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Epigenetic Regulations Of T2D In HSD Fed Drosophila Melanogaster And Development Of Antidiabetic Condition With Food Additives: Cinnamon Cassia

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Abstract

Drosophila melanogaster is increasingly utilized as a versatile and most potent model genetic organism as an alternative to *rodent* and *C. elegans* models to study human diseases including metabolic disorder like diabetes. There are seven insulin–like peptides in insulin signaling pathway of *Drosophila melanogaster* and is very similar to the human insulin pathway to study different aspects of the diabetic status. In this study we discuss features of metabolic syndrome, and related abnormalities including insulin resistance and metabolic disorders. This study will focus on the diet-dependent development of metabolic disorder in fruit flies by feeding them with high sugar diet (HSD). Furthermore, regular observation of morphogenetic changes pertaining to weight, locomotor gait, memory, longevity, and insulin signaling pathways etc., additionally intended to observe the antidiabetic effects of food additives such as powder of *Cinnamomum cassia* on the diabetic larvae. The study also evaluates the potential antidiabetic effect of the food additive *Cinnamomum cassia*, known for its bioactive compound cinnamaldehyde. Imbalanced diet disrupts metabolism, homeostasis in *Drosophila melanogaster* and promotes insulin- resistant phenotypes. Therefore, the fly system may be a useful alternative tool in the exploration of molecular mechanisms of insulin resistance and the development of pharmacologic treatments. This study focuses on identifying epigenetic changes in insulin/insulin-like growth factor (IIS) which is required for both energy metabolism and growth of larval tissues and to understand regulatory mechanism of the genes *Drosophila insulin* like peptides (DILP- 1 to DILP- 8) expressed in the brain of *Drosophila.* In *Drosophila* ***growth*** and metabolism is mediated by these DILPs. This study analyzes metabolic disorder, alteration in insulin signaling pathway, antidiabetic effects of food additives and disease intervention.

Keywords: *Drosophila melanogaster,* Insulin Signaling Pathway, HSD, Diabetes, DILPs, *Cinnamomum cassia,* Tri-Acetyl Glyceride Assays, Fecundity, Locomotion, Trehalose.

1 | INTRODUCTION

Diabetes is a chronic metabolic disease which prevails among more than 400 million people worldwide. The primary causative causes of this disease are defects in insulin secretion or diminished cellular sensitivity to insulin secretion of the patients suffering from diabetes, the number in the range of 90–95% falls under type II diabetes

(T2D) [1]. Several studies have shown that T2D is caused by hereditary component influenced by environmental and epigenetic factors [2]. There are several studies revealed that 100s of genes (TCF7L2, SLC30A8, HHEX, ADAMTS9, CDC123/CAMK1D, CDKAL1, CDKN2A/B, IGF2BP2, JAZF1, NOTCH2, RBMS1, THADA, TSPAN8/LGR5, PPARG, etc.) have been associated with T2D risk. The incidence of T2D is rising worldwide and thus requires further research into novel therapeutic strategies [3]. Epigenetic modifications, which govern gene expression but do not modify the DNA sequence, have recently been identified as potential targets in the management of T2D [4]. During the last two decades, *Drosophila melanogaster* has been one of the most popular model organisms for studying many human diseases, including neurodegenerative diseases, diabetes, renal disorders, and cardiovascular diseases. The reason for choosing *Drosophila* as a model for insulin research is that it has short life span of 60 days with eating and fasting periods, easy to raise and maintain in the laboratory and it has completely sequenced genome. *Drosophila* conserves 75% of all human diseases related genes and possesses ortholog to mammals in tissues, organs, obesity, and metabolic complications. It has been found that the environmental components of caloric restriction (CR) respond similarly in *Drosophila as* that of mammals. Results in extended lifespan and delayed onset of age- related pathologies, like that of mammals, when *Drosophila* has been subjected to CR which is reduction of food intake, just above that of causing malnutrition [5]. *Drosophila* possesses a similar system of blood control as that seen in humans. Thus, the fruit fly is considered an excellent model organism to study metabolic disorders, such as diabetes [6]. During feeding, the sugars from the food travel through the digestive, intestinal tracks, and gut epithelial cells and are getting converted into trehalose which are transported into the blood of *Drosophila* which is called hemolymph and the excess sugars are stored in the form of triglyceride (TAG) or glycogen in the muscles and fat bodies. It plays a role like that of the liver and adipose tissues in mammals, the critical role in the regulation of metabolism [7].

