

Cognitive and Locomotor Behaviours of Methanol Leaf Extract of *Cucurbita pepo* (Pumpkin) in *Drosophila melanogaster* Model of Alzheimer's Disease

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Abstract

AD is characterized by progressive memory loss, cognitive decline, amyloid buildup, and neurofibrillary tangles, but the underlying causes and mechanisms remain unclear. The fruit fly (*Drosophila melanogaster*) is a valuable model organism for studying complex biological processes and neurodegenerative diseases like AD. *Cucurbita pepo* (pumpkin) is a nutrient-rich vegetable with various bioactive compounds, including antioxidants, vitamins, and minerals. This study seeks to investigate the phytochemical profile and neuroprotective potential of *C. pepo* methanol leaf extract in mitigating the behavioral functions in A β -42 expressing *Drosophila melanogaster*, a model of Alzheimer's disease. The bioactive compounds in *C. pepo* were identified through qualitative and quantitative phytochemical analyses. A β -42-expressing *Drosophila* flies were treated with different concentrations of *C. pepo* extract (75.5 mg, 113.3 mg, and 151 mg), and their behavioral responses were compared to those of Donepezil-treated and GAL4 control groups using negative geotaxis, learning and memory retention assays. The extract was rich in Alkaloids (37.15 $\pm$ 0.30g/mg), Steroids (28.42 $\pm$ 1.17 g/mg), Flavonoids (23.62 $\pm$ 0.57g/mg), AD flies exhibited significant motor and cognitive deficits, which were notably reversed upon treatment with *C. pepo* extract. The 75.5 mg of the extract has a significant increased ability to climb than the A β -42 dose demonstrated the highest efficacy, comparable to Donepezil, in restoring locomotor performance while the 113.3 mg of the extract has a significant increased learning ability than the A β -42, while the 75.5 mg, 113.3 mg and 151 mg of the extract have a significantly increase in memory ability than the A β -42. The methanol extract of *Cucurbita pepo* demonstrated significant neuroprotective effects in a *Drosophila* model of Alzheimer's disease.

Keywords: Alzheimer Disease (AD), *Cucurbita pepo*, Donepezil, A β -42, *Drosophila melanogaster*, Fruit Fly, Methanol.

Introduction

Alzheimer's disease (AD), the most prevalent form of dementia, is a neurodegenerative disorder with no cure at the moment. Currently, 55 million people worldwide suffer from AD and other dementias. This number is projected to rise to 78 million by 2030. Alzheimer Disease main symptoms include progressive memory loss and cognitive decline, accompanied by characteristic brain changes like amyloid buildup and neurofibrillary tangles. However, the underlying causes and mechanisms driving these symptoms' variability among patients remain unknown. Moreover, effective treatments for AD are still inadequate. Recent attention has focused on advanced imaging and genomic techniques, which hold promise for uncovering AD's pathology, enabling early detection, and informing treatment strategies (Li *et al.*, 2022). As Alzheimer's disease (AD) diagnoses continue to surge, the healthcare system is facing an unprecedented burden. Accurate diagnosis of AD relies on identifying two hallmark features: extracellular amyloid plaques and intracellular neurofibrillary tangles, which are the classical neuropathological signs of the disease (Hashemi *et al.*, 2023).

While the complex and multifaceted pathophysiology of Alzheimer's continues to be studied, major attention has been given to the genetic, environmental, and social factors, which play a role in the development of this neurological degeneration. Primarily, aging is considered to be the dominant risk factor for the development of Alzheimer's disease; only 1–6% of cases take the form of early-onset AD (EOAD), which is characterized by onset between the ages of 30–65 (Alzheimer's Association, 2022). Late-onset AD (LOAD) is much more common, with a current prevalence of around 6.5 million cases in the United States and a projected increase to 13.8 million by the year 2065 (Alzheimer's Association, 2022).

The fruit fly is a premier model organism for studying complex biological processes in multicellular eukaryotes, offering a unique combination of genetic, anatomical, behavioral, and economic advantages. Notably, it was one of the first organisms to have its entire genome sequenced making it an invaluable tool for scientific research (Adams *et al.*, 2000). Fruit fly genetics is being harnessed to tackle some of the most challenging and costly medical issues of our time, including neurodegenerative diseases like Alzheimer's. By applying a wide range of biological techniques to fly models of Alzheimer's, researchers are gaining new understanding of disease mechanisms and identifying potential therapeutic targets. This approach can also help interpret human genome studies by testing whether specific genes linked to neurodegenerative diseases have a significant impact on disease symptoms in fly models, allowing for more focused research (Moloney, *et al.*, 2010).

Pumpkin (*Cucurbita pepo*) is a nutrient-rich vegetable from the *Cucurbitaceae* family, boasting an impressive array of vitamins, minerals, and bioactive compounds. These include healthy fats like unsaturated fatty acids, essential proteins (albumin and globulins), and a range of antioxidants like α and γ tocopherols, β -carotene, and phytosterols, making it a valuable source of nutrition (Muhammad *et al.*, 2023). Extensive scientific research has

revealed the potentials of *Cucurbita pepo* (pumpkin) in treating various health conditions. As pumpkin is widely consumed globally, its neuroprotective effects on lipopolysaccharide (LPS)-induced neuroinflammation remain unclear, warranting further investigation (Zhang *et al.*, 2021). Research has shown that consuming a diet high in antioxidants can significantly lower the risk of developing chronic diseases, including diabetes, cancer, and heart related diseases (Ahmad *et al.*, 2020). According to recent researches, consuming antioxidants has been linked to a reduced risk of developing neurodegenerative diseases, including Alzheimer's disease. Similarly, low levels of antioxidants in the body can lead to oxidative stress, which has been linked to the development of depression (Afzal *et al.*, 2022).

