add 2.140 grams of *Bacopa monnieri* extract powder to the food along with the dry ingredients. Stir with a glass stirring rod until the suspension is spread throughout before adding the wet ingredients.

- *Bacopa monnieri* Concentration Calculations: $4.62 \cdot 10^{-3}$ g/mL (14)
    - Total amount of food needed for research
        - 30 mL corn syrup + 390 mL distilled water + 1.88 mL propionic acid = 421.88 mL wet ingredients
        - 6.75 g yeast + 3.90 g soy flour + 28.5 g yellow cornmeal + 2.25 g agar = 41.4 g dry ingredients
        - 1 g = 1 ml dry ingredients
        - 41.4 mL + 421.88 mL = 463.28 mL
    - Grams of *Bacopa monnieri* required

- $0.00462 \frac{g}{ml} \cdot 463.28$ ml = 2.140 g

Continue the rest of the steps, as usual. Be sure to separate the food vials with *Bacopa monnieri* from the regular food so the experiment's results are not compromised.

Culturing and Maintaining Flies (13):

To maintain the flystock the vials of flies must be incubated and hydrated, to do this the food must be kept damp but the sides of the vials should be dry. It is also important to make sure that the temperature must be maintained at 22°C so that the fly life cycle can last up to two weeks. The fly vials must be checked regularly to ensure that they are not overpopulated. If there is minimal food and overcrowding of the *Drosophila melanogaster*, it is best to assume that the vial is overpopulated. If removing flies from a vial, they should be anesthetized so that they can be separated into two different vials. While removing flies from a vial to prevent overpopulation, only adult flies should be removed from the vial, but the fly eggs should remain inside. Flies should transfer vials every 3 weeks if they are not being experimented on. To expand a fly stock, they must be tapped to encourage reproduction. Flies must be tapped into different vials once larvae are seen to make sure that the new flies can mate and reproduce.

There are many threats to the fly stocks, some of these threats include dryness, mites, fungus/mold, and contamination. To prevent dryness the food must be kept refrigerated, and food older than 3 weeks should not be used. If there is dryness in the food of the vial it can be treated using distilled water, only a little should be used to fill in cracks and spaces. To prevent mites from getting into the fly stock utensils, tools, and stations must be wiped with 70% ethanol before sorting the flies. To prevent fungus and mold propionic acid is added to the food, in case there is moldy food the flies in the vial must be tapped into a new vial immediately. To prevent contamination in the lab it is important to check fly appearances when sorting, the food must be covered at all times. In case a fly stock is contaminated, all stocks must be thrown away to prevent it from spreading.

To tap flies into new vials, obtain both the old vial with the flies that need to be tapped and a fresh food vial from the refrigerator. With the fly vial in one hand and the fresh vial uncapped and upside down in the other, vigorously tap the fly vial on the lab bench. This momentarily brings the flies to the bottom of the vial. Quickly, pop off the flug and place the fresh vial at the top of the fly vial to prevent flies from escaping. Carefully, hold the two vials together and quickly flip them so that the fresh vial, which should now contain the flies, is on the bottom and the old vial is on top. To transfer the flies into the new vial, continue to hold the two vials together and tap them on the lab bench. Tap the new vial to bring the flies to the bottom, remove the old vial from the top, and quickly insert a flug to prevent flies from escaping. Label the new vial with the researcher's initials, the date, and type of flies/stock number and store them at 22°C. In order to expand a stock, tap flies into new vials every four days. To maintain a fly stock, tapping them into new vials every three weeks will suffice.

To dispose of flies, they can be freezed at -80°C, and disposed of in the trash bin. If a vial needs to be kept, after being frozen the flies can be tapped out of the vial, the vial can then be washed so that it could be reused.

Cold Sorting (13):

To gender sort, it is important to distinguish male flies from female flies. Female flies are typically larger, have a light and pointed tip, and have no sex combs. Virgin female flies have a dark spot on their abdomen. On the other hand, male flies are typically smaller, have a dark and rounded tip, and have sex combs.

To cold sort, set the cold sorter to 4°C. Ensure that it reaches this temperature before proceeding to the next step to keep the flies anesthetized for the whole procedure. Tap the flies into an empty vial by following the procedure detailed in the "tapping flies" section above. Submerge the vial in an ice bath for about five minutes, or until the flies are asleep. Check on them regularly and take them out as soon as they are asleep.

Place a piece of weighing paper on the cold sorter plate, and gently tap the asleep flies onto it. Use a magnifying glass to look at the flies and determine their sex. Use a feather brush to move them around and see the defining characteristics of a male or female fly. After sorting them into groups based on their sex, transfer them into a new vial based on gender. Discard the flies that will not be used in the experiment. Follow the discarding method outlined in the “disposal of flies” section.