Drosophila melanogaster has insulin producing cells (IPCs) in their brain, functionally identical to that of the β cells of islets of Langerhans of the pancreas of human. There are 14 IPCs of the fruit flies in their brain that can represent the mammalian pancreas. The fruit fly's IPC produces *Drosophila* insulin like peptides (DILPs) to decrease blood glucose level. The Adipo kinetic hormone is secreted from corpora cardiac or CC cells, which reside within the neuroendocrine ring gland of *Drosophila,* which is counterpart of glucagon of human [8] and is responsible for upregulation of blood glucose level. DILPs and AKH are the two hormones in *Drosophila* antagonize mammalian insulin signaling pathway to regulate the blood glucose homeostasis. The fruit fly *Drosophila* consists of an open circulatory system, the hemolymph which is provided with generous quantity of trehalose and glucose. Trehalose (2 glucose molecules) which is synthesized in the fat body and is corresponding to the adipose and liver tissues of mammals. In adult flies IPCs govern the closure of K+ ATP channels and opening of Ca2+ channels which stimulates the secretion of DILPs.

Drosophila has insulin and glucagon hormones, the properties of these are almost the same as that of mammalian hormones and perform the same functions. There are eight identified genes namely DILP1 (Drosophila insulin like peptide), DILP2 DILP3, DILP4, DILP5, DILP6, DILP7 and DILP8 encode *Drosophila* insulin-like peptides (DILPs). Of these DILP2, DILP3 and DILP5 are playing a vital role in the regulation of glucose and fat storage. Moreover, these cause the insulin but it fails to activate the signaling and thus leads to a state called insulin control of metamorphosis, longevity, and size of the body [9]. Insulin signaling pathway in *Drosophila is* ortholog with mammals. The proteins Chico and Lnk are recruited when D1LPs 1-7 bind to insulin receptor (InR). The P13Ks activated by Chico and Lnk convert PIP2 to PIP3 within the plasma membrane. PTEN and Susi are negative regulators of PIP3 formation. PDK1and Akt kinases are recruited to the plasma membrane by PIP3, which enables PDK1 to phosphorylate akt responsible for regulating many signaling pathways necessary for cell

growth and survival through action on downstream substrate like Foxo (transcription factor) involved in metabolism and stress responses. Tsc1/Tsc2 complex is necessary to suppress TOR (Target of rapamycin) signaling pathway and to modulate growth and metabolism and inactivates Sgg to induce glycogen synthase [10].

Drosophila has hepatocyte nuclear factor-4 (HNF-4) which is the orthologue of human HNF- 4α. Human HNF-4α gene is critical for the formation and function of beta cells of the pancreas. HNF-4 protein controls glucose homeostasis through regulation of expression of glucokinase gene, which is the main protein to sense blood glucose levels in the body [11]. *Drosophila* contains 2 GCK which include Hex-C, which is expressed in the fat body, and Hex-A expressed in IPCs that are ortholog to the hepatic and pancreatic cells of mammals. Fruit flies fed with HSD culminate in the development of hyperglycemia that leads to metabolic and neurodegenerative defects characterized that of diabetic patients and also development of oxidative defects, apoptosis, or autophagy [12].
Since thousands of years traditional ayurvedic medicine has been used to treat various human diseases including diabetes. Many medicinal plants, natural products and food additives are potential treatments for diabetic control. Despite, due to inadequate knowledge and research works in the molecular mechanisms involved in traditional medicine in treating diabetes people are unable to practice such an effective ayurvedic medicine. Hence, this study focuses on analyses of the mechanism and basic principles of traditional food additives such as powder of *Cinnamomum cassia* in maintaining glucose homeostasis in diabetic *Drosophila* a genetic model, as it has ortholog with the insulin signaling pathway of human [13]. *Cinnamon cassia* is a spice that contains bioactive compounds, of which cinnamaldehyde is one that has been proven to have antidiabetic properties by enhancing insulin sensitivity, increasing glucose uptake, and inhibiting alpha-glucosidase and alpha-amylase, which catalyze starch hydrolysis. Being a spice of natural origin, *cinnamon cassia* is considered safer than synthetic drugs. It has been found that *cinnamon cassia* possesses blood glucose-lowering and insulin sensitizing properties in both in vitro and in vivo experimental models. This makes it a very desirable candidate for studying T2D because of its effects on insulin signaling pathways. The effects that this phenomenon manifests in *Drosophila give* rise to insights relevant to human T2D, with the conservation of the mechanisms of insulin signaling and glucose metabolism across species [14].

2| MATERIALS AND METHODS

2.1 | Preparation of *Drosophila* culture media:

Drosophila culture medium was prepared by adding 50gms of jaggery and 50gms of rava to 500 ml of boiling water with constant agitation. To this mixture, 10 gms of agar along with 7.5 ml of propionic acid were added to make the medium congeal and avoid fungal contamination. This medium was poured into sterilized culture bottles up to a depth of 2 cm. Once the media had cooled to room temperature, the water droplets were removed, and a small amount of yeast granules was added to each jar. Then flies of both genders were transferred gently into the bottles, which were sealed with sterilized cotton at their opening ends.