Statement of the Problem

While current diagnostic procedures for Alzheimer's disease (AD) are informative, they are too time-consuming for routine use. To accurately diagnose AD, researchers rely on biotechnology and bioinformatics to identify disease biomarkers. Decades of research have shed light on AD's molecular and cellular mechanisms, including the hallmark accumulation of amyloid-beta plaques and tau tangles. Yet, despite significant progress, the exact causes and precise pathogenesis of AD remain unknown. This knowledge gap highlights the need for ongoing research into this complex condition. The typical Alzheimer's treatment options mostly include drugs that provide temporary relief from symptoms without addressing the underlying causes. As the population ages, the demand for effective therapies increases. In this regard, the study of natural chemicals discovered in pumpkin (*Cucurbita pepo*) extract shows potential as an alternative medicine. The study of natural chemicals discovered in pumpkin (*Cucurbita pepo*) extract shows potential as an alternative medicine. However, there is a significant lack of thorough investigations examining the therapeutic potential of these extract in Alzheimer's disease, both in the laboratory and in animal models. This study aims to advance the growing field of Alzheimer's research by investigating the effects of pumpkin extract on a fruit fly model of the disease.

Aim

This study seeks to investigate the phytochemical profile and neuroprotective potential of *C. pepo* methanol leaf extract in mitigating the behavioral functions in A β -42 expressing *Drosophila melanogaster*, a model of Alzheimer's disease.

Objectives

- i. To Identify and quantify the phytochemical compounds present in the methanol leaf extract of *Cucurbita pepo*.
- ii. To determine the effects of Methanol extract of *C. pepo* Leaves on locomotor behavior of A β -42 strain of *Drosophila melanogaster*.

- iii. To examine the effect of Methanol extract of *C. pepo* Leaves on learning and memory behavior in A β -42 strain of *Drosophila melanogaster*.

Materials and Methods

Materials

Chemicals and Equipment

- **Reagents/Chemicals**

All reagents for this research were of analytical grade. These reagents include; Methanol, Donepezil, Quinine Hydrochloride (Sigma Aldrich; CAS Number: 6119-47-7), Distilled Water, High-Density Polyethylene (NALGENE VWR Catalog No. 62868-021), Acetate solution, ferric chloride, lead acetate, Hydrochloric Acid, Dragendroff's, Chloroform.

- **Apparatus/Equipment**

Hotplate, Soxhlet Apparatus, Rotary Evaporator (RE300) and Microplate reader (NeoMedica NW-67), Microscope. Stereo scope (Zeiss), Small paint brushes for pushing flies, 28.5 x 95 mm Polystyrene na Vial (Capitol Vial, Inc.), Mini-Alarm Timer/Stopwatch (VWR International), Sharpie pens, Adhesive Tape, Digital measuring scale for precise dilution by weight, Quick Disconnect Connectors, 15 mL Centrifuge Tube (VWR International) cut at the 2 mL mark from closed end to fit with the Quick Disconnect Connector adaptor for light source, Single-channel 200 μ L pipetter to load quinine solution onto filter paper lining the chamber, T-Maze (Simple Behavioral Systems;). Aluminum Foil, Light Source (Zeiss), A lamp with red light and Filter Paper.

- **Experimental Animal**

Drosophila melanogaster strain of Alzheimer's Disease UAS-A β 42 and elav-Gal-4 were used in this study. To obtain flies expressing A β 42 pan-neuronally (experimental group: elav-GAL4; UAS-A β 42) GAL4-UAS transgene expression system was used with the pan-neuronal elav-GAL4 driver (Bloomington stock #458). Because the elavc155-GAL4 transgene is located on the X chromosome, I collected virgin females from elav-GAL4 transgenic flies and crossed them with male UAS-A β 42 transgenic flies.

- **Plant Material**

The pumpkin (*Cucurbita pepo*) was bought from Keffi market. The fresh leaves of pumpkin (*Cucurbita pepo*) were rinsed with distill water to remove all debris, shade-dried for seven days and subsequently pulverized using an electric blender. The leaf was grinded into powder for extraction.



Figure 1: *Cucurbita pepo* (Pumpkin) leaf

Methods

Drosophila Diet Preparation (850ml)

850ml of distilled water was divided into 500ml and 350ml portions. The 500ml portion was added to a cooking pot and boiled. A small quantity of the boiled water was used to dissolve 10g of yeast. Then, 8g of agar-agar was added to the remaining boiling water and for 10 minutes with periodic stirring. Out of 350ml, 200ml portion of water was used to dissolve 50g of cornmeal. The dissolved cornmeal was added to the boiling agar-agar mixture and stirred for 10 minutes with periodic stirring. The dissolved yeast was added to the seething mixture and stirred periodically while allowing it to seethe for 15 minutes. The food was then cooled to 65°C, and 1g of Nipagin previously dissolved in 10ml of ethanol was added and stirred. After cooling to about 35°C (semi solid state), aliquots of the media were supplemented with the appropriate concentration of extract/drug and dispensed into vials (10-30cm) or as required. The remaining food was refrigerated until when needed. Flies could be added as soon as the media solidified and cooled (Sani *et al.*, 2023).

Drosophila Stock Preparation and Culture

Collection of Virgin Female Flies

To obtain flies expressing A β ₄₂ pan-neuronally (experimental group: elav-GAL4; UAS-A β ₄₂) or control flies (elav-GAL4), I used the GAL4-UAS transgene expression system with the pan-neuronal elav-GAL4 driver (Bloomington stock #458). Because the elav-GAL4 transgene is located on the X chromosome, I collected virgin females from elav-GAL4 transgenic flies and crossed them with male UAS-A β ₄₂ transgenic flies.

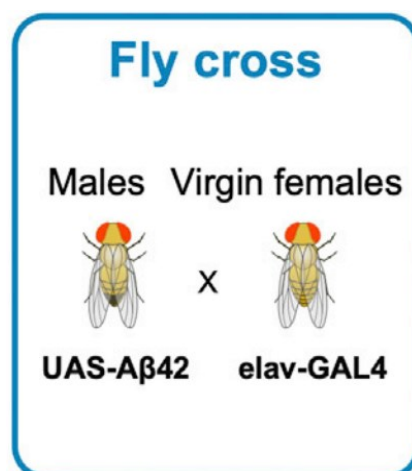


Figure 2: Fly Crossing (Sekiya and Iijima, 2021).