Negative Geotaxis Assay (Figure 2):

Cold sort 10 male flies from one of the four experimental groups and transfer them into new food vials. Wait until the flies are seven days old to begin the climbing assay.
Use a ruler to measure 8 cm from the bottom of a clear fly vial and mark this point with a marker. Tap the Drosophila from their food vial to an empty vial and seal the top with another empty vial to keep the flies from escaping. Tape the two vials together with clear tape to easily see which flies pass the 8 cm mark. Set up a video camera and a timer next to the vial in such a way that the timer is visible in the recording. Start the video, set the timer for 10 seconds, and harshly tap the vial so the flies fall to the bottom. Start the timer immediately after the flies reach the bottom of the container. Once the 10 seconds are up, stop the recording. Dispose of these flies by following the discarding method outlined in the “disposal of flies” section. Repeat steps 1-6 for five times for each of the ten trials per group, for each of the four groups. Ultimately, this will result in 200 climbing assays. Analyze the video by recording the total number of flies in the vial and the total number of flies that crossed the 8 cm line. If a fly crossed the line and then went below, it does not count. Similarly, if a fly crossed the line multiple times, it only counts once. If a fly is on the line at the end of the 10 seconds, it does not count. Calculate the percentage of flies that passed the line for each group in each trial.

Safety, Possible Risk Involved, and Disposal of Materials:

Wear closed toed shoes at all times in the laboratory to prevent glass, heavy objects, or chemicals from coming into contact with your feet. Wear goggles and a lab coat when conducting the experiment. This keeps the skin and eyes safe from chemicals and

glassware. Use hot hands when making fly food to prevent burning yourself. Tie back long hair to keep it from getting caught in machinery or in chemicals.

The materials used will be disposed of by either being placed in the glass disposal bin, or in the sink to be washed. The chemicals being used will also be disposed of safely by making sure that they go in the waste disposal containers. To dispose of propionic acid, unused product should be left in the container and can not be disposed in household garbage. For the disposal of *Bacopa monnieri* powder, the product should be left in the original container and not mixed with other waste. Additionally, *Bacopa monnieri* should not be disposed in household garbage.

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Figures and Captions

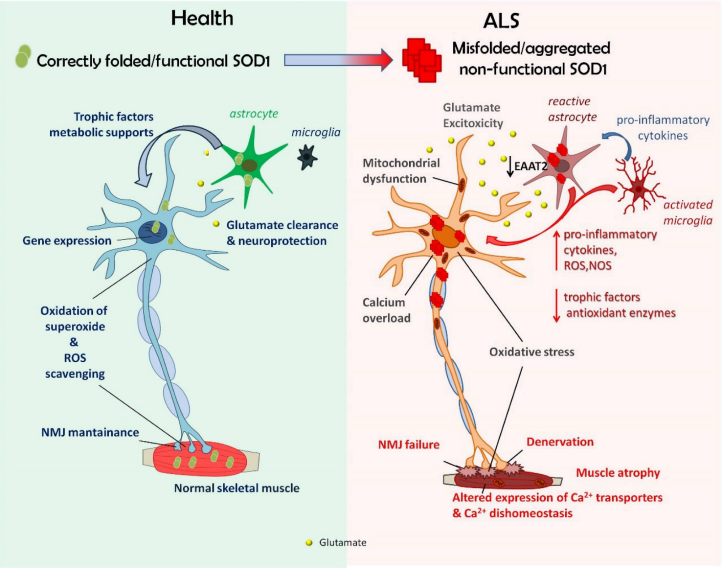


Figure 1: Comparison of regular SOD1 gene to the misfolded SOD1 gene (15). The figure above represents how the misfolding of the SOD1 gene increases the amount of reactive oxygen species and oxidative stress in the brain.

