

A novel Alzheimer's Disease therapeutic model: Attenuating hyperphosphorylated Tau and Amyloid β ($A\beta$) aggregates by characterizing antioxidative, anti-inflammatory, and neuroprotective properties of natural extracts

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SUMMARY

A recent line of drug failures to cure or manage Alzheimer's disease prompted research towards discovering ways to delay the progression of this disease. Oxidative damage and neuro-inflammation were the key pathways implicated in the pathogenesis of Alzheimer's disease. In this study, 30 natural extracts from plant roots and leaves with extensive anti-inflammatory and anti-oxidative properties were consumed by *Drosophila melanogaster*. In this plant extract medium, the GAL4-UAS system was used to overexpress amyloid beta ($A\beta_{42}$) human transgene in all the neurons of two transgenic *Drosophila* lines. Using Kaplan-Meier lifespan plots, 12 extracts that increased *Drosophila's* lifespan the most were mixed in specific ratios to make 5 different combinational extracts. Several assays were performed to evaluate the efficacy of these combinational extracts on delaying the progression of Alzheimer's disease. $A\beta_{42}$ -overexpressing flies fed regular cornmeal were used as a control group, whereas $A\beta_{42}$ -overexpressing flies fed the extract medium was used as the experimental group. In the climbing assays the experimental group showed an increased motor activity. In the lifespan assays, Kaplan-Meier plots revealed that the control group experienced a sharp decline while the experimental group showed a gradual decline. Performance index from olfactory training to evaluate the associative memory also indicated improved learning ability in the experimental group.

INTRODUCTION

Alzheimer's disease is a progressive, cognitive impairment disease that leads to decreased motor and verbal ability. Major characteristics of Alzheimer's disease are deposition of abnormal amyloid β ($A\beta$) protein plaques, formation of neurofibrillary tangles of tau protein, and decline in neurotransmission in brain leading to neuronal dysfunction (1). Although few current medications can

effectively treat Alzheimer's disease, there are some medications available to treat the symptoms and may delay clinical decline, with benefits to cognition and function (2). Previous studies reported that plant extracts contain active compounds that are effective in treating various diseases. For example, *Cryptolepis sanguinolenta* is traditionally used for the treatment of malaria, upper respiratory and urinary tract infections, diarrhea, and hypertension (2). In addition, *Terminalia ivorensis* is known to reduce yellow fever, pile, stomach ulcers, wounds, and infections (3). Moreover, there are many studies that have shown plant extracts possess antioxidant and anti-inflammatory properties (4-6). For example, garlic and cinnamon extracts are known to inhibit $A\beta$ aggregations in the brain and thereby improving learning, lifespan, and memory disorders in mice (1). Many studies also confirm that plant extracts have significant potential to suppress oxidative stress and therefore could treat Alzheimer's disease. However, very few studies have explored this potential of individual plant extracts or combination of extracts to treat Alzheimer's disease.

For this study, thirty plant extracts with extensive anti-inflammatory and anti-oxidative properties were organically grown and obtained from an Oregon Benefit Company, Mountain Rose Herbs (7-11). Extensive research on each extract's ability to suppress oxidative stress and neuroinflammation were conducted to evaluate their potential to treat Alzheimer's disease.

D. melanogaster has become a model organism and equipped us to study and research human diseases for the past 100 years. As *Drosophila* cemented its position in disease research, we used a GAL4-UAS system to produce flies overexpressing human amyloid $A\beta_{42}$ in all the neurons. GAL4 is a transcriptional activator that binds to UAS enhancer sequences found in DNA. For this study, GAL4 gene is placed under the control of a driver gene, while the UAS controls the expression of the target gene $A\beta_{42}$ human transgene. These flies exhibit all neurodegenerative symptoms like motor skills, memory disorders, and life-span issues (12).

Thirty plant extracts with extensive anti-inflammatory and anti-oxidative properties were used on the crosses (F1

generation) of two transgenic *D. melanogaster* lines, one expressing human A β_{42} carrying the “Arctic” mutation (a familial Alzheimer’s disease mutation) and other expressing GAL4 in all neurons under the control of neuronal synaptobrevin. (Figure 1).

