**Group 1-16: Effects of Chinese herbal extracts on Amyotrophic Lateral Sclerosis (ALS) - a study using a Drosophila melanogaster model**

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**Abstract**

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterised by a loss of function in motor neurons, and hence a loss of function in muscles, which is fatal. This study aims to investigate the effects of ginseng root and ginkgo leaf on symptoms of ALS in a *Drosophila melanogaster* ALS model. To induce symptoms of ALS, two models were used; the G85R mutation, and hydrogen peroxide. Fly locomotor function was studied using a climbing assay to determine the degree of neurodegeneration, in which the number of flies climbing past a certain height (10 cm, 15cm) within 30 seconds was counted. Our findings revealed that the flies with the G85R mutation and those fed with hydrogen peroxide showed decreased locomotor function and signs of neurodegeneration, and that ginseng root and ginkgo leaf were able to restore or even stimulate locomotor function, implying that these herbs could possibly be used to treat or prevent neurodegeneration.

## 1. Introduction

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is a neurodegenerative disease characterised by a loss in function of motor neurons, which causes a loss in function of muscles (NINDS, 2013). This is generally fatal within 2 to 5 years of diagnosis, due to respiratory problems caused as the diaphragm weakens. The number of ALS cases is expected to rise from 222,801 in 2015 to 376,674 in 2040, an increase of 69% mostly due to ageing populations (Arthur et al., 2016), hence it is vital to be able to treat ALS. Currently, there are only 2 FDA-approved drugs to treat ALS, riluzole and edaravone. Riluzole is used to slow down the disease progression of ALS, extending life expectancy for patients, but causes numerous side effects including rashes, nausea and jaundice (MedlinePlus, 2019). Edaravone only helps to improve daily function in patients by treating symptoms of ALS, but does not slow down disease progression (Cruz, 2018).

In our study we used herbs commonly used in Traditional Chinese Medicine (TCM) to treat symptoms of ALS. The two herbs we chose to use are *Panax ginseng* and *Ginkgo biloba*. *Panax ginseng* was used in the form of ginseng root, and it is a neuroprotective herb due to the presence of the active ingredient ginsenosides (Huang, Li, Pu, Zhang & Wang, 2019). Ginseng has been

studied in a transgenic mice ALS model, where it was shown to delay the onset of symptoms and extend lifespan, due to its ability to reduce neuronal death and increase expression of nerve growth factor, as well as its antioxidant properties (Jiang, DeSilva & Turnbull, 2000). *Ginkgo biloba* was used in the form of leaf extract, and it is a neuroprotective herb that also has neuroregenerative properties. It demonstrated antioxidant properties, and was able to stimulate the growth of neural stem cells, promoting neurogenesis (Raghavan & Shah, 2014). *Ginkgo biloba* extract has been used successfully in treating central nervous system (CNS) disorders in animal models, and as a symptomatic treatment for dementia in numerous studies. Thus, both herbs have potential for treating neurodegenerative diseases, and we hope to investigate their potential for treating ALS.

The model organism we used is *Drosophila melanogaster*, commonly known as the fruit fly. It is cheap and easy to culture, and 75% of disease causing genes have a functional homolog in the *D. melanogaster* genome, allowing similar disease pathways to be simulated (Pandey & Nichols, 2011). To model the disease pathway of ALS, we used 2 methods; the G85R mutation, and hydrogen peroxide.

The G85R mutation is a mutation of the SOD1 gene in the fly genome, which codes for the SOD1 protein. The SOD1 gene is highly conserved between humans and *D. melanogaster*, and is the first ALS-linked gene to be discovered in 1993 due to SOD1 mutations being found in ALS patients (Pansarasa et al., 2018). In particular, the hallmark of SOD1-associated ALS is the presence of misfolded SOD1 protein in small aggregates in the nuclei of glial cells of the spinal cord. The G85R mutation in *D. melanogaster* was found to induce neurodegeneration, locomotor deficits, and shortened lifespan, as it rendered the SOD1 protein enzymatically inactive (Şahin et al., 2017). In a mice model, it was also found to cause SOD1 to deposit as insoluble aggregations in motor neurons, matching the hallmark of SOD1-associated ALS. Hence, the G85R mutation is able to simulate the disease pathway of SOD-1 associated ALS.