2.2 | Induce Diabetic-like phenotypes in *Drosophila* larvae with high sugar diet

The *Drosophila* stocks were maintained on the conventional yeast-agar medium. In this experiment, one set of third-instar larvae was fed on the control diet having a 0.5M concentration of sucrose solution (HSD), and the other set in which third-instar larvae received a 1M sucrose solution to mimic the diabetic state. The weight of the larvae was noted before and after treating with HSD. Then larvae were incubated for 24hrs in the respective sucrose solution to develop a diabetic condition after which transferred to culture media. Flies were reared at 25°C.

2.3 | Measuring wet weight of the fly

Third instar larvae were collected and rinsed well with PBS (phosphate buffered saline) and dried on a Kim wipe. The larvae were weighed before and after treating them with HSD using an analytical balance.

2.4 **| Collection and extraction of *Cinnamon cassia* and then treating first/ second/ third generation diabetic larvae with *Cinnamon cassia***

The extraction was prepared by dissolving 5g of *Cinnamon cassia* powder in 50 ml of 10% ethanol. Then, the mixture was incubated for 72 hours. After incubation, the solution was filtered using Whatman filter paper. Then again 20 to 30 ml of ethanol was added and kept in a hot air oven for sterilization. After that the *cinnamon* powder was extracted and 5mg of it was added in the culture media in each culture bottle. The third instar larvae were divisible into 20 in a group and placed each group of larvae in each bottle to feed the same.

2.5 | Subjecting the *Drosophila* to Tri-acetyl glyceride assays

Drosophila flies were assayed. The flies were homogenized for 3 minutes at 40Hz in 1mL of 0.05% Tween prepared from a stock of Tween-20 in a 70°C water bath for 5 minutes. Samples were spun at 5000rpm for 1 minute. 500µl of the supernatant was transferred to a new microfuge tube. The supernatant was spun again at 14000rpm for 3 minutes. 50ul of sample was added to 200µl of TG solution in a 96 well plate. The plate was incubated at 37°C for 5 minutes. After that, absorbance was measured at 630nm against a standard curve to find out the quantity of fat. TAG analysis was carried out on flies in control, high sugar diet, and dietary supplement with food additive.

2.6 |Trehalose measurements in *Drosophila larval* homogenate

There are 8–15 third instar larvae weighing approximately 5–10 mg were collected and placed on ice for homogenization. Larvae were homogenized using a homogenizer in 100 µl of cold trehalase buffer. The homogenate was incubated at 70°C in a water bath for 10 minutes. The extract was centrifuged at 5000 rpm for 5 minutes at 4°C. Twenty microliters of the supernatant was mixed with 20 µl of porcine trehalase used to measure digested trehalose. For the standard, glucose at 25 mg/ml was used for the reaction, whereas trehalose, at 25mg/ml, was used for the test reaction. A volume of 40 µl of trehalase buffer was added to the mixture. All the samples were incubated overnight at 37°C. The samples were then centrifuged at 4°C for 5 minutes at 5000 rpm. Trehalose concentration was measured by glucose assay kit. For that, added 1 ml of glucose reagent to 20 µl of each sample. Samples were read in a spectrophotometer at 540 nm. The experiment was repeated three times to ensure accuracy.

2.7 | Measurement of Fecundity

For the fecundity assays, 40 total pairs per treatment group were collected less than 12hrs post exclusion and placed in 8-dram vials, one male and one female per vial. A sugar-charcoal media (5 mL) covered with 75µL of yeast solution was added, containing 1 g yeast/mL of 1% acetic acid. Pairs were allowed to oviposit for a duration of 24 hours and subsequently required transfer to fresh vials at 24-hours interval for a total of 10 days. The number of eggs was enumerated every 24 hours.

2.8 | Measurement of Locomotor performance

Vertical locomotion (climbing) was measured. 10 flies (n = 8 vials) were placed in a vial and left to recover for 24 hours. The following days, flies were tested for height reached for four seconds after being tapped to the bottom of an empty vial. The experiment was performed five times at 30 second intervals and the mean height of all flies over all trials had been determined per vial.

2.9 | Isolation of RNA from Fat Bodies of 3rd Instar *Drosophila* Larvae and analysis of Dilp2 (*Drosophila* insulin like peptide 2) expression.