The modified method of Sekiya and Iijima (2021), was adopted for crossing the flies. Briefly, vials of elav-GAL₄ flies for collection of virgin females and vials of UAS-Aβ₄₂ for male was prepared under standard laboratory condition of 24°C–25°C, 40%–50% humidity with 12h light/dark cycle. After the adult flies were enclosed around day 10–11. 30 virgin females of elav-GAL₄ and 15 males of UAS-Aβ₂ were placed into the culture, in the vials. After 4–5 days, the parental (Fo) flies were transferred to new vials to increase the number of vials. The F₁ males from control (elav^{c155}-GAL₄) and the experimental group (elav-GAL₄; UAS-Aβ₄₂) were collected 10 days after setting up the cross. The flies were anaesthetized and 50 flies were placed per plastic vials (25 mm diameter) containing fly food. These flies were 0 day old. The vials were changed every 3–4 days to obtain age-matched flies from the experimental and control groups for each experiment.

Experimental Design

The groups were divided as follows:

Table 1: The Experimental design

Groups	Profile
Gal-4	Negative control, wild strain
Aβ-42	Positive control, mutant strain induced with AD and + untreated
10μl of Donepezil	Standard control, mutant strain induced with AD + Drug
75.5 mg of <i>C. pepo</i>	Mutant strain induced with AD + extract
133.3 mg of <i>C. pepo</i>	Mutant strain induced with AD + extract
151 mg of <i>C. pepo</i>	Mutant strain induced with AD + extract

Behavioral Assays

Advances in genetic methods have enabled the study of genes involved in human neurodegenerative diseases using *Drosophila* as a model system (Bonini and Fortini, 2003). Most of these diseases, including Alzheimer's, Parkinson's and Huntington's disease are characterized by age-dependent deterioration in learning and memory functions and movement coordination (Lloyd and Taylor, 2010). Behavioral assays, including the negative geotaxis assay (Benzer, 1967) and the aversive phototaxis suppression assay (APS assay) (Le Bourg and Buecher, 2002), were utilized in this research to show that some of the behavioral characteristics associated with human neurodegeneration can be recapitulated in flies. In the negative geotaxis assay, the natural tendency of flies to move against gravity when agitated is utilized to study genes or conditions that may hinder locomotor capacities. In the APS assay, the learning and memory functions were tested in positively-phototaxis flies trained to associate light with aversive bitter taste and hence avoid this otherwise natural tendency to move toward light (Seugnet *et al.*, 2009).

Negative Geotaxis Assay

The Negative Geotaxis Assay was done following the modified method of Mershin *et al.* (2004), flies from the experimental group (elav-GAL4; UAS-A β ₄₂) and control group (elav-GAL4) were collected and aged. A total of 150 flies were used per group, with 30 flies placed into each plastic vial (25 mm diameter) containing fly food. This resulted in 5 vials per group. Flies were tapped to the bottom of the vials and transferred to fresh fly food vials every 3 days. The negative geotaxis assay was performed once, starting when the flies were 2-weeks-old. On Day 14, flies were tapped to the bottom of the food vials and transferred to new, empty plastic vials (25 mm diameter) marked with genotype and a scale representing height. The vials were laid horizontally for 5 minutes to acclimate the flies to their new environment.

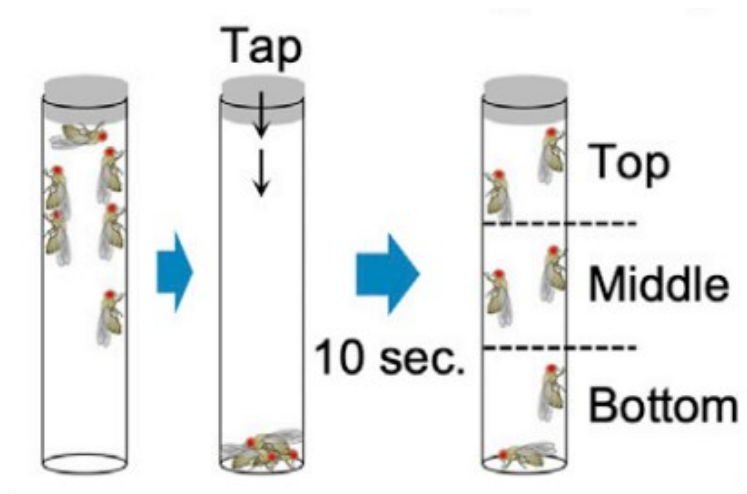


Figure 3: Negative Geotaxis Assay (Sekiya and Iijima, 2021).

First Trial: The vials were tapped against a foam pad or layered Kimtowels to knock the flies to the bottom. The vials were then stood upright in front of a white board. After 10 seconds, a photograph was taken to capture the distribution of flies within the vial. Healthy flies exhibited negative geotaxis by climbing up the vial walls. The recording time of 10 seconds was chosen as most control flies reached the top of the vial within this period. The recording time was adjusted based on experimental conditions. The vials were laid horizontally for 3–5 minutes before proceeding to subsequent trials.

Second to Fifth Trials: Steps from the first trial were repeated for a total of five trials. After the assay, flies were transferred back to their original plastic vials containing fly food and maintained. Flies were categorized based on their position within the vials (top, middle, bottom) using the captured photographs. The percentage of flies in each section of the vial was calculated, averaging the results from five trials per vial. The average percentages and standard errors were computed for 5–6 vials per treatment.

Aversive Phototaxis Suppression Assay (APS Assay)

APS assay method was Adapted from Le Bourg and Buecher (2002) with modifications, this assay exploits the positive phototactic behavior in flies to train them to associate light with aversive stimuli (in this case, bitter taste of quinine). After a training/conditioning phase, wild type flies were able to associate the lighted area with aversive taste and avoid it. Flies with compromised learning capacities failed to make this association. Furthermore, the APS assay can also be used to measure short term memory function of flies (Seugnet *et al.*, 2009), by subjecting already trained flies to the same test 6 hours post conditioning to test their ability in remembering the learned task.

Preparing the Quinine Solution: Quinine hydrochloride was dissolved in distilled water to make a 0.1M stock solution (1.98g in 50mL distilled water). The stock solution can be kept at -20°C for up to a year in small aliquots. The working solution was prepared of 1 μ M by diluting the stock solution in distilled water.

Preparing the T-Maze: T-maze consists of the center column with the trap door and two independent chambers, a "dark" chamber and a "lighted" chamber (Figure 3).