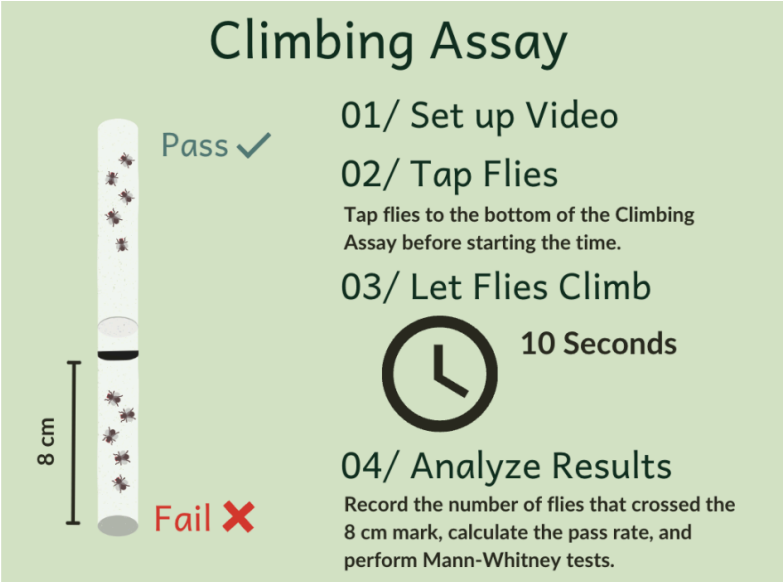
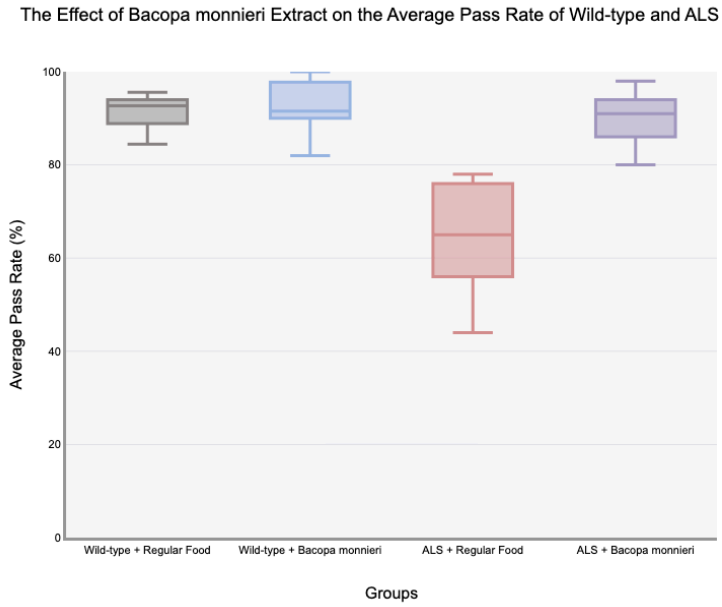


Figure 2: Negative geotaxis assay procedure. The figure above represents how a negative geotaxis assay is conducted. The flies that cross the 8 centimeter mark are considered passed, while the rest of the flies are considered failed. A negative geotaxis assay is conducted for approximately 10 seconds, which should be timed with an iPhone.



Graph 1: Pass rates of flies from the negative geotaxis assay for the four groups. The x-axis are the four different groups and the y-axis is the average pass rates (Table 1). In the graph, the median, minimum, maximum, first quartile, and third quartile pass rates are represented.

Tables and Captions

Effect of *Bacopa monnieri* Extract on the Pass Rate of *D. melanogaster*				
Trial	**Pass Rate (%)**			
	Wild-type + Regular Food	**Wild-type + *Bacopa monnieri***	**ALS + Regular Food**	**ALS + Bacopa monnieri**
1	94.00	94.00	62.00	92.00
2	93.33	90.00	76.00	92.00
3	84.44	82.00	76.00	96.00
4	88.89	88.00	68.00	98.00
5	88.00	91.11	52.00	84.44
6	94.00	100.00	74.00	90.00
7	92.00	92.00	78.00	86.00
8	90.00	91.11	62.00	86.00
9	95.56	97.78	56.00	80.00
10	93.33	98.00	44.00	94.00
Mean	**91.36**	**92.40**	**64.80**	**89.84**
Median	**92.67**	**91.56**	**65.00**	**91.00**

Table 1: Pass rates of flies from the negative geotaxis assay for the four groups. For each trial, five different climbing assays were conducted and then averaged into one cell for each of the vials, and a total of 10 vials of flies were tested per group. Overall, 50 trials were conducted for all four groups. The mean and median pass rates are also represented in the yellow cells.

Mann-Whitney U-test Stastical Results		
Groups	**p-value**	**Significance**
Wild-type + Regular Food vs. Wild-type + *Bacopa monnieri*	0.7045	Not Significant
Wild-type + Regular Food vs. ALS + Regular Food	0.0002	Significant
ALS + Regular Food vs. ALS + *Bacopa monnieri*	0.0002	Significant
Wild-type + Regular Food vs. ALS + *Bacopa monnieri*	0.6216	Not Significant

Table 2: Mann-whitney U-test results. The table represents the p-values and significance for each of the four Mann-Whitney tests that were conducted. For each of the Mann-Whitney tests, the significance level of 0.05 was used. A p-value for a Mann-Whitney test below 0.05 was considered significant, while a p-value for a Mann-Whitney test above 0.05 was considered not significant.