Third instar larvae were harvested, washed, and kept contamination-free. The dissecting tools and workspace were sterilized to prevent RNase contamination. The larvae were anesthetized on ice or with a light CO2 stream. The fat bodies were teased out gently under a dissection microscope and transferred directly into a microcentrifuge tube containing pre-chilled TRIzol reagent. The tissues were treated with 1 ml of TRIzol reagent per 100 mg of tissue. The tissues were homogenized using a pestle or a mechanical homogenizer until completely lysate homogenate was left at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. Chloroform (200 µl per 1 ml of TRIzol used) was added. The tube was closed tightly. It was mixed vigorously by vortexing for 15 seconds. It was centrifuged at 12,000 rpm for 15 minutes at 4°C. The upper aqueous phase was transferred carefully to a new tube free from contamination from the interphase or organic phase. Isopropanol (0.5 ml for every 1 ml of TRIzol used) was added to the aqueous phase. The tube was gently inverted several times to mix the solution. The mixture was left to incubate at -20°C for at least 30 minutes to precipitate the RNA. The sample was centrifuged at 12,000 rpm for 10 minutes at 4°C. Supernatant was removed, and the RNA pellet was washed with 75% ethanol. The tube was gently mixed by vortexing followed by centrifugation at 7,500 rpm for 5 minutes at 4°C.

The RNA pellet was air-dried for about 10-15 minutes to avoid over drying. The RNA pellet was resuspended in the appropriate volume of RNase-free water or TE buffer. If dissolved, the sample was heated to 55-60°C for 10-15 minutes. On a spectrophotometer, RNA concentration and purity were checked. The ratios of A260/A280 and A260/A230 were expected to be close to 2.0 for pure RNA. Using an isolated mRNA and a reverse transcription kit, this mRNA was converted into complementary DNA, whose expression of Dilp2 (drosophila insulin like peptide 2) was then assessed via qPCR. The obtained DNA was also run on an agarose gel for further electrophoresis. RNA samples were always handled gently, with caution against degradation. Operations were conducted relatively fast; as much as possible, samples were kept on ice. Quantities of reagents were apportioned in proportion to the tissue amount handled. Chloroform was handled in a fume hood or well-ventilated area due to its toxicity. By following these steps, high-quality mRNA was successfully isolated from the fat bodies of 3rd instar *Drosophila* larvae for subsequent Dilp2 (drosophila insulin like peptide 2) gene analysis.

3| RESULTS

3.1 | Induce Diabetic-like phenotypes in *Drosophila* larvae with high sugar diet

It has been demonstrated that third-instar *Drosophila* larvae gain weight upon feeding with an excess amount of sucrose from a diet rich in sugar, and *Drosophila* demonstrates symptoms like those of diabetes. The larvae and pupa emergence on the day before the treatment on sucrose-rich diet was used for the assessment of survival rate. When compared with the control, the larvae that fed with sucrose were typically heavier. This study, in its own merit, is supported by earlier research where at an early stage following diet with high sugar, obesity that results in increased weight.