The other method used was hydrogen peroxide. Hydrogen peroxide is a commonly used oxidative stressor, and it is well-established as a reactive oxygen species (ROS) generator as it slowly decomposes to form the highly reactive hydroxyl radical (Wu et al., 2018). Oxidative stress has been strongly associated with ALS, with increased oxidative stress in ALS pathogenesis and

increased oxidative damage to protein in ALS post-mortem tissue (Barber, Mead & Shaw, 2006). In fact, edaravone's therapeutic properties for ALS have been attributed to its known antioxidant properties, as oxidative stress is one of the main mechanisms through which motor neurons are killed in patients with ALS (Cruz, 2018). Additionally, oxidative damage is one of the reasons why the SOD1 protein may be misfolded and form protein aggregations, hence becoming neurotoxic as stated previously (Pansarasa et al., 2018). Hence, we hope to use hydrogen peroxide to investigate the role oxidative stress plays in neurodegeneration, and to simulate symptoms of ALS.

**Objectives and Hypotheses**

Our objectives are to

1. Induce symptoms of ALS in *D. melanogaster* using the G85R mutation and hydrogen peroxide,
2. Treat/reduce severity of symptoms of ALS in *D. melanogaster* using ginseng root and ginkgo leaf.

Our hypotheses are that

1. The G85R mutation or hydrogen peroxide will induce neurodegeneration and locomotor deficits in *D. melanogaster*,
2. Ginseng root and ginkgo leaf can improve the locomotor function of *D. melanogaster* showing symptoms of ALS.

## 2. Materials and Methods

### 2.1 Materials

Wild type flies, premixed fly food powder, dried yeast extract and fly vials were purchased from the Carolina Biological Supply Company (Burlington, NC) while the G85R mutant flies were purchased from Bloomington Drosophila Stock Center (Bloomington, IN) under the stock number 33608. Dried ginseng root and ginkgo biloba leaf extract tablets were purchased from a local traditional Chinese medicine store. Hydrogen peroxide and a plastic tube were obtained from the SRC.

### 2.2 Organisation of flies into experimental groups

The flies were first anesthetised by placing them in a freezer for 1 minute in order to transfer them into different fly vials. For the experiments conducted using the hydrogen peroxide model, the wild type flies were split into four groups, with about 30 flies each. The first group was a negative control, fed normal fly food. The second group of flies had 0.2% hydrogen peroxide solution added to their fly food to induce symptoms of ALS. The last 2 groups consisted of wild type flies with 0.2% hydrogen peroxide solution, and ginseng or ginkgo extracts added to their fly food. For the experiments conducted using the mutant fly model, the mutant flies were split into two groups, with about 10 flies each. The first group of flies were left untreated to act as a control and the second group of flies had ginseng extract added to their fly food. Flies were grown in the plant culture room at SRC biology lab.

### 2.3 Preparation of herbal treatment

The dried ginseng root was blended into fine powder while the gingko biloba tablets were grounded into fine powder using mortar and pestle. 30ml of hydrogen peroxide solution was then microwaved for 30 seconds before 0.3g of the herb powder was added to it. The mixture was then stirred at 75$^{o}$C using a magnetic stirrer and hot plate for 5 minutes. For the experiment involving the mutant flies, 30ml of water was added instead of hydrogen peroxide solution to prepare the ginseng herbal extract.

### 2.4 Preparation of fly medium

5ml of premixed fly food powder was added to a fly vial before 5ml of water was added and yeast extract was sprinkled on top. For the experiment on the wild type flies, 0.2% hydrogen peroxide solution was added instead of water to induce ALS symptoms in the flies. For the experimental groups that received the herbal treatment, 5ml of the herbal extract prepared was added to the fly vial instead. For the mutant flies experimental setup, the control group were fed ordinary fly food with water added but 5ml of the ginseng herbal extract was added to the fly food medium of the experimental group.