These transgenic flies clearly exhibit lifespan, memory, motor, and neurodegeneration issues associated with A β and Tau aggregation (13). In this research, all the extracts were ranked based on their efficacy to increase the sporadic life span of F1 generation when compared to the control group. The twelve best extracts were combined in different proportions and their efficacy to reduce the progression of Alzheimer’s disease in the F1 generation is tested. Despite many natural disease inhibitor properties in these extracts, no studies have evaluated the potential of such combination therapies to treat Alzheimer’s disease. Due to the anti-inflammatory and antioxidative properties of these natural plant extracts, we hypothesized A β_{42} positive *Drosophila* would have extended lifespans, increased motor ability, and less memory impairment when treated with natural plant extracts in their food when compared to A β_{42} positive *Drosophila* raised on normal cornmeal medium. We took the initiative to study the effects of selected natural extract combinations to stop Alzheimer’s disease progression.

RESULTS

Phase 1: Ranking efficacy of individual plant extracts to extend lifespan of *D. melanogaster* using Kaplan-Meier lifespan plots

The cooked extract was mixed with cornmeal food, and the F1 generation (crosses) of two transgenic *D. melanogaster* lines, one expressing human A β_{42} and other the other expressing GAL4 in all neurons are produced under the plant extract medium along with a control group that has just the cornmeal food. The two fly lines are maintained at a male to female ratio of 5:10 across 30 extracts with 3 different concentrations (1mg/mL, 2mg/mL, 3mg/mL of fly food). The number of live F1 generation flies were recorded every day for 2 months. During this period, visible decline in motor skills was also recorded as an observation.

The percent survival of each extract group was calculated using Kaplan-Meier method. The average life of flies was 60 days. This study of the project comprised of A β_{42} overexpressing flies fed the plant extract, A β_{42} overexpressing flies fed the regular cornmeal, A β_{42} not expressing flies fed regular cornmeal. At the end of day 55, extracts were ranked based on percentage of survival (Figure 2). The extracts that increased life expectancy by at least 15 days compared to control flies (A β_{42} -) that were fed regular cornmeal were chosen to make the plant extract combinations for phase 2 study.

We identified 12 extracts that extended the average lifespan of A β_{42} -overexpressing flies the most (Figure 3). The 12 extracts were combined in different proportions and crosses (F1) from the same transgenic fly lines were raised

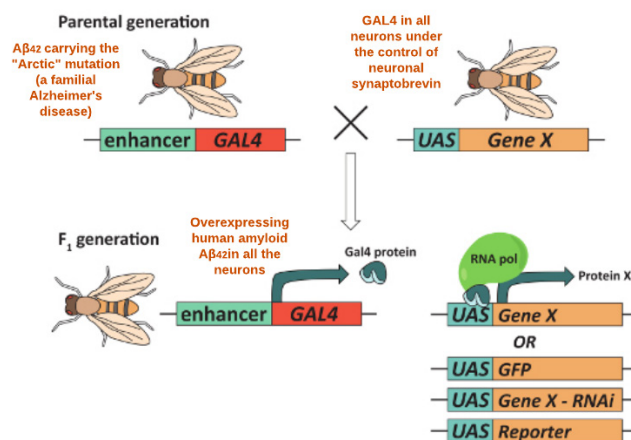


Figure 1. GAL4/UAS system

on mixed plant extract mediums along with control group flies (A β_{42} -) raised on regular cornmeal.