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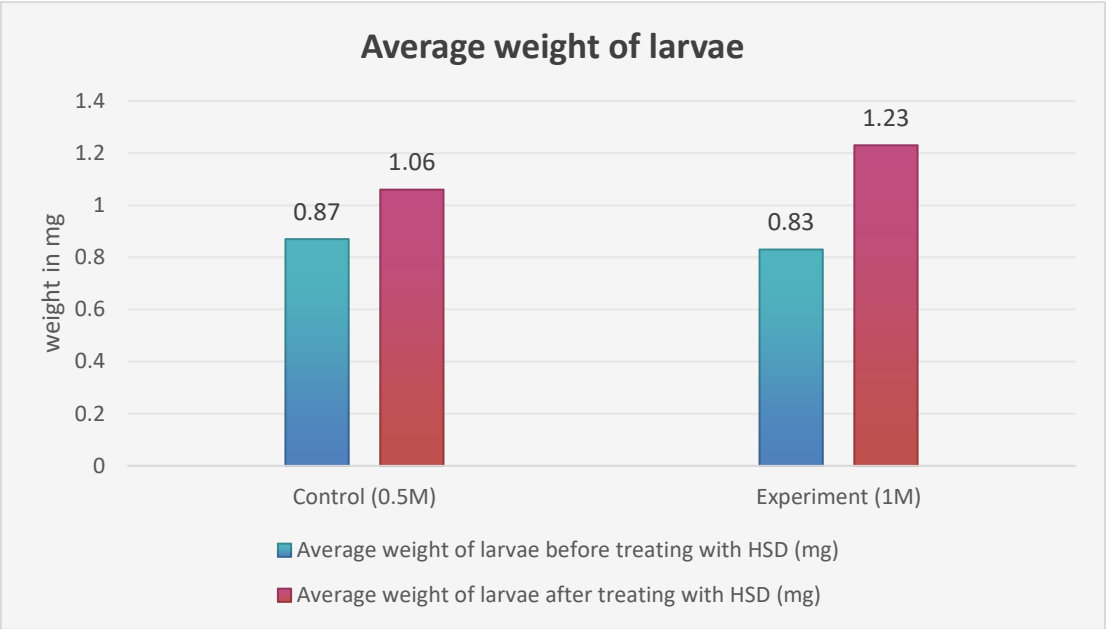
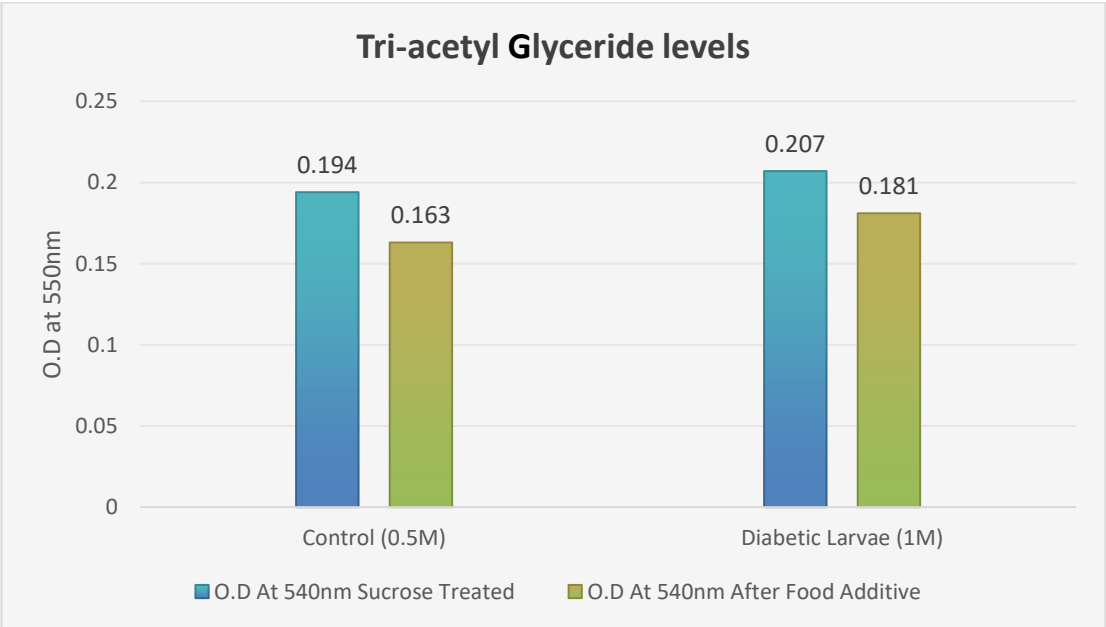


Graph 1: Average weight of larvae before and after treating with sucrose.

3.2 | Subjecting the larvae to Tri-acetyl glyceride assays

The O.D. values increase significantly when flies are treated with high sugar (both 0.5M and 1M), indicating a potential stress and adverse effect on the fly's physiology, mimicking diabetic conditions. When *cinnamon* is added to the diet of diabetic flies, the O.D. values observed to be decreased (0.207 and 0.163 for the 0.5M sugar and 0.207 to 0.181 for the 1M sugar). This result gives an indication that *cinnamon* can moderate the negative consequence of high sugar intake by providing metabolic health that reduces stress of the flies.