### 2.5 Climbing assay

The climbing assay was conducted to investigate the locomotor function of the flies. It utilises the negative geotaxis response of flies, which is an innate motor reflex that causes flies to climb upwards when stimulated. A plastic tube of height 20 cm was used, and markings at heights 5cm, 10cm, 15cm and 17.5cm were made. The flies were transferred to the plastic tube after being anaesthetised by freezing for 1 minute, then allowed to recover from anaesthesia for 10 minutes, following which the tube was tapped from the bottom to stimulate climbing. The flies were filmed for 30 seconds, before the video were analysed to determine the number of flies crossing each marking within 30 seconds. A higher height climbed by the flies would indicate a better locomotor function and less severe neurodegeneration. Two days after the flies were separated into their experimental groups, the climbing assay was conducted over the next 3 days. One reading for the number of flies climbing past each marking was obtained each day and the average reading was calculated using the 3 daily readings obtained.

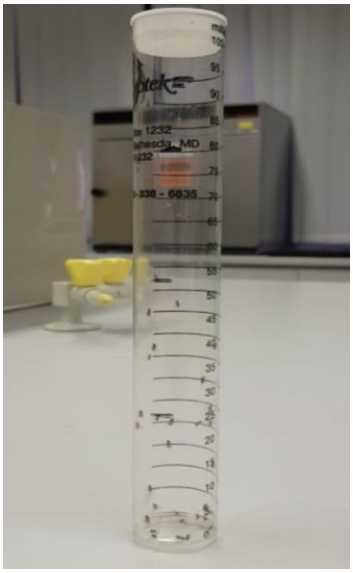

Fig 1. Image of climbing assay being conducted

### 3. Results and discussion

Due to shipping restrictions, only 2 vials of wild-type flies or 1 vial of mutant flies could be obtained per order (hence the limited numbers of flies per group used). There were insufficient flies for experiments to have multiple groups, and due to time constraint imposed by circuit breakers, only 1 cycle of experimentation could be carried out. **Due to lack of replicates, all results presented here are only preliminary, and there are no error bars for most graphs**.

### 3.1 Hydrogen peroxide ALS model

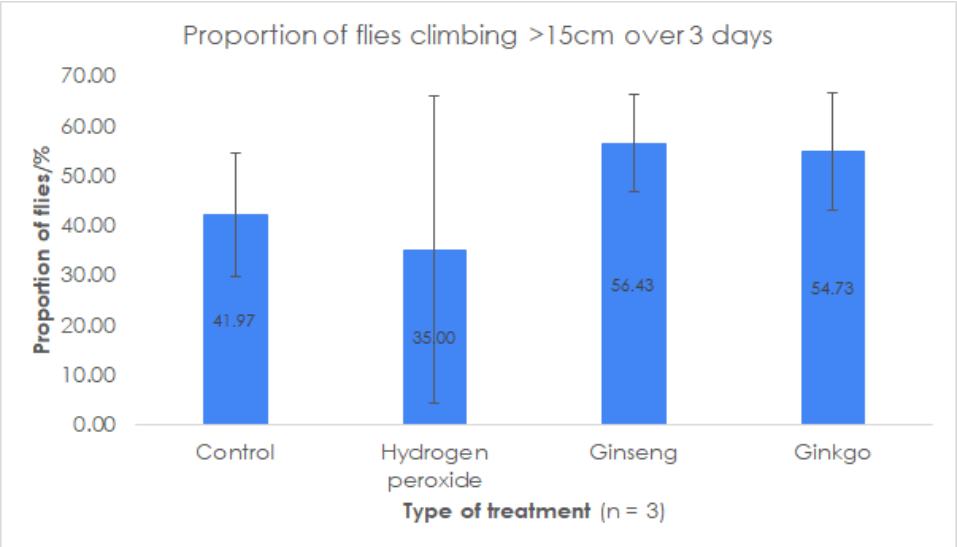


Fig 2. Proportion of flies that climbed more than 15cm over 3 days

For an overall comparison between the different setups, the average proportion of flies climbing more than 15 cm for each setup was calculated based on daily readings obtained over 3 days. It was observed that the hydrogen peroxide treatment reduced the locomotor function of the flies, with a smaller average proportion of flies climbing more than 15 cm compared to the control group. Both the ginseng and ginkgo treatment were not only able to mitigate the effects of the hydrogen peroxide on the flies, but also improved their locomotor function, with more flies from both herb treatment groups climbing more than 15 cm on average than flies in the control group.