ALDC (ALzheimer’s Disease Control) combinations

ALDC1:

Tribulus (17.86%), Ashwagandha (16.07%), Brahmi (12.5%), Milk Thistle (12.5%), Beetroot (8.93), White Peony Root (7.14%), Licorice (7.14%), Thyme (3.57%), Cacao (3.57%), Bee Pollen (1.79%)

ALDC2:

Tribulus (17.86%), Ashwagandha (16.07%), Brahmi (12.5%), Milk Thistle (12.5%), Beetroot (8.93%), White Peony Root (7.14%), Licorice (7.14%), Sea Buckthorn (5.36%), Thyme (3.57%), Parsley (3.57%), Cacao (3.57%), Bee Pollen (1.79%)

ALDC3:

Tribulus (18.87%), Ashwagandha (16.04%), Brahmi (13.21%), Milk Thistle (11.32%), Beetroot (9.43%), White Peony Root (7.55%), Licorice (6.6%), Sea Buckthorn (6.6%), Thyme (3.77%), Parsley (2.83%), Cacao (1.89%), Bee Pollen (1.89%)

ALDC4:

Tribulus (20.83%), Ashwagandha (10.41%), Brahmi (10.41%), Milk Thistle (8.83%), Beetroot (7.29%), White Peony Root (7.29%), Licorice (7.29%), Sea Buckthorn (7.29%), Thyme (6.25%), Parsley (5.21%), Cacao (5.21%), Bee Pollen (4.17%)

ALDC5:

Tribulus (10.53%), Ashwagandha (10.53%), Brahmi (9.47%), Milk Thistle (9.47%), Beetroot (9.47%), White Peony Root (8.42%), Licorice (8.42%), Sea Buckthorn (8.42%), Thyme (7.37%), Parsley (6.32%), Cacao (6.32%), Bee Pollen (5.26%)

