# Behavioural analysis of *Drosophila melanogaster* as a model using methanolic extracts of seeds - *Thymus vulgaris, Salvia hispanica, Nigella sativa* and *Anethum graveolens* – Individual & Cumulative effect

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## Abstract

The global epidemic of overweight and obesity is affecting both high- and increasingly low-income nations. The World Health Organization (WHO) estimates that 1.9 billion persons were overweight in 2016, with 650 million of them being obese. Numerous non-communicable diseases (NCDs), including as diabetes mellitus type 2 (T2DM), cardiovascular disorders, metabolic syndrome, and cancer, are associated with overweight and obesity, increasing the burden and raising the risk of a global healthcare system collapse. Comparing current obesity and overweight medications to allopathic ones, a variety of negative effects have been linked to them. The development of novel, practical, and efficient pharmaceutical and non-pharmacological treatments is crucial to control over weight and obesity. Drosophila melanogaster's exceptional sensitivity to varying toxicant concentrations makes it a popular model for toxicity research. This study aimed at assessing the impact of methanolic extracts of individual seeds and their cumulative effect on LD50 value, survival, fly weight, locomotion and fecundity in D. melanogaster, a model organism. The seeds chosen for the study were - *Thymus vulgaris* (Thyme), *Salvia hispanica* (Chia), *Nigella sativa* (Black cumin or Kalonji) and *Anethum, graveolens* (Dill). Concentration range of less than or equal to 250 mg / 10 g of fly food (LD50 value is 284.8 mg/10 g fly medium) was used for individual seed extracts and cumulative seed extracts in various analysis. Survival rate of flies fed with High Fat Diet (HFD) along with cumulative seed extract had increased significantly ($p < 0.05$) when compared to that of flies fed with HFD alone. Compared to individual seed extracts, cumulative methanolic seed extract might have a greater protective effect on *D. melanogaster*. Also, the cumulative methanolic seed extract may help in controlling over weight compared to the individual seed extracts. A notable increase in locomotor ability was observed for flies treated with cumulative extract. During the 14-day test period, the reproductive capacity of flies was not adversely affected because both the exposed and unexposed groups were able to emerge adequately. The safety of the selected seeds in *Drosophila melanogaster* was discovered and confirmed by this study.

**Keywords:** *Drosophila melanogaster*, high fat diet, survival rate, LD50, locomotor ability, fecundity

## Introduction

An imbalance between the generation of reactive oxygen species (ROS) and antioxidant defenses leads to oxidative stress, which can harm tissues. An excess of ROS can weaken the stability of a number of biomolecules, such as proteins, lipids, and DNA. Ageing, diabetes mellitus, rheumatoid arthritis, neurological disorders, cataracts, cardiovascular illnesses, respiratory diseases, and other human diseases can all be attributed to increasing oxidative stress caused by decreased stability of biomolecules [1, 2].

Obesity is associated with the pathophysiology of many diseases that are impacted by a condition of excessive oxidative stress [3, 4]. Because of the side effects of several anti-obesity drugs and synthetic antioxidants, researchers are searching for safe and effective natural bioactive compounds that can address the conditions of oxidative stress and obesity. Plants are regarded as one of the most significant and fascinating topics that has to be investigated in order to find and create safer and more advanced medication options. Assays for lethal concentration and effects on survival and longevity in various organisms are standard methods of determining a plant substance's toxicity. A common toxicity metric used to calculate how much of a drug is required to kill half of a set of experimental organisms in a specific amount of time is called Lethal Concentration 50, or LC50 [5]. These assays and other preclinical studies must be undertaken prior to any biologically active drug being clinically evaluated for therapy.

*Drosophila* is used as a model for toxicity studies because of its remarkable sensitivity to different levels of toxicants [6]. *Drosophila* has also been used to model some human diseases because approximately 65% of the genes linked to human diseases have functional orthologs in flies. [7, 8]. In particular, all of the tissues, organs, and systems seen in human obesity and related metabolic disorders are also found in *Drosophila*. It is also a helpful model for assessing how therapeutic drugs work biologically to treat a variety of human illnesses. [9]

The fruit fly has emerged as a vital model for fundamental research because of its quick generation time, modern genetic tools, low cost, and ease of laboratory upkeep [10]. *D. melanogaster* has been useful as a model in the study of aging. Because of its quick generation time and post-mitotic cells, which make it a useful model for ageing research, life span studies is a crucial indicator of the ageing rate in *Drosophila* [11]. Embryo, larva, and pupa are the three life stages of the holometabolous fruit fly, *Drosophila melanogaster* [12].