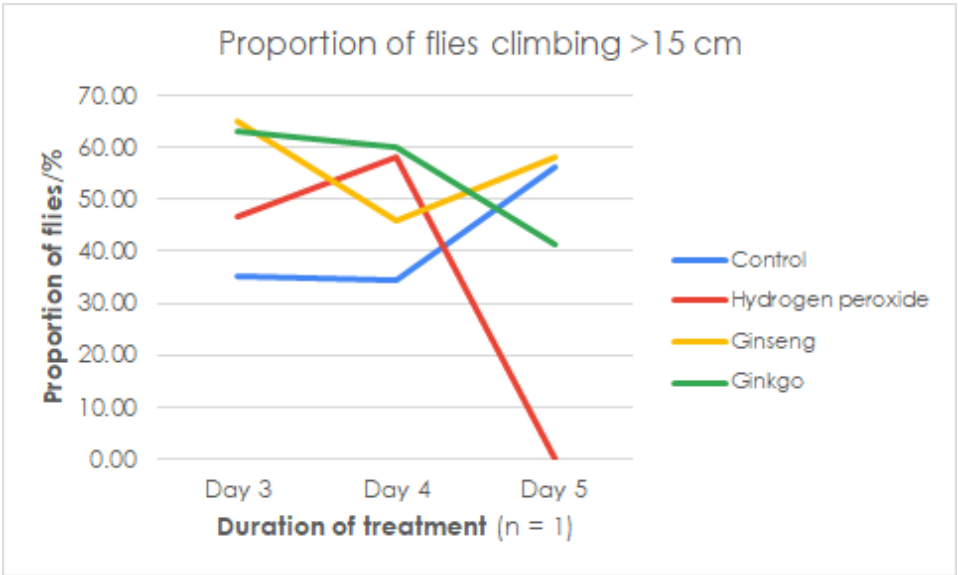


Fig 3. Proportion of flies that climbed more than 15cm over the 3 days

This graph shows the overall trend in performance for each group of flies over 3 days. The increase in locomotor function over time for the control group indicates that as the flies grow, their locomotor function should naturally increase. However, the locomotor performance for the hydrogen peroxide group drastically fell from day 4 to 5, indicating severe neurodegeneration that compromised their locomotor function. In comparison, ginseng and ginkgo both were able to lessen or prevent this fall in performance and mitigate the impact of hydrogen peroxide on locomotor function, with ginseng even performing consistently better than control.

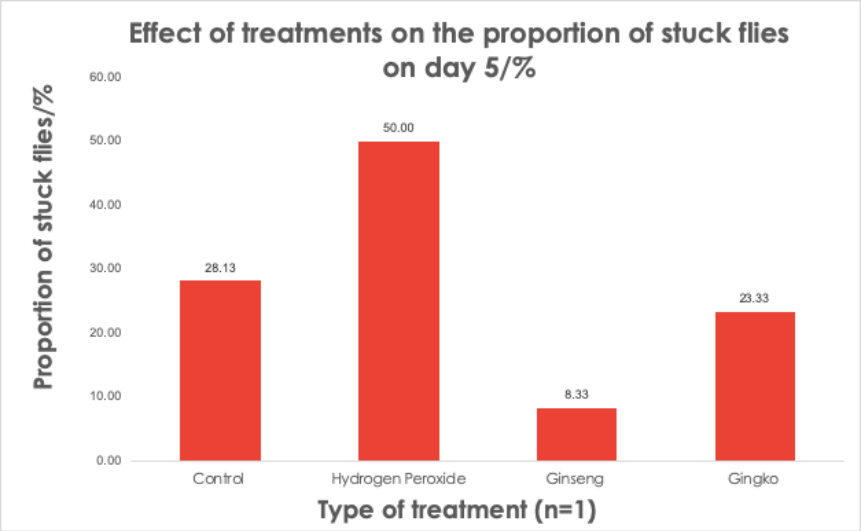


Fig 4. Proportion of flies stuck and unable to climb at all on day 5 of experimentation