Chamber preparation: Two 15mL plastic centrifuge tubes were cut at 2 mL mark from the bottom closed end with a manual saw. The connector adapters were fit at each end, and the connection was seal with parafilm. The adapter at the end of the tube serves as the input slot for the gooseneck light source. One tube was connected with the light source and this tube will be the "lighted" chamber. The other tube was wrapped with aluminum foil to serve as the "dark" chamber. 180 μ L of quinine solution was added to the filter paper and place in the light chamber. The T-maze was assembled by screwing the lighted and dark chambers

on each side of the center column, with the trap door in the middle closed (Figure 4). Then the light source was inserted into the lighted chamber.

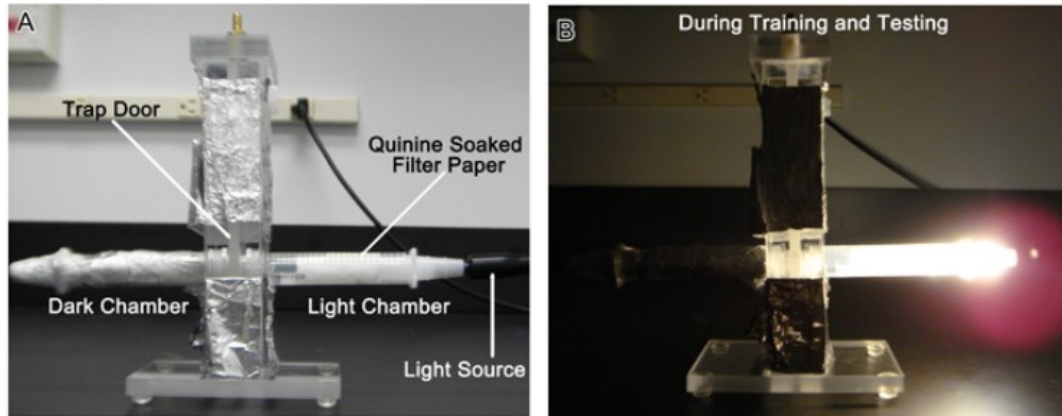


Figure 4: T-Maze Setup for Aversive Phototaxis Suppression Assay.

The overall experimental setup for the APSA experiment with the light source connected to the "lighted" falcon tube, lined with filter paper and the "dark" tube on the left (covered in foil), separated by a trap door. During the training and testing phase, the fly is in the dark chamber and the trap door opened after the light is turned on in the lighted chamber.

Training Flies to Suppress Phototaxis with Aversive Stimuli

The dark chamber was unscrewed from the T-maze and a single fly was transfer into this chamber then the chamber was immediately screwed back to the maze. The lights in the room were turn off and the red lamp turn on. The fly was allowed to acclimatize in the dark chamber for 30 seconds and the light was slowly switched on illuminating the lighted chamber. Slowly open the trap door that separates the two chambers. If the fly walks to the lighted chamber within 10 seconds, it is considered positively phototactic and is ready to be trained for the assay. Failure in phototaxis indicates problems in the visual system and flies with negative phototaxis need to be excluded from the assay. For Aversive Phototaxis Suppression (APS) training, the fly that demonstrated positive phototaxis was tap back to the dark chamber, close the trap door and turn off the light. Allow 30 seconds of acclimatizing. During this time the filter paper with quinine solution was inserted into the lighted chamber. Slowly open the trap door and turn on the light. The fly was allowed to walk into the quinine coated lighted chamber. After one minute, tap the fly back to the dark chamber and repeat these 9 more times. (Typically, wild type flies avoid the lighted chamber after 3 to 5 training trials). Immediately after training, five test trials was conducted. In each test trial, allow 10 seconds after light is turned on for the trained fly to walk to the lighted chamber. Failure to walk to the lighted vial was recorded as "Pass", which is equivalent to "task learned through reinforcement". The Pass rate over five consecutive trials was recorded as PCo (0 hr post conditioning).

Assessing Short-Term Memory Function in Flies: After the flies were trained and initial PCo was recorded, each fly was placed back into its original food vial and kept for six hours. After six hours post training, each fly was subjected to 5 trials again in the same way as before, and the number of times the fly avoids (pass) or goes into (fail) the lighted vial was recorded. This pass rate was recorded as PC6 (6 hrs post conditioning), which is an indicator of short-term memory.

Statistical Analysis

Data analysis was performed using SPSS version 21, with one-way ANOVA and Duncan's post hoc test used to determine statistically significant differences ($P < 0.05$) among groups. Results were visualized as bar charts using GraphPad Prism version 10.

Phytochemical Analysis

Qualitative Phytochemical Screening

The qualitative phytochemical screening was carried out according to the procedure stated by Vishnu *et al.* (2019).

Test for Phenol

5ml of extract, 3 ml of 10% acetate solution was added and mixed gently. The formation of bulky white precipitate will indicate positive for phenol (Vishnu *et al.*, 2019).

Test for Tannins

5ml of extract, few drops of 5% ferric chloride solution were added. The production of dark green color will indicate the presence of tannins (Vishnu *et al.*, 2019).

Test for Flavonoids

1ml of the extract was taken and 10% of lead acetate was added. The formation of yellow precipitate will indicate positive inference for flavonoids (Vishnu *et al.*, 2019).

Test for Alkaloids

5ml of extract, 2 ml of HCl was added and 1ml of Dragendorff's reagent was added. An orange or red precipitate will indicate positive results for alkaloids (Vishnu *et al.*, 2019).

Test for Glycosides (Borntrager's test)

2ml of the extract, 3ml of chloroform was added and shaken. The chloroform layer was separated and 10% ammonia solution was added. The pink color formation will indicate the presence of glycosides (Vishnu *et al.*, 2019).

Test for Cardiac Glycosides (Keller-Killani test)

5ml of the extract was mixed with 2ml of glacial acetic acid and a drop of ferric chloride solution was added followed by the addition of 1ml of conc. H_2SO_4 . A brown ring in the interface will indicate the presence of deoxy sugars of cardenoloides (Vishnu *et al.*, 2019).

Test for Saponins

0.5mg of extract was vigorously shaken with few of distilled water. The formation of frothing will indicate positive for saponins (Vishnu *et al.*, 2019).