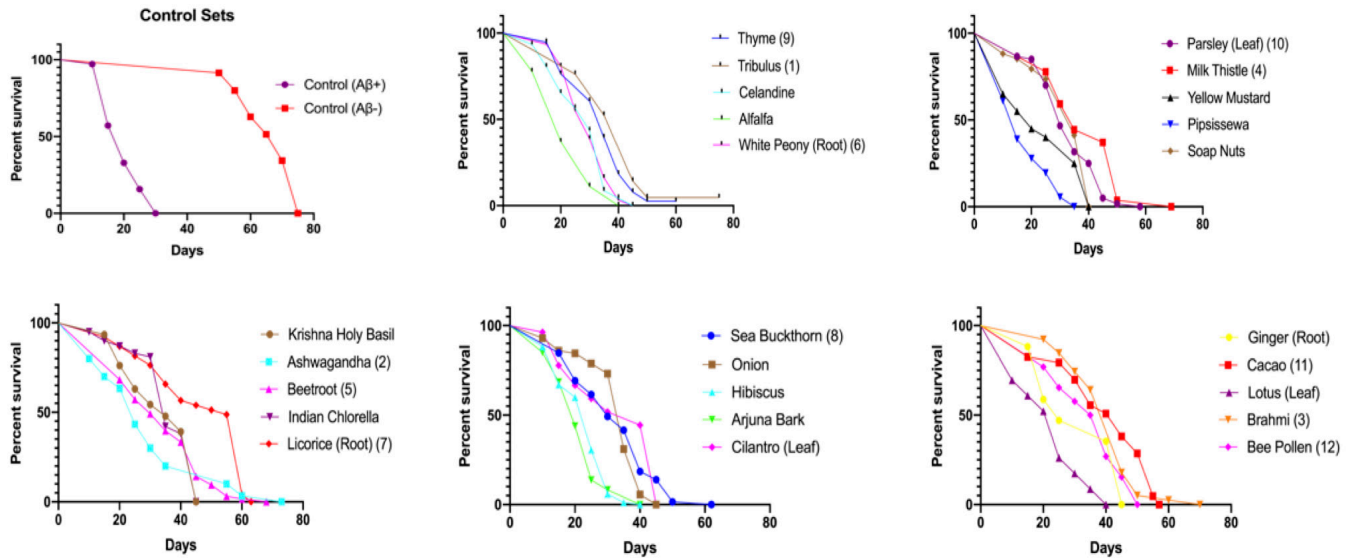


Figure 2. Lifespan activity of control and $A\beta_{42}$ overexpressing flies that were fed individual plant extracts and regular corn meal **A)** lifespan activity of $A\beta_{42}+$ and $A\beta_{42}-$ that were fed regular corn meal. **B)** lifespan activity of $A\beta_{42}+$ flies ($n = 200$) that were fed Thyme, Tribulus, Celandine, Alfalfa, White Peony with their respective rankings (1-10). **C)** lifespan activity of $A\beta_{42}+$ flies ($n = 200$) that were fed Parsley, Milk Thistle, Yellow Mustard, Pipsissewa, Soap Nuts with their respective rankings (1-10). **D)** lifespan activity of $A\beta_{42}+$ flies ($n = 200$) that were fed Krishna Holy Basil, Ashwagandha, Beetroot, Indian Chlorella and Licorice with their respective rankings (1-10). **E)** lifespan activity of $A\beta_{42}+$ flies ($n = 200$) that were fed Sea Buckthorn, Onion, Hibiscus, Arjuna Bark and Cilantro with their respective rankings (1-10). **F)** lifespan activity of $A\beta_{42}+$ flies ($n = 200$) that were fed Ginger, Cacao, Lotus, Brahmi and Bee Pollen with their respective rankings (1-10).

Lifespan assay

Kaplan-Meier analyses clearly indicates ALDC4 has the largest lifespan increase (**Figure 3**). For the flies overexpressing $A\beta_{42}$ fed on regular cornmeal, there is a rapid decline in survival rate between 20 to 30 days of age. The percent survival of the same flies fed on the extracts combinations is not different until day 35, but from day 35 to 55 the percent survival decline is very gradual and ALDC4 exhibited the most gradual of them all. The gradual decline is statistically significant between ALDC4 and other ALDC combinations, $P < 0.05$, whereas among the ALDC combinations, the significance is not different from each other.

Climbing assay

To evaluate motor functionality in $A\beta_{42}$ -overexpressing flies climbing assays were used. In the fly group fed on the regular cornmeal, 15% of 10-day-old flies, 30% of 20-day-old flies, and 60% of 30-day old flies showed decreased motor activity (**Figure 4**). However, in the fly groups fed on plant extract combinations, only 35% showed a decreased motor activity at 40–45 days of age. Of the combinations, ALDC4 showed the least decline in motor skills. The results clearly indicate that the intake of plant extract combinations delayed the decrease in motor functionality in $A\beta_{42}$ -overexpressing flies.

Single olfactory memory assay

Drosophila's associative memory persists for hours or days, depending on short term or long-term olfactory rewarding or punishing training (20-21). To evaluate the effect

of extract combinations on memory impairment, a single olfactory memory assay was used. Performance index (PI) was calculated as the number of flies selecting Geranium Essential Oil odor minus the number of flies selecting Valerian Root odor and divided by the total number of flies. Each PI is the average of PIs from reciprocal experiments with the two odors swapped, thus eliminating non-associative effects. We observed a significant decrease in memory/learning functionality in $A\beta_{42}$ -overexpressing flies fed on the regular cornmeal compared to control flies (carrying $A\beta_{42}$ transgene but not expressing it) fed on regular cornmeal (**Figure 5**). However, that decrease was suppressed in $A\beta_{42}$ -overexpressing flies fed on the plant extract combinations.

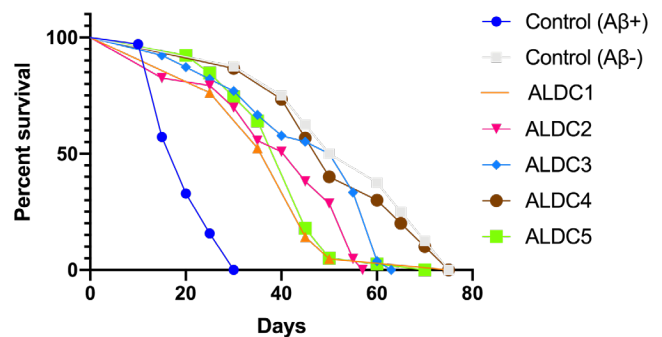


Figure 3. Lifespan activity of control and $A\beta_{42}$ overexpressing flies ($n = 300$) that were fed plant extract combinations (ALDC) and regular corn meal