Analyzing and comparing the life-history traits (e.g., locomotor behaviour, survival, life span, mating competitiveness and fecundity) of DM with human-genome is one of the primary benefits that a biologist would find interesting [13].

Under the influence of tested substances, the locomotor behaviour (movement) of *Drosophila melanogaster* can be observed. This behaviour can be caused by a variety of factors, such as genetic makeup, evolutionary constraints, and environmental impact [14]. Research on the fruit fly's locomotor behavior can reveal information on the internal physiological conditions of the creature as well as its adaptive reactions to outside stimuli [15].

In *Drosophila*, fecundity is a measure of reproduction that is used to estimate the number of potentially viable embryos (eggs) laid by the animal. It can be used to directly estimate the number of young flies that emerged within a given period of time and is also a commonly used proxy for estimating animal fitness [13, 16]. In order to determine how well the plant extract affected *Drosophila* fertility we can count the number of young flies that emerged every 24 hours after mating an equal number of exposed and unexposed male and female flies (1:1) and let them lay eggs for 24 hours. The central nervous system (brain), peripheral nervous system, and muscles govern movement in model organisms, including flight and locomotion in fruit flies, just as they do in humans [17, 18]. This study therefore investigates the effects of methanolic extract of seeds: S1-*Thymus vulgaris* (Thyme), S2-*Salvia hispanica* (Chia), S3-*Nigella sativa* (Black cumin or Kalonji) and S4-*Anethum graveolens* (Dill) on LD50 value, survival, fly weight, locomotion and fecundity in *D. melanogaster* - individually and cumulatively. The study was an experimental design.

**Materials and Methods - Preparation of Seed Extract:**

Seeds procured from National Seeds Corporation Ltd., Bengaluru were dried, powdered and soaked in methanol-water (70:30) for 3 days. Homogenised the contents on a hot plate at 40$^{o}$C using magnetic stirrer and centrifuged at 4000 rpm for 15 minutes. The liquid supernatant was

refrigerated in airtight containers for further analysis. Stock solution of 10% of the seed extract was prepared using methanol-water (70:30).

**Drosophila strain, Culture Conditions**

The wild-type *Drosophila melanogaster* (DM) mutant ($w^{1118}$) were obtained from Drosophila Stock Centre, Manasagangothri University Campus, Mysuru. Flies were maintained at normal temperature and humidity under 12h dark/light cycle. The *Drosophila melanogaster* flies were cultured by feeding them with normal diet (ND) prepared using 100g of jaggery,100g of Rava (Semolina) and 10g of agar in 1000 ml of water. 2-3 ml of propionic acid was added to the mixture and stirred thoroughly for uniform consistency. The mixture was heated on a medium flame for 30 mins, after the medium cools down yeast granules (15g) mixed in water were added, stirred and poured in the vials [19]. A High Fat Diet (HFD) was prepared by supplementing the control diet (ND) with 10% (w/w) olive oil.

**Determination of 14 days LD50**

The lethal dose (LD50) was determined using a protocol previously described by Iorjiim et al. [20]. Briefly, sixty (60) flies (both sexes) of 1-3 day old were anesthetized under light ice, counted and exposed to 10 graded concentrations (10 mg, 20 mg, 50 mg, 100 mg, 200 mg, 250 mg 300 mg, 350 mg, 400 mg, 450 mg) of the methanolic seed extract in 10 g food supplemented with olive oil (HFD) for 14 days. Experiment was repeated in five independent biological replicates for each concentration.

Young flies 1-3 days old were preferred. To obtain the young flies of known age the culture bottles with pupae were strictly emptied of all flies and the date noted and labeled accordingly. Adult flies of known age were then harvested from the newly hatched population. Cumulative fly death was recorded every 24 hours for the duration of the treatment. During the experimental period, flies were transferred onto new vials containing fresh food every 2 days.