Test for Steroids

2ml of extract, 2ml of chloroform and 2ml of concentrated H_2SO_4 was added. The appearance of red color and yellowish-green fluorescence will indicate the presence of steroids (Vishnu *et al.*, 2019).

Quantitative Analysis of the Plant Extract**Total Phenolic Content**

Total phenolic content was calculated using the procedure outlined by Mauryan and Singh (2010). Essentially, this is a color reaction that yields a blue color that can be detected using spectrophotometry. The total phenolic content was assessed using the Folin Ciocalteu Reagent, and the standard expression for gallic acid was mg/g gallic acid equivalent (GAE). Methanol was created with concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml. A 0.5 ml sample of the concentrated plant extract (1 mg/ml) in methanol was produced, and 2.5 ml of the 10-fold diluted Folin Ciocalteu Reagent was added to it in a test tube. Add the mixture to 2ml of sodium bicarbonate (7.5%). Before measuring the absorbance at 760 nm, the tubes were left at room temperature for 30 minutes while covered with parafilm. Three copies of the reading were taken.

Estimation of Total Tannins

Determination of total tannins content was evaluated for total phenolic content by using Folin-Ciocalteu's reagent (Rajeev *et al.*, 2012). Plant extract (10mg) was dissolved in 10ml of distilled water to make final concentration of 1mg/ 1ml. Different aliquots of gallic acid (0.5, 0.4, 0.3, 0.2 and 0.1 mg) was dissolved in 1ml of distilled water to make the final concentrations 100, 80, 60, 40 and 20 μ g/ml. After preparing 3.5% v/v sodium carbonate solution, 0.1ml gallic acid solution of every concentration (100-20 mg/l) was taken into different volumetric flask. Then 7.5 ml of distilled water and 0.5 ml FC reagent was added to it. After 5mins, 1ml 3.5% sodium carbonate solution and 10ml distilled water was added to that flask. After 30mins, UV absorbance was determined at 725nm. Total tannins content of the extract was measured using gallic acid standard calibration curve and stated as mg gallic acid equivalent, GAE/100g of dried plant extract.

Total Flavonoid Content

The total flavonoids content was determined according to the method describe by Zengnin *et al.* (2011). A standard solution (10-50 µg/ml) of rutin was added to 75 µl of sodium nitrite (NaNO_2 , 5%) solution and mixed properly. After 5-6 minutes, 0.5 ml of aluminum chloride (AlCl_3 , 100g/l) was added. 0.5 ml of sodium hydroxide (NaOH , 4%) was added after 5 minutes. The final volume was kept at 2.5ml with distilled water and thoroughly mixed. The absorbance of the mixture was determined at 510nm against the same mixture without the sample as blank. Total flavonoids content was expressed as mg rutin/g dry weight (mg/g DW) through the calibration curve of rutin. All samples were analyzed in triplicates.

Total Alkaloid Content

The total alkaloids content was determined according to the method describe by Raheleh *et al.* (2013). 100ppm solution of atropine (1mg in 10 ml of distilled water) was used as standard. From the stock solution, exactly 0.5, 1.0, 1.5, 2.0 and 2.5 ml of atropine solutions was transferred to five different separating funnels. To each separating funnel, 5 ml of phosphate buffer (pH 4.7) and 5 ml Bromocresol green (BCG) solution was added and mix vigorously. The formed complex mixture was extracted with chloroform (5ml). The chloroform fraction was collected in a 10 ml volumetric flask and make up the volume with chloroform. Absorption at a wavelength of 470nm of each flask was measured and a calibration graph was drawn. For the presentation of sample, the plant extract (1mg/ml) was dissolved in 2N HCl and then filtered. The pH of the extract was adjusted to neutral with 0.1N NaOH. 1ml of this solution was transferred to a separating funnel and to the mixture of Bromocresol green solution along with 5ml of phosphate buffer was added and mixed properly. The form mixture was extracted further with 5ml of phosphate chloroform and transferred to 10ml volumetric flask and make the volume with chloroform. The absorbance of the complex in chloroform was measured at 470nm.

Estimation of Total Steroids

Estimation of total sterols in the extract was done by the method of Attarde *et al.* (1976) with little modification. The Liebermann-Burchad (LB) reagent was employed for the quantitative estimation of sterol. It was prepared by adding 0.5ml of concentrated sulphuric acid in 10ml of acetic anhydride. To 1ml of each of the extract (1mg/ml), chloroform was added to make the volume to 5ml in a test tube. A volume of 2 ml of LB reagent was added and mixed well. These tubes were then covered with black paper and kept in the dark for 15 minutes to avoid any exposure to light. The reaction mixture turned green which was measured spectrophotometrically by using UV-Vis Spectrophotometer at 640nm against the blank. Beta-sitosterol was used as the standard to prepare a calibration curve. All the test was carried out in triplicates and the results was expressed as Beta-sitosterol equivalent (µg of Beta-sitosterol/mg of extract).

Determination of Cardiac Glycosides

The procedure described by Sofowora (1995) was used. 10ml of the extract was pipetted into a 250ml conical flask. 50ml chloroform was added and shaken on vortex mixer for 1 hour. The mixture was filtered into 100ml conical flask. 10ml of pyridine and 2ml of 29% of sodium nitroprusside was added and shaken thoroughly for 10mins. 3ml of 20% NaOH was added to develop a brownish and yellow color. Glycosides standard (Digitoxin) was used at concentration range from 0-50mg/ml from stock solution and the absorbance was read at 510nm.

Determination of Total Saponins

The total saponins content was determined by spectrophotometry as described previously by (Uematsu *et al.*, 2000) with little modification. 2ml of each extract, 1ml of reagent A (p-anisaldehyde 0.5 in ethyl acetate 99.5) and 1ml of reagent B (H₂SO₄ 50% in ethyl acetate 50%) was mixed and homogenized in a vortex. The methanol extract of *C. pepo* and standard (Digitoxin) was dissolved in 2ml ethyl acetate. Then, 1ml of reagent A and 1ml of reagent B was added. The mixture was stirred and incubated at 60°C for 10 minutes in a water bath. The solution was cooled at room temperature for 10mins and the absorbance of the color developed solution was recorded at 430nm. Ethyl acetate was used as a control for the measurements of absorbance. Solution containing 75-175 µg standard (digitoxin) in 2ml ethyl acetate was used to obtain a calibration curve. The total saponins was determined from the calibration curve and expressed as milligrams of digitoxin equivalent per grams of extract (mgDE/g).