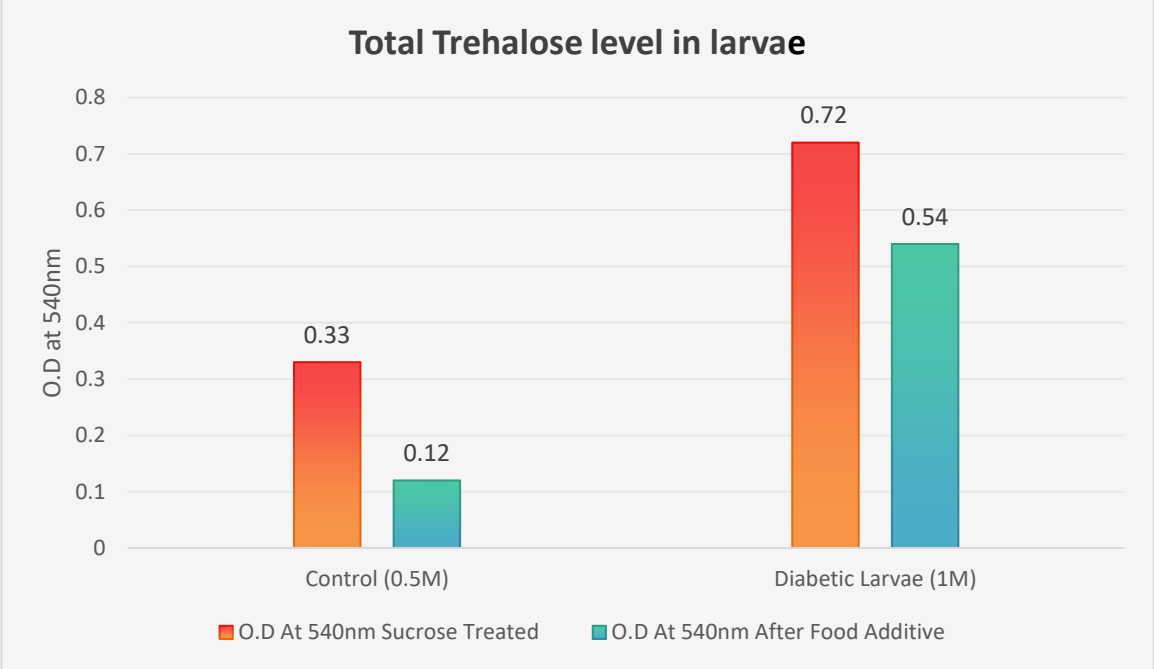


Graph 2: Tri-Acetyl Glyceride level in *Drosophila melanogaster*

3.3 | Total Trehalose measurement in *Drosophila* larval homogenate

Elevated sucrose intake (1M) led to higher trehalose level, evident from increased O. D. values in diabetic larvae compared to the control group. The food additive effectively reduced trehalose level in the both groups,

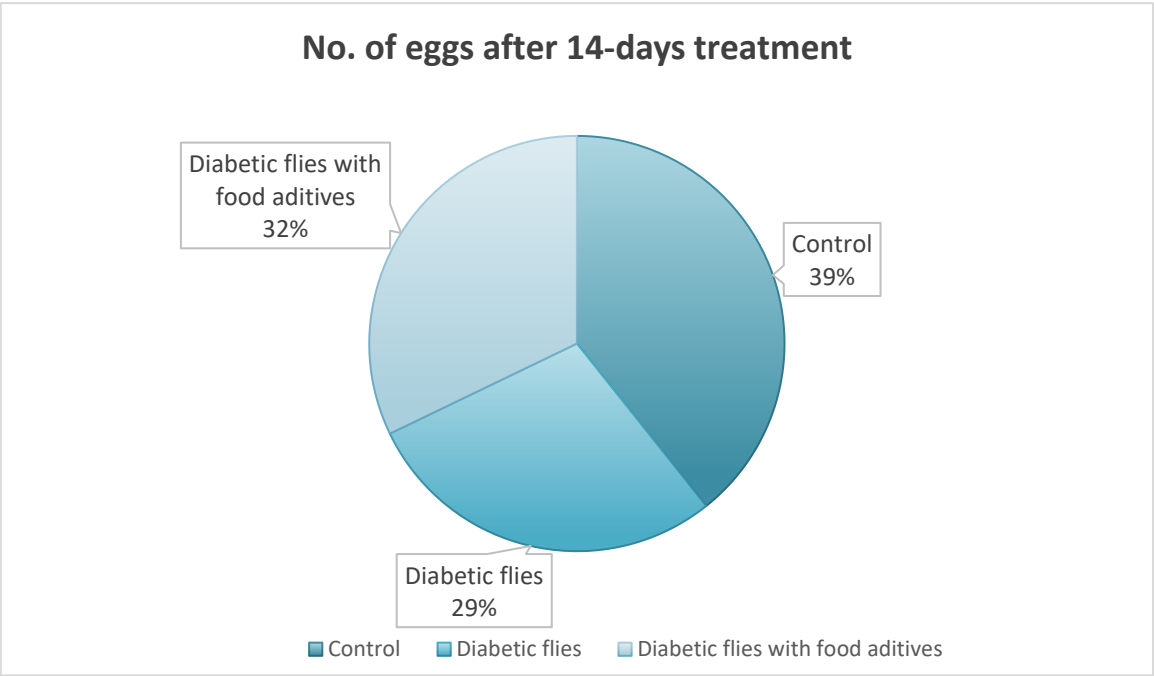
with a stronger effect. This suggests that the cinnamon helps regulate sugar metabolism and reduce hyperglycemic conditions in larvae.