**Survival assay**

The flies were divided into seven groups containing 60 flies each. Control group (I) flies were placed on normal diet alone, group II flies were placed on HFD, while groups III - VI flies were placed on HFD diet containing methanolic extracts (0.2%) of the seeds - *Thymus vulgaris* (S1), *Salvia hispanica* (S2), *Nigella sativa* (S3) *and Anethum graveolens* (S4), group VII flies were placed on HFD with cumulative seed extracts (equal proportion of S1, S2, S3 and S4) [21, 22]

**Group I** - flies fed with ND
**Group II** - flies fed with HFD
**Group III** - flies fed with HFD + 0.2% of methanolic extract of S1/10g of fly food
**Group IV** - flies fed with HFD + 0.2% of methanolic extract of S2/10g of fly food
**Group V** - flies fed with HFD + 0.2% of methanolic extract of S3/10g of fly food
**Group VI** - flies fed with HFD + 0.2% of methanolic extract of S4/10g of fly food
**Group VII** - flies fed with HFD + 0.2% of cumulative methanolic extract (CE) of S1+S2+S3+S4/10g of fly food

Every two days throughout the trial, the flies were moved to fresh food-filled vials. The vials holding the flies were kept at room temperature while the flies were subjected to various treatments for seven days. Every experiment was conducted using five separate vials for each experimental group. For seven days, fly mortality was calculated every 24 hours, and the mean survival rate was represented as a percentage of live flies. Following the therapy period, the data were analysed and presented as percentage survival and cumulative mortality. Based on the number of fatalities reported, survival analyses were computed and assessed using the log-rank Mantel-Cox test.

Percentage survival = (Number of surviving flies / Total number of flies per vial) x100

## Fly Weights

A weighing machine was used to weigh all the flies in different group vials and an average of all the flies was produced to get the weight of each fly. The weights were compared to the control setup after each diet's measurements were completed in triplicate [23].
10 flies fed with ND and HFD for 3 days in separate vials were transferred into pre-weighed empty vials (1 and 2) and weighed using a weighing balance. The mean weight of a single fly was calculated by dividing the total fly weights by the number of flies per vial.
On day 4, flies fed with HFD (10 separate vials) were treated with 2 different concentrations (0.1% and 0.2%) of the seed extracts (S1, S2, S3, S4 and CE) for next 10 days (day 4 through day 13). The weight of a single fly was estimated on the 13$^{th}$ day after 10 days treatment following the same procedure [24]. Each biological replicate was weighed three times. (n=3)

## Negative Geotaxis - Climbing Assay

The negative geotaxis experiment was used to assess the locomotor (climbing) abilities of treated and control flies after 7 days of treatment [21, 25]. Ten flies were immobilised under moderate cold anaesthesia and placed independently in marked vertical glass columns (length 15 cm; diameter 1.5 cm) for each of the control and treatment groups (Experimental groups I through VII as mentioned in survival assay). Following a 20-minute recovery time, the flies were gently tapped to the column's bottom. The quantity of flies that ascended to the column's 6 cm mark after 6 seconds, as well as those that stayed below it, were counted. The percentage of flies that fled past the 6-cm barrier in 6 seconds was used to express the data. Three assays were performed on each biological replicate, separated by one minute. Each group's score was calculated by averaging the three trials for both the treated and control fly groups.

Percentage negative geotaxis
= [number of flies that cross the 6 cm mark / total number of flies] x 100 per group

## Fecundity (Reproductive effect)

The fecundity of Drosophila was examined following the flies' exposure to the test material according to the procedure previously outlined by Charpentier et al. [26, 27] with little modification. Ten flies - five males and five females - were separated from each of the five-day treatment groups (IIIa, b to VII a, b) and group I & II (Table 1) while under ice anaesthesia. After 20 minutes of complete recovery, the flies were placed in vials with fresh fly food that was untreated and given a 24-hour period to mate and lay eggs. The flies were taken out after the 24-hour period. Over the course of 14 days, the experimental setup was checked for the potential emergence of new flies every 24 hours. The effect of the seed extract on fecundity of

**Table 1:** Diet groups for Fecundity Assay

| | | Concentration of methanolic seed | |
|---|---|---|---|
| **Group** | **Diet** | **0.1% (a)** | **0.2% (b)** |
| I | ND | | |
| II | HFD | | |
| III | HFD | S1 | S1 |
| IV | HFD | S2 | S2 |
| V | HFD | S3 | S3 |
| VI | HFD | S4 | S4 |
| VII | HFD | CE (S1+S2+S3+S4) | CE (S1+S2+S3+S4) |

**Note:** ND-Normal diet; HFD-High Fat Diet; S1-*Thymus vulgaris*, S2-*Salvia hispanica*, S3-*Nigella sativa*, S4-*Anethum graveolens* and CE-(S1+S2+S3+S4).

*Drosophila melanogaster* was measured by the average number of flies that emerged during the course of the experiment, which lasted for 14 days. This number is a direct indicator of the viable egg deposited.