On the 5th day of treatment, half of the flies in the hydrogen peroxide experimental groups were stuck and did not climb up the tube at all after stimulation, showing a severe decline in motor function and indicating a limited remaining lifespan. However, both ginseng and ginkgo were able reduce the incidence of such cases by more than half compared to the hydrogen peroxide setup, with ginseng even reducing such cases to 8%, lower than even the control group.

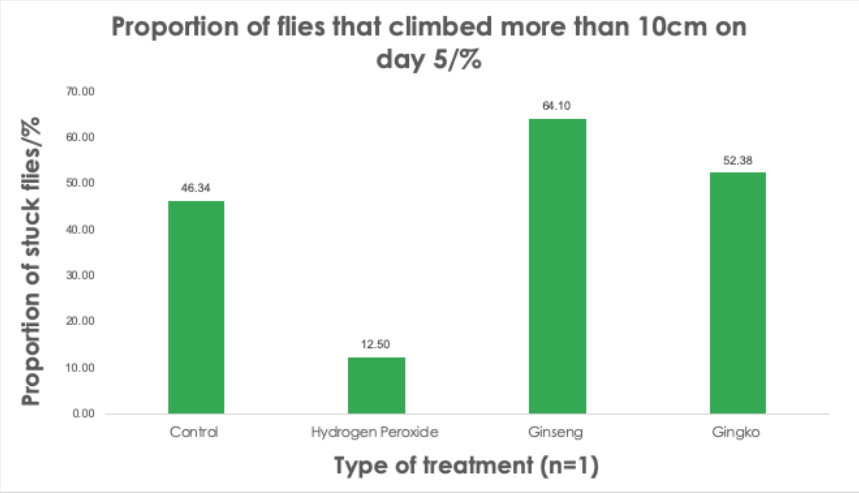


Fig 5. Proportion of flies able to climb more than 10cm on day 5

On the 5th day of treatment, only 12.50% of the flies in the hydrogen peroxide group managed to climb more than 10 cm, or halfway up the tube. Both herbs were able to increase this proportion of flies by more than 4 times, even raising it above that of the control group. This suggests that the herbs were able to lessen the severity of neurodegeneration, ensuring a basic level of locomotor function for the flies, which might help improve standard of living for ALS patients.

### 3.2 Results for G85R mutation fly model

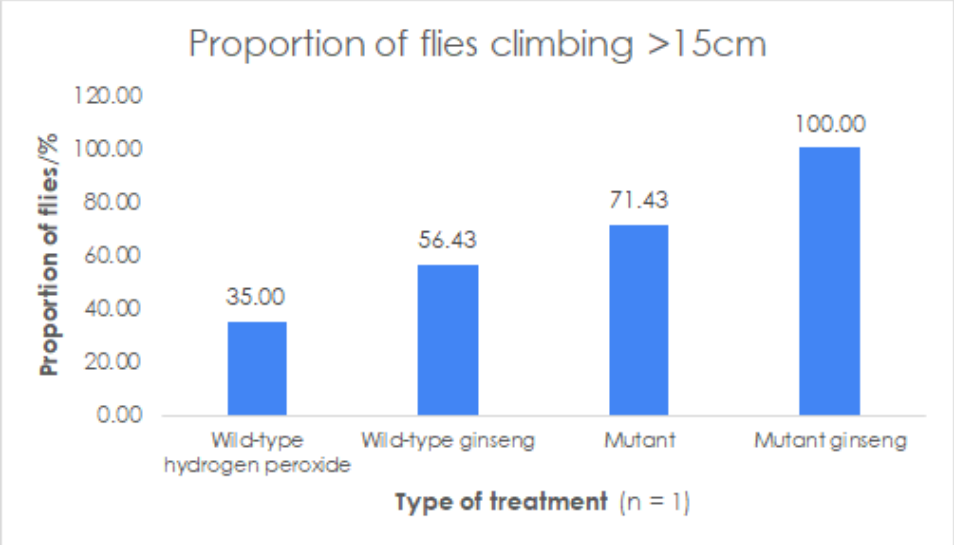


Fig 6. Proportion of flies climbing more than 15 cm.