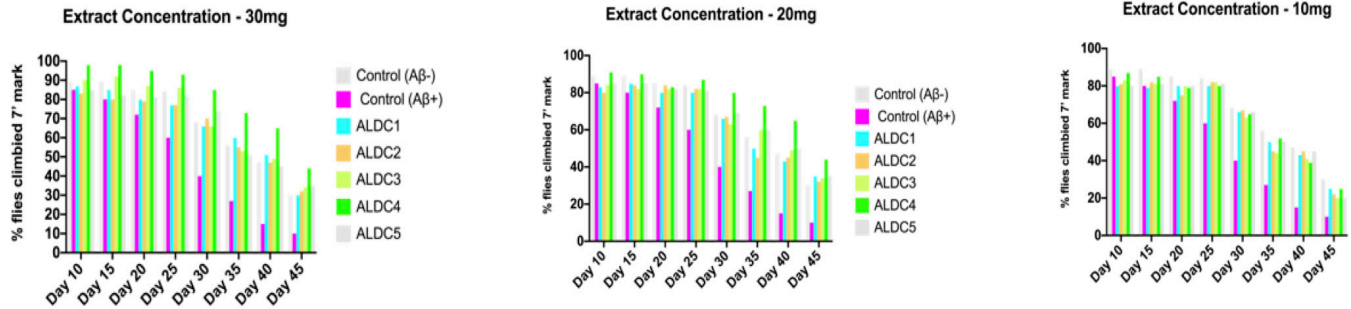


Figure 4. Locomotor activity of control and Aβ₄₂ overexpressing flies (n = 75) that were fed plant extract combinations (ALDC) and regular corn meal locomotor activity at extract combinations concentration of A) 10 mg B) 20 mg C) 30 mg. All the results are expressed as means +/- SEM for five trials. Means with Δ and O are significantly different, *p* < 0.05, however means with the same icons are not different from each other.

The suppression is most prominent in the fly group fed the extract combination ALDC4.

DISCUSSION

In patients with Alzheimer’s disease, it is certain that aggregation Aβ₄₂ peptide occur during the preliminary stages of neurodegeneration (14-15). The results of this study indicate that plant extracts contain compounds that have anti-Alzheimer’s disease effects. To the best of our knowledge, efficacy of mixed plant extracts on Alzheimer’s disease has not been reported. It is well established that oxidative damage and neuro-inflammation are the key pathways implicated

in the pathogenesis of Alzheimer’s disease (16). In this study, I have characterized that natural plant extracts with extensive anti-inflammatory and anti-oxidative properties can help battle the progression of the disease. The promising neuroprotective properties of these extracts can protect the brain against neuronal damage and/or cell death associated with oxidative stress (17). Many promising therapeutic agents like polyphenols, hydroxycinnamic acids, flavones, and procyanidins found in these extracts protect the brain against β-amyloid-induced neurotoxicity and neuronal damage (18).

However, the neuroprotective effects of the extracts in animal and clinical studies involving Alzheimer’s disease