## Results & Discussion

### 14 days LD50

The concentration of the individual seed extracts and duration for treatment employed in this work were predetermined from a pilot study (data not shown). The concentration of cumulative methanolic seed extract of (S1+S2_S3+S4) that is capable of killing 50% (LD50) of the population in *D. melanogaster* was found to be 284.8 mg/10 g fly medium. (Fig. 1). Concentration range of less than or equal to 250 mg / 10 g of fly food was considered as safe for individual seed extracts and cumulative seed extracts for various analysis.

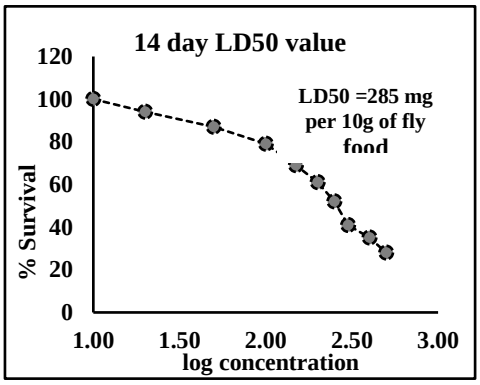



**Fig. 1:** LD50 of cumulative methanol extract of (S1+S2+S3+S4) in *D. melanogaster*. Data are presented as mean ± SD of five independent biological replicates

### Survival assay

Results of the study revealed that there is decrease in the survival rate for the flies fed with HFD (group II) for a period of 7 days. However, survival rate has increased for the treated groups (group III to VII) (Fig. 2a). This suggested that the methanolic extract of the seeds had increased the survival rate of the flies in comparison to the flies fed with only HFD. Also, it was observed that there was no significant difference in % survival of flies treated with individual seed extracts when compared to that of flies treated with HFD - group II ($p > 0.05$).

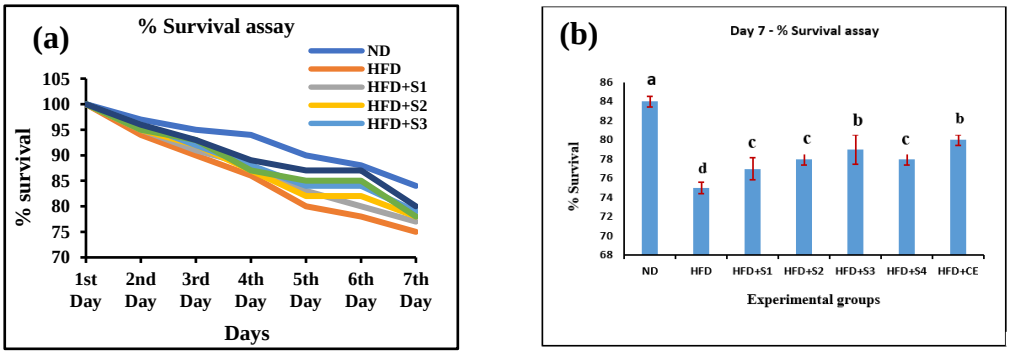



**Fig 2: (a)** Survival rate (%) of *Drosophila melanogaster* fed with ND, HFD and HFD supplemented with methanolic extract of the seeds S1, S2, S3, S4 and CE for 7 days. **(b)** Day 7 survival rate (%) of *D. melanogaster* fed with ND, HFD and HFD supplemented with methanolic extract of the seeds S1, S2, S3, S4 and CE. ND-Normal diet; HFD-High Fat Diet; S1-*Thymus vulgaris*, S2-*Salvia hispanica*, S3-*Nigella sativa*, S4-*Anethum graveolens* and CE-(S1+S2+S3+S4). Data are presented as mean ± SD (n=5).

Survival rate of group VII flies - cumulative seed extract - had increased significantly ($p < 0.05$) when compared to group II (Fig 2b). This infers that the cumulative methanolic seed extract may have better protective influence on *D. melanogaster* compared to the individual seed extracts.

## Fly Weights

Weight analysis showed an increase in the weight of the DM flies fed with HFD in comparison to the average weight of flies fed with ND. Weights were taken at the end of day 3 and day 13. With the passage of time (from day 3 to day 13) the weight increased, as can be seen in Fig. 3. Weight of flies treated with seed extracts was found to decrease significantly when compared to the flies fed only with HFD. The difference in weight of the flies treated with 0.1% and 0.2% concentrations of the seed extract was not significant. However, the decrease in the weight of the flies treated with cumulative seed extract (0.2%) is significant ($p < 0.05$) when compared to that of flies treated with HFD alone. Thus, the cumulative methanolic seed extract may help in controlling over weight compared to the individual seed extracts.