Determination of Total Glycosides Content (TGC).

Balet's reagent colorimetric method was used for the determination of the total glycosides content of the extract as previously reported by Tonight *et al.* (2016) with modification. The extract (1mg/ml) was dissolved with 6ml of distilled water and 1ml of 12.5% (w/v) lead acetate solution was added. The mixture was made up to 10ml with distilled water and filtered. The filtrate (5ml) was transferred to a volumetric flask, 1ml of 4.77% (w/v) Na₂HPO₄ solution was added and made up to 10ml with distilled water and filtered. Baljet's reagent (10ml) was added to 1ml of the clear filtrate and the mixture was allowed to stand for 1hour and diluted with 20ml of distilled water. The absorbance of the mixture was read against the blank at 495nm with a UV-vis Spectrophotometer. The total glycosides content was determined from the calibration curve and expressed as milligrams of digitoxin equivalent per gram of extract (mgDE/g).

Results

Phytochemical Analysis of Methanol extract of *C. pepo* Leaves

The qualitative phytochemical analysis indicates the presence of several phytochemicals in the *C. pepo* Leaves methanol extract. As shown in table 2 below, these phytochemicals

include Cardiac glycosides, Glycosides, Flavonoids, Alkaloids, Tannins, Steroids, Saponins, and Phenols. Three phytochemicals are found in abundance Alkaloids, Flavonoids and Steroids. While Phenols and Saponins are present in moderate quantity and Cardiac glycosides, Glycosides and Tannins are less.

Table 2: Qualitative Phytochemical Composition of Methanol extract of *C. pepo* Leaves

Phytochemicals	Concentration
Cardiac Glycosides	+
Glycosides	+
Flavonoids	++
Alkaloids	+++
Tannins	+
Steroids	++
Saponins	++
Phenols	++

+ = present; - = absent

In figure 5 below, the quantities of phytochemicals were as follows; Alkaloids ($37.15 \pm 0.30 \text{ g/mg}$), Steroids ($28.42 \pm 1.17 \text{ g/mg}$), Flavonoids ($23.62 \pm 0.57 \text{ g/mg}$), Saponins ($13.07 \pm 0.64 \text{ g/mg}$), and Phenols ($17.99 \pm 1.47 \text{ g/mg}$), and Cardiac Glycosides ($6.10 \pm 0.62 \text{ g/mg}$), Glycosides ($4.76 \pm 0.61 \text{ g/mg}$), and Tannins ($10.33 \pm 1.50 \text{ g/mg}$).

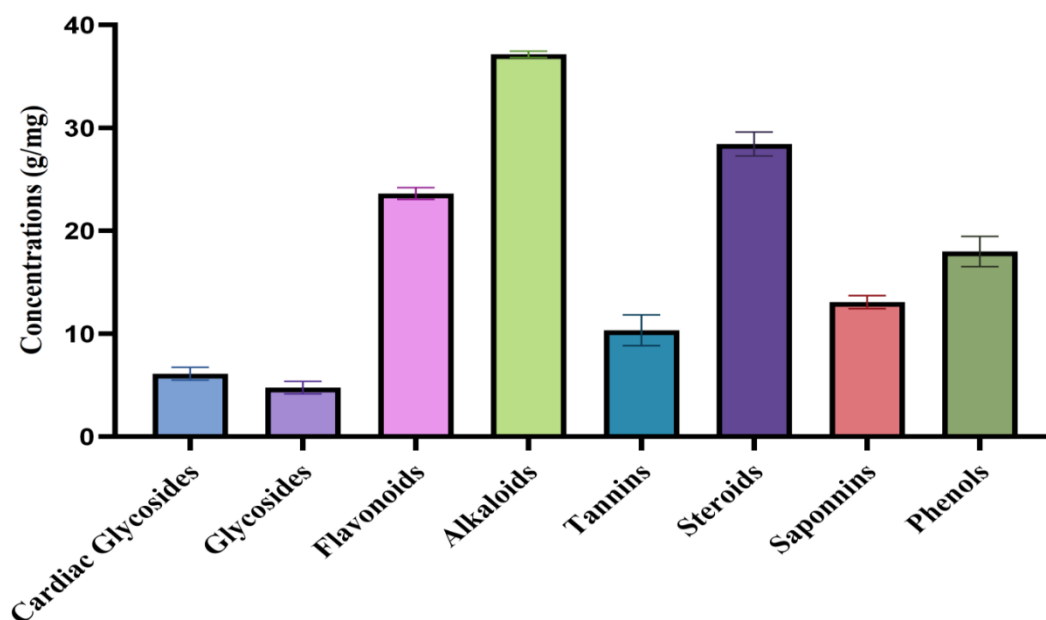


Figure 5: Quantitative Phytochemical Content of Methanol extract of *C. pepo* Leaves

Effects of Methanol extract of *C. pepo* Leaves on Climbing Ability in *D. melanogaster*

Negative Geotaxis Assay is a behavioral assay used to assess the climbing ability and locomotor function of fruit flies. The fruit flies are placed in a vertical tube, and their ability to climb upwards against gravity is measured. The Gal-4 is the control group that has normal ability to climb, the A β -42 has a decreased ability to climb, The Donepezil (Standard) has an increased ability to climb against the gravity, The 113.3 mg and 151mg of the extract are not significantly increased as compared with the A β -42. The 75.5mg of the extract has a significant increased ability to climb than the A β -42 (figure 6).