As can be seen from the graph, the ginseng group outperformed the corresponding hydrogen peroxide or mutant group in terms of proportion of flies climbing more than 15 cm. This suggests that although the hydrogen peroxide or mutation causes a decrease in locomotor function, ginseng

was able to mitigate this effect and prevent neurodegeneration, even being able to restore locomotor function.

### 3.3 Discussion of ALS models

In comparison, the mutant fly group performed far better than the hydrogen peroxide treated fly group, which may be attributed to decreased competition for nutrients, since there were fewer mutant flies per vial compared to wild-type flies. However, the overall trend still remains the same, with locomotor function being restored or even stimulated by ginseng.

For the hydrogen peroxide model, it could be seen that the locomotor performance of the flies on days 3 and 4 was above average, suggesting that hydrogen peroxide did stimulate locomotion initially. This could be due to the hypothesis that hydrogen peroxide is involved in motor neuron signaling pathways in *D. melanogaster*, and plays a role in regulation of locomotion (Grover et al., 2009). Feeding the flies hydrogen peroxide may have altered the signaling of motor neurons, inducing increased fly activity and locomotion. However, over time, flies were unable to cope with oxidative stress induced by ingesting hydrogen peroxide and the constant stimulation of motor neurons, causing neurodegeneration to take place.

For the mutant model, given that the G85R mutation causes the SOD1 protein to become enzymatically inactive, and that the SOD1 protein breaks down highly reactive superoxide radicals which are a product of metabolism (Genetics Home Reference, 2020), the mutant flies should be more susceptible to oxidative stress without functional SOD1 as superoxide radicals accumulate in the fly. Hence, from our results, we can observe that oxidative stress induced by hydrogen peroxide, and the mutation had a similar effect in decreasing locomotor function, which may suggest that the mechanism of the mutation modelling ALS could be oxidative stress, which warrants further investigation.

## 4. Conclusions

Preliminarily, we observed that hydrogen peroxide and the G85R mutation were able to induce neurodegeneration and decrease locomotor function in *D. melanogaster*, and that both ginseng and ginkgo were able to mitigate the effects of hydrogen peroxide or the mutation, reducing the cases

of severe neurodegeneration and preserving basic locomotor function, even stimulating locomotor function beyond that of the control. This suggests their potential for treating neurodegeneration, and as a possible supplementary treatment for ALS to slow disease progression and mitigate symptoms, improving standard of living for ALS patients.

### 4.1 Limitations

As mentioned previously, experiments could only be carried out for 1 cycle due to time constraints and shipping restrictions on flies, so no replicates have been carried out yet, limiting reliability of results presented here and preventing conclusive statements. Statistical tests could not be carried out and error bars could not be added for most graphs, hence we cannot evaluate the true significance of our data.

Due to shipping restrictions on the number of mutant flies, the sample size for mutant fly experiments was small, and there were insufficient flies to test the ginkgo herb at the same time, which is why the ginkgo herbal extract was not tested on the mutant fly model.

Finally, the number of flies in each experimental group could not be standardised as it was impossible to accurately transfer an exact number of flies into a vial, hence sample sizes differed between fly groups.

### 4.2 Future work

We hope to obtain more flies to carry out more cycles of experimentation in the future, which would allow us to carry out replicates and conduct statistical tests to determine significance of our results. At the same time, we would also be able to test the ginkgo herb extract on the mutant flies.

To further extend our research, walking assays measuring flies walk speed and directional changes can be carried out to investigate the passive motor function of the flies, and protein assays investigating the SOD1 protein or other ALS-linked proteins can be carried out to investigate the mechanisms through which oxidative stress may cause ALS.

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