Graph 3: Estimation of Total Trehalose level in larvae of control, experimental, and infested with *cinnamon*

3.4 | Measurement of Fecundity

Diabetic flies lay fewer eggs compared to the control group, indicating reduced fecundity due to the high sucrose diet. The addition of food additives improves fecundity (36 eggs), but it does not restore it to the level of the control group (44 eggs). This suggests that the food additive partially mitigates the negative impact of a high-sucrose diet on fecundity in *Drosophila*.



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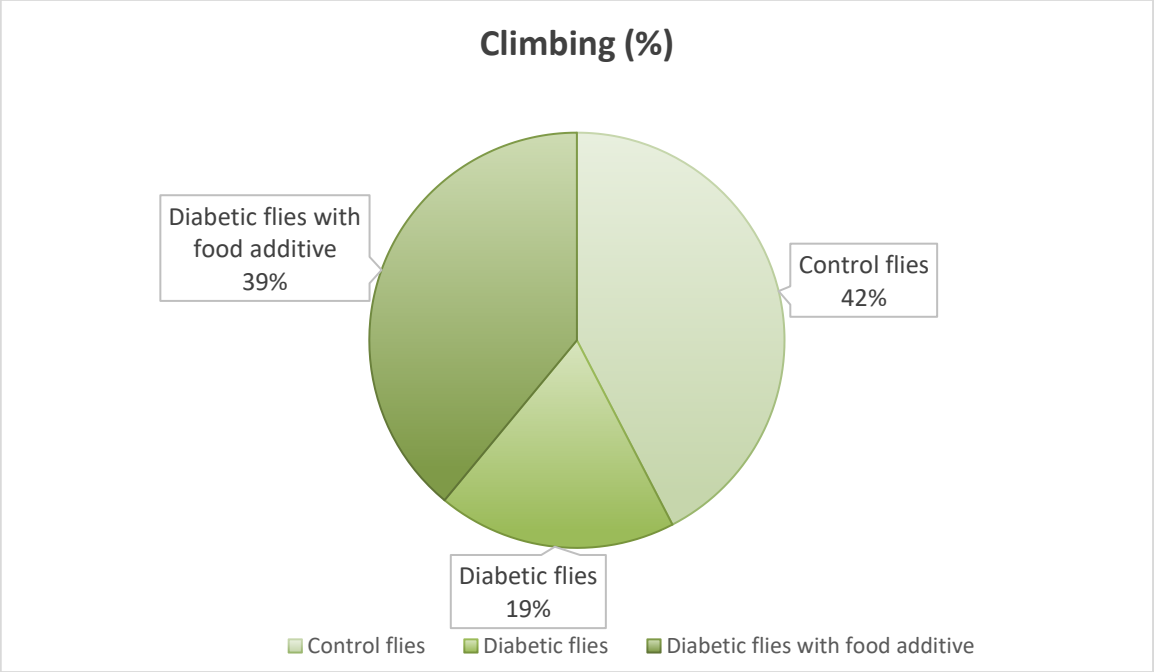
Graph 4: Fourteen-day cinnamon powder extract exposure increased fecundity in *Drosophila*

3.5 | Measurement of Locomotory performance

Control flies had a climbing percentage of 83.33%, suggesting healthy locomotory activity, but diabetic flies had a significantly lower climbing ability, with a percentage of 36.6%, indicating reduced locomotor performance. However, diabetic flies treated with food additive displayed enhanced locomotory performance compared to diabetic flies, with a climbing percentage of 76.66%, indicating that the food additive had a beneficial effect. Cinnamon cassia, a dietary supplement, improves the locomotory performance of flies with diabetes, but does not totally restore it to control level.



Graph 5: Locomotory performance decreased climbing ability in *Drosophila*

3.6 | Isolation of RNA from Fat Bodies of 3rd Instar *Drosophila* Larvae and analysis of Dilp2 expression.

The presented image illustrates the outcomes of gel electrophoresis conducted to examine the expression levels of the Dilp2 gene across control, diabetic, and diabetic flies that received treatment with a food additive (FA). The left segment of the image emphasizes the lanes displaying bands in response to a particular stain, whereas the right segment depicts the same gel exposed to UV light, facilitating enhanced visualization of the bands. The bands have different intensities across the lanes, which would be indicative of different Dilp2 gene expression in control, diabetic, and treated flies. The expression of the Dilp2 gene in diabetic flies varies with the control. This shows an effect of diabetes on the gene's activity. Diabetic flies treated with the food additive (FA) show a possibly altered gene expression pattern, indicating that the additive may modulate Dilp2 expression.