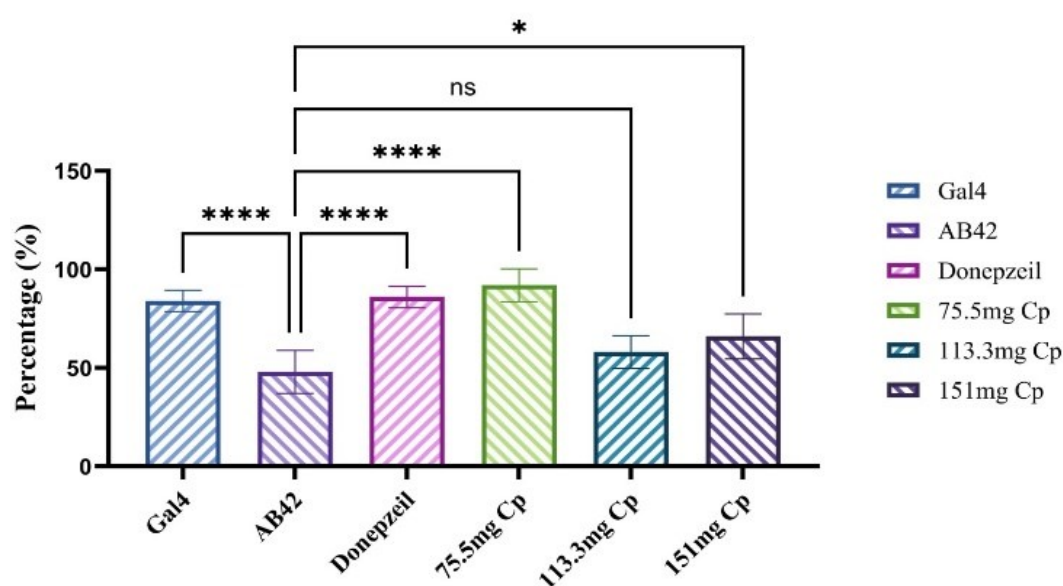


Figure 6: Result of Negative Geotaxis Assay (Climbing assay)

Effects of Methanol extract of *C. pepo* Leaves on the Learning and Memory of *D. melanogaster*

Aversive Phototaxis Suppressive Assay is a behavioral assay used to assess the learning ability of fruit flies. In this assay, fruit flies are trained to associate a specific visual cue (light) with an aversive stimulus (quinine). The Gal-4 is the control group that has increased learning ability, the A β -42 has a significantly decreased learning ability, The Donepezil (Standard) has an increased learning ability than the A β -42, The 75.5 mg and 151mg of the extract does not significantly increase in learning ability. The 113.3 mg of the extract has a significant increased learning ability than the A β -42 (figure 7 and 8).

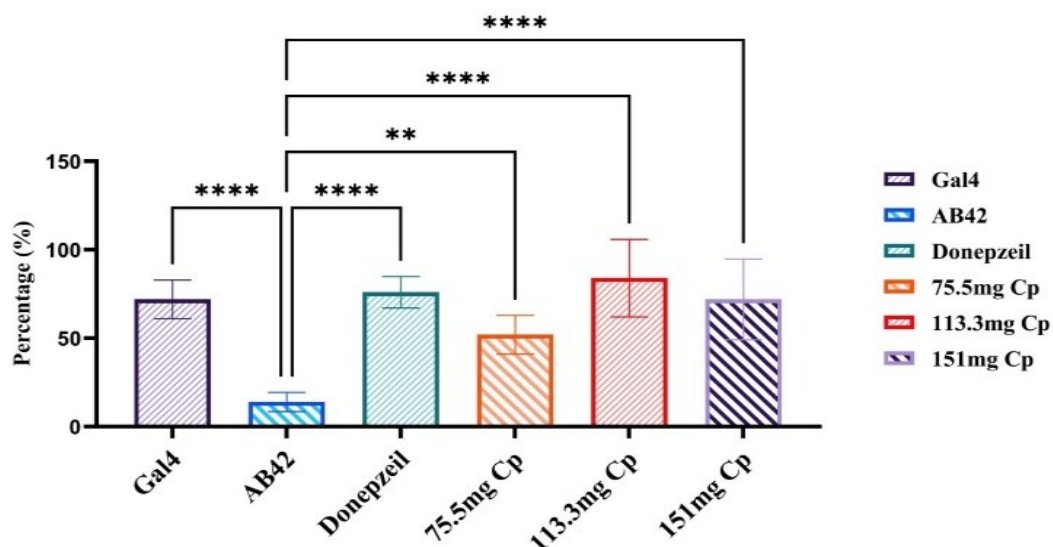


Figure 7: Result of Aversive Phototaxis Suppressive Assay (Learning)

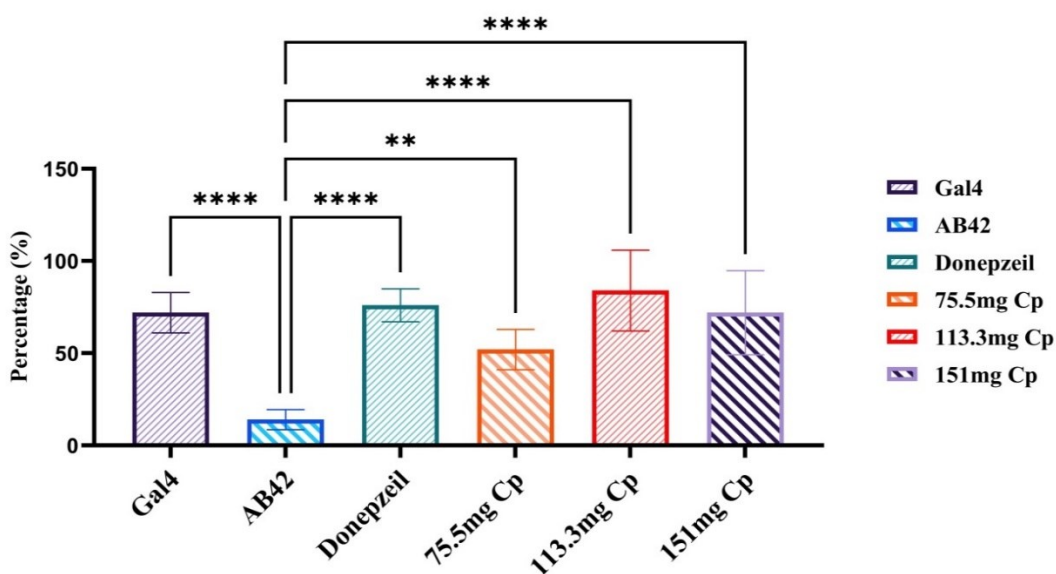


Figure 8: Result of Aversive Phototaxis Suppressive Assay (Memory)