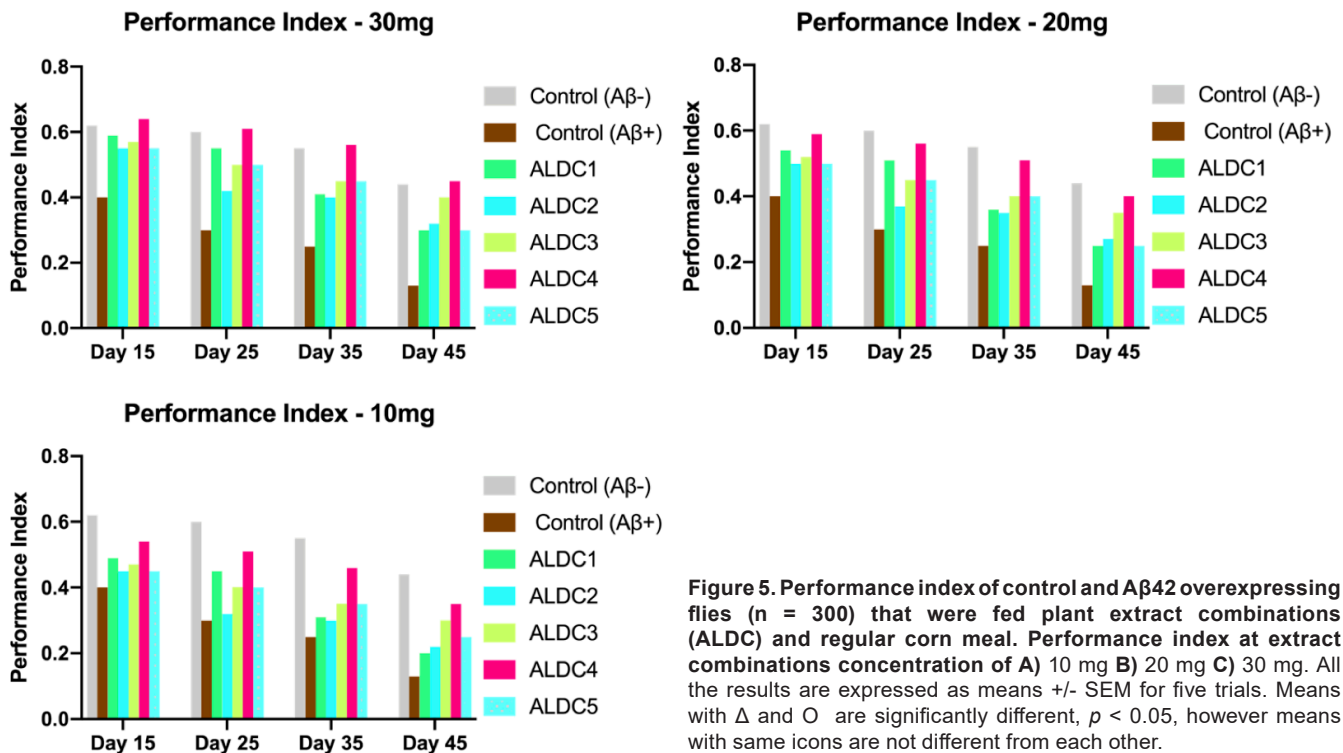


Figure 5. Performance index of control and Aβ₄₂ overexpressing flies (n = 300) that were fed plant extract combinations (ALDC) and regular corn meal. Performance index at extract combinations concentration of A) 10 mg B) 20 mg C) 30 mg. All the results are expressed as means +/- SEM for five trials. Means with Δ and O are significantly different, *p* < 0.05, however means with same icons are not different from each other.

are under-researched. In the recent years, it was thought that these extracts suppress $A\beta_{42}$ and neurofibrillary tangles and therefore protect neurons by preventing the destruction of neuronal membrane triggered by aggregation. Many reports support the claim that these extracts contain many neuroprotective properties which are believed to protect against neuronal death (17).

In this study, individual, and combinations of natural plant extracts improved motor, memory, cognitive, learning abilities and lifespan in $A\beta_{42}$ -overexpressing flies. This improvement is thought to be caused by the suppression of amyloid β ($A\beta$) deposits, and accumulation of neurofibrillary tangles of hyperphosphorylated tau protein. Interestingly, oxidative stress and neuroinflammation is reported to have significant role in the onset of Alzheimer's Disease (11-13). All the extracts used to make the ALDC combinations are reported to have the ability to suppress oxidative damage and neuro-inflammation. Thus, the ALDC combinations may have improved the memory and motor abilities of $A\beta_{42}$ -overexpressing flies by suppressing oxidative damage and neuro-inflammation caused by $A\beta_{42}$ peptide. Flies that were fed ALDC combinations showed a gradual decline in the survival percent, whereas the control group showed a sharp decline. Also, in the climbing assay the control group showed a decreased motor activity in 30-day old flies, while the ALDC fed flies showed decreased motor activity after day 45. Moreover, single olfactory memory assay results confirmed decreased memory activity from day 15 for the control group whereas, ALDC fed flies showed improved memory even after day 45. In conclusion, this study clearly reported to have delayed the progression of Alzheimer's Disease using natural extract combinations. These results provide useful information that may lead to novel drug innovations for Alzheimer's Disease.