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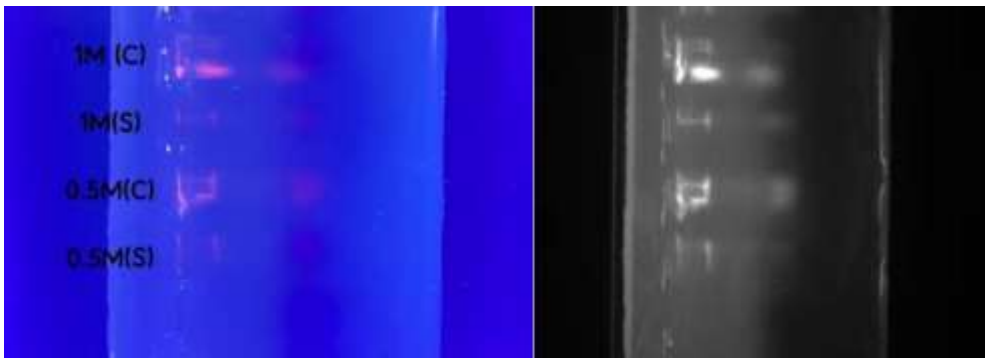

Fig. 1: The expression of the *Dilp2 gene* in Control, Diabetic and Diabetic flies treated with food additive (cinnamon).

DISCUSSION

Metabolic studies in the *Drosophila melanogaster* model organism are very extensive as it is highly amenable to genetic manipulation in necessary pathways, especially glucose and lipid metabolism pathways [15]. In this experiment, the high sugar diet elicited diabetic phenotype of the larvae by causing high levels of triglycerides, disrupted glucose and trehalose metabolism, a decrease in the reproductive performance of the larvae, and poor locomotory capability. This would then be consistent with other species that have recorded observations under induced hyperglycemic or diabetic states, thus underlining the value of *Drosophila* as a model for studying diabetes and metabolic diseases. Positive outcomes were observed with the supplementation of *Cinnamon cassia* powder, suggesting a potential role in counteracting some of the negative consequences arising from a diet high in sugar. Some of the traditional uses of *Cinnamon* are related to therapeutic properties that include anti-inflammatory and anti-hyperglycemic effects. Such improvements in metabolic markers and physical activity after supplementation with *Cinnamon cassia* indicate potential benefits in mitigating diet-induced metabolic derangements. However, further research is required to clearly define the molecular mechanisms through which *Cinnamon cassia* produces such effects. In terms of gene expression for Dilp2, the present study determined that the high-sugar diet had changed the insulin-like peptide signaling in the fat bodies of *Drosophila.* Dilp2 is involved in the regulation of glucose homeostasis and lipid metabolism. The observed variation in the expression of Dilp2 (drosophila insulin like peptide 2) cells indicates that changes in insulin-like signaling pathways could underlie the diabetic-like condition caused by a diet rich in sugar. Thus, this study further emphasizes the applicability of *Drosophila* as a model to understand the molecular basis of diabetes and the action of potential therapies.

Cinnamomum cassia contains several bioactive compounds, among which cinnamaldehyde [18] and eugenol [19] play a significant role in its antidiabetic properties. Cinnamaldehyde has been found to enhance insulin sensitivity, improve glucose uptake, and regulate key enzymes involved in glucose metabolism. Eugenol, on the other hand, contributes to glucose homeostasis through its antioxidant and anti-inflammatory effects, reducing oxidative stress-related insulin resistance. The combined action of these phytochemicals is responsible for lowering blood glucose levels, supporting the traditional and scientific recognition of *Cinnamomum cassia* as a potential complementary therapy for diabetes management.

CONCLUSION

This study demonstrated that this diet resulted in widespread physiological changes of the larvae, including changes in weight, impaired locomotor functions, disturbances in glucose metabolism, and reproductive dysfunctions. The results indicate that the high-sugar diet indeed triggered a diabetes-like phenotype in the larvae.

The therapeutic effects of intervention using the *Cinnamon cassia* were found to have the potential in neutralizing or reducing some of the adverse effects of a high-sugar diet. Further analyses, namely triglyceride levels, reproductive efficiency, and tests for motor activities indicated that supplementation with *Cinnamon cassia* may enhance metabolic and physiological activities as well as reduce fat deposition linked to the high-sugar diet. Further, the molecular analysis that included the expression of a key insulin-like peptide, Dilp2 supported the hypothesis that a high-sugar diet might alter metabolic signaling pathways in *Drosophila* and could be modulated by treatment with *Cinnamon cassia*. The recent studies [16],[17] establish *Drosophila* as a model system in which to study T2D. This model represents a simple and unique platform on which to identify gene products and drugs that can ameliorate the complications of T2D. Future studies will dissect the functional significance of different metabolic pathways in the production of toxic lipid mediators of sugar-induced pathophysiology.

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