Discussion

Phytochemicals are plant secondary metabolites that play critical roles in health promotion and disease prevention, especially in oxidative stress-related diseases like Alzheimer's (Ahmed *et al.*, 2021). Flavonoids, alkaloids, and phenols are well-documented for their antioxidant and neuroprotective properties, while tannins and saponins contribute to anti-inflammatory and membrane-stabilizing effects. Oxidative stress markers, including MDA, SOD, CAT, and GPx, are essential indicators of redox balance, particularly in neurodegenerative diseases like Alzheimer's. Elevated AChE activity is a hallmark of

cholinergic dysfunction in Alzheimer's disease, making it a crucial target for therapeutic intervention (Khan *et al.*, 2018). Thus, the present research evaluates these parameters and provides insights into the therapeutic potential of *C. pepo* leaves methanol extract. Findings from the research shows the abundance of alkaloids, flavonoids, and phenols in *C. pepo* extract these aligns with previous studies highlighting the neuroprotective effects of these compounds. Flavonoids like rutin have been shown to inhibit AChE and reduce oxidative stress in Alzheimer's models (Kooshki *et al.*, 2023). Alkaloids, such as isorhamnetin, exhibit strong radical-scavenging activity and can modulate neuroinflammation (Kooshki *et al.*, 2023).

The Negative Geotaxis Assay is a widely employed method for assessing the locomotive abilities of *Drosophila melanogaster* (Minocha *et al.*, 2022). It measures the innate tendency of flies to climb against gravity following a mechanical stimulus, which serves as a proxy for neuromuscular coordination and motor function. In neurodegenerative disease models, such as Alzheimer's disease (AD), impaired locomotive function is a hallmark symptom resulting from neural damage caused by oxidative stress, neuroinflammation, and the accumulation of toxic aggregates like A β -42 (Nitta *et al.*, 2022). Thus, the result of this assay in the present study shows the therapeutic potential of *C. pepo* to restore motor coordination in the affected *Drosophila*.

In the current study, the A β -42 expressing group (AD model) showed a significant reduction in climbing ability compared to the Gal-4 group (control). This is consistent with the literature indicating that A β -42 expression impairs neural circuitry, resulting in motor dysfunction (Kenchappa *et al.*, 2024). Treatment with Donepezil (a standard acetylcholinesterase inhibitor used in AD therapy) and *C. pepo* methanol leaf extract at doses of 75.5 mg and 151 mg significantly improved the climbing performance, suggesting neuroprotective and neuro-modulatory effects. However, the dose of 113.3 mg did not show a significant improvement compared to the A β -42 group, indicating a possible dose-dependent response.

These findings are in line with previous studies that demonstrated the protective effects of phytochemicals against lead-induced neurotoxicity (Eickelberg *et al.*, 2022). The improvement in climbing ability reflects the potential of *C. pepo* extract to modulate motor deficits through antioxidant and anti-inflammatory mechanisms (Sharma and Shanti, 2022).

The Aversive Phototaxis Suppression Assay (APS) is used to evaluate learning and memory in *Drosophila* (Pokkunuri *et al.*, 2022). This assay leverages the fly's natural phototactic behavior (attraction to light) and its ability to associate light with an aversive stimulus (quinine). Deficits in learning and memory are critical hallmarks of AD and are directly linked to A β -42-induced synaptic dysfunction and neuronal loss (Zhao *et al.*, 2021). Thus, the APS assay provides a robust model for assessing cognitive deficits and evaluating therapeutic interventions. The current results revealed a significant decrease in learning and memory performance in the A β -42-expressing group compared to the Gal-4 control. This finding

aligns with studies demonstrating that A β -42 disrupts synaptic plasticity and memory formation pathways, including the inhibition of key proteins such as CREB and BDNF (Ong *et al.*, 2020). Treatment with Donepezil and *C. pepo* methanol leaf extract at a dose of 113.3 mg significantly restored learning and memory functions, underscoring their potential therapeutic benefits. The observed cognitive improvement following *C. pepo* extract treatment can be attributed to its phytochemical constituents, such as flavonoids and phenols, which have been reported to enhance memory through the modulation of oxidative stress and inflammation (Baghel *et al.*, 2021). Notably, the neuroprotective properties of *C. pepo* are consistent with the findings by Singh *et al.* (2020), who demonstrated that plant-based extract rich in antioxidants improved cognitive performance in *Drosophila* AD models. These findings support the potential of *C. pepo* methanol extract as a source of novel therapeutic agents for neurodegenerative diseases (Chaudhuri *et al.*, 2021).

Conclusion and Recommendations

Conclusion

The present research demonstrates the therapeutic potential of *Cucurbita pepo* leaves methanol extract in mitigating oxidative stress, improving motor coordination, and enhancing learning and memory in a *Drosophila melanogaster* model of Alzheimer's disease. The abundance of phytochemicals such as alkaloids, flavonoids, and phenols in the extract aligns with previous studies highlighting their neuroprotective effects. The extract's ability to restore motor function and cognitive performance in *Drosophila* suggests its potential as a source of novel therapeutic agents for neurodegenerative diseases. Notably, different doses of the extract showed varying degrees of efficacy in restoring motor and cognitive functions, with some doses exhibiting effects comparable to the standard treatment, Donepezil. These findings support further investigation into the mechanisms underlying the neuroprotective effects of *C. pepo* and its potential applications in the treatment of Alzheimer's disease.

Recommendations

- Isolation of each lead bioactive compound for further research in-vivo is required.
- Further studies are needed to optimize the dose of *C. pepo* extract and to evaluate their safety and efficacy in clinical trials using human specimen.
- Further research needs to be carried out for molecular docking and dynamics of the lead bioactive compounds.

Study Limitations

This study has the following limitations:

- The study did not evaluate the cholinergic system's role in Alzheimer's disease, which is an important area of research.

- The study did not explore the genetic factors contributing to Alzheimer's disease, which may be essential for understanding the disease's underlying mechanisms.
- The study did not employ GC/MS to identify specific chemicals for molecular docking and dynamics evaluation, which may have provided more comprehensive insights into the molecular interactions involved.

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