## Negative Geotaxis - Climbing Assay

Climbing ability decreased for the flies treated with HFD when compared to those fed with ND. Though the increase in locomotor ability was not significant ($p > 0.05$) for individual seed extracts compared to flies fed with HFD, a notable increase was observed for flies treated with cumulative extract (Fig. 4). This further demonstrated the safety of this seed extract on motor co-ordination in *Drosophila melanogaster*.

## Fecundity

The rate of emergence of the flies was observed for the experimental groups treated with methanolic extract of the seeds after 14 days treatment. This result (Fig. 5a, b) revealed no statistically significant difference ($P > 0.05$) between the treated groups (72 – 75%) in comparison with control and HFD groups (76%). This means that while taking this seed extract, flies reproductive capacity is not affected negatively in the testing period of 14 days since both the exposed and unexposed groups were able to emerge appropriately.

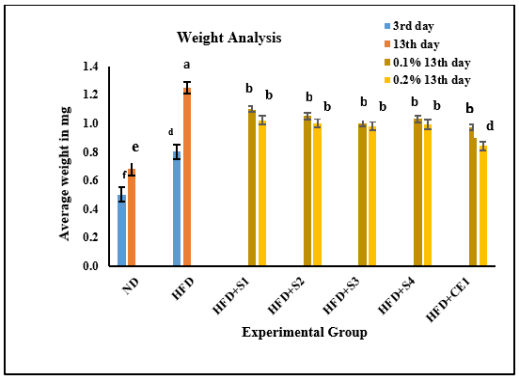



**Fig 3:** Weight Analysis to depict increase in weight of flies fed with HFD when compared to flies fed with ND, and decrease in weight with treatment of methanolic extracts of the seeds.

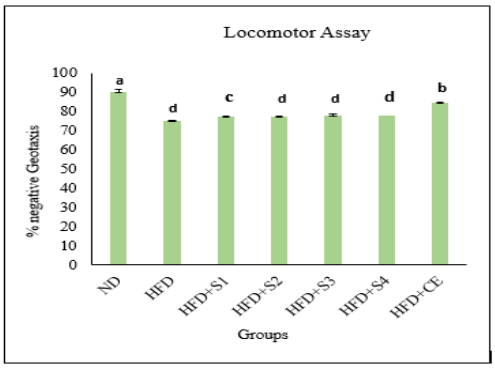
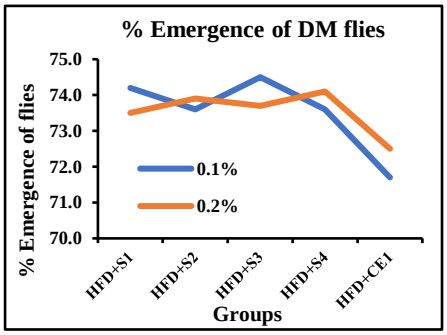



**Fig 4:** Percentage locomotor performance (Negative Geotaxis) of *Drosophila melanogaster* fed with ND, HFD, HFD supplemented with individual seed extracts (0.2%) and cumulative extract (0.2%). Values are represented as mean ± SD. (n=3) Values are not significant ($p > 0.05$).



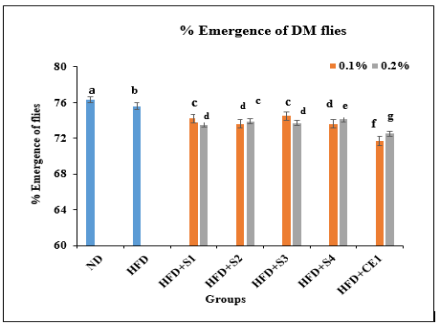




**Fig. 5a:** % Emergence of young flies from eggs of DN flies fed with high fat diet (HFD) supplemented with 0.2% concentration of individual seed extracts (S1, S2, S3, S4) and cumulative extract CE (S1+ S2+ S3+S4) **5b:** % Emergence of young flies from eggs of DM fed with normal diet (ND), high Fat Diet (HFD) and HFD supplemented with 0.2% concentration of individual seed extracts (S1, S2, S3, S4) and cumulative extract (CE-S1+ S2+ S3+S4). S1-*Thymus vulgaris*, S2-*Salvia hispanica*, S3-*Nigella sativa*, S4-*Anethum graveolens*.

## Conclusion

The safety of the selected seeds in Drosophila melanogaster was experimentally confirmed by this research study. The relatively low LD50 value for both individual and cumulative extracts of the seeds on *D. melanogaster* ensures the safety of these seeds in toxicological studies. Further explorations on this work may throw light on the use of these seeds in diet for better human well-being.

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