Although advances have been made in unrevealing AD neuropathology, only a few treatment options currently exist. Various potential therapeutic or preventive compounds have been tested in clinical trials, yet most have failed to show a clear benefit. Results of this research project clearly support natural extracts individually and when mixed in different proportions can significantly help with battling the progression of Alzheimer's Disease. As the extracts used in this study were obtained naturally from plants, it is believed that they might minimize the side effects or symptoms of toxicity. Henceforth, plant extracts can be a useful source of drugs for treatment of Alzheimer's disease and can improve the quality of life of Alzheimer's disease patients. However, chemical complexity and the difficulty in quantifying dose are the two main problems associated with plant extracts. These problems can cause individuals accidentally consume toxic compounds within plants or overdose. Hence, further research is warranted to understand the effects of the compounds in these extracts on the nervous system to improve motor and cognitive deficits at the molecular level.

MATERIALS AND METHODS

Extract preparation

All plant extracts were organically grown and obtained from an Oregon Benefit Company named Mountain Rose Herbs. Raw natural plant leaves/roots are dried aerially, and portions are milled into fine powder. Each powdered extract is dissolved in deionized water at room temperature and cooked at 170°F for 15 minutes. The cooked extract is mixed with a cornmeal-molasses-yeast medium and used as fly food.

The w1118 control (Stock 3605) and chico1 (Stock 10738) flies were obtained from the Bloomington Drosophila Stock Center at Indiana University. The flies used in this study were derived from an Amherst, Massachusetts's population established by P.T. Ives in 1975 (Rose and Charlesworth 1981, Rose, 2002 #149). This population has been cultured at moderate to large population sizes and controlled densities (50–80 eggs per vial) for more than 700 generations with discrete generations cultured every 2 weeks.

Flies were reared by placing approximately 5 males and 10 females in a 28.5 x 95 mm plastic cylinder with Buzz Plugs. The housing at the bottom is the cornmeal-molasses food with yeast sprinkled (prepared by Lab Express). These flies could lay eggs and two weeks later, approximately 30 flies/vials were recovered after eclosion, which were then used for lifespan studies or other experiments.

Climbing assay

The *D. melanogaster* climbing assay was performed (15) by placing flies in a graduated cylinder measuring 1.5 inches x 9 inches. Twenty flies were transferred to the cylinder and very lightly tapped so all the flies collect to the bottom surface. Six climbing trails were run for each mixed extract and video-recorded the events for two minutes. The video is analyzed to record how many flies climbed past the target 7-inch mark.

Feeding and lifespan assay

Flies were fed fresh individual and mixed plant extract (20-21) corn meal medium (1 mg/mL, 2 mg/mL, 3 mg/mL) or regular corn meal post-eclosion. All flies were maintained at 25°C under a 12-hour light-dark cycle for all experiments. The number of live flies were recorded in each vial for 60 days.

Single olfactory memory assay

Twenty flies raised on each mixed plant extract food medium were trained to associate an odor with a 65V electric shock. After few minutes, the same flies were exposed to another odor without the electric shock. In the T-Maze, the flies were taken to a choice point where they were exposed to both odors without any electric shock for about two minutes. The distribution of flies towards each odor were recorded and performance index for each odor is calculated. Individual performance index is obtained by averaging the performance index of two experiments, one with shock pairing odor from Valerian Root, and the other shock pairing with odor from Geranium Essential Oil.

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