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Effect of *Stevia rebaudiana* pure and crude leaf powder on the climbing ability of *Drosophila melanogaster*

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Abstract

The act of locomotion is crucial for various purposes, including finding food, locating a mate, escaping predators, defending territory, and responding to stress. Consequently, locomotion is essential to most animal behaviours. Thus, for animals to survive, move, and reproduce effectively, a balance between energy intake and expenditure is crucial. Sugar substitutes, which mimic the taste of sugar but often with fewer calories, are one such category of products. These substitutes are typically about 200 times sweeter than sugar and can be either synthetic or natural. *Stevia rebaudiana* powder is the most popular natural sweetener permitted for use in various products. The basic principle involves placing a known number of flies in a vial and tapping it firmly against a hard surface, causing the flies to fall to the bottom. Due to their innate behavior, the flies will attempt to climb to the top of the vial, moving against gravity. The results of both products, i.e., stevia extract and dried stevia leaves, were compared and discussed.

Keywords: *D.melanogaster*, diet, climbing assay, stevia, stevia dried leaf, mated flies.

Introduction

Every activity an organism undertakes to ensure its survival and reproduction requires energy. For instance, tasks such as movement, courtship, and general motion are vital. The act of locomotion is crucial for various purposes, including finding food, locating a mate, escaping predators, defending territory, and responding to stress. Consequently, locomotion is essential to most animal behaviors (Jordan et al., 2007). Animals derive their energy from their diet, which can be

classified qualitatively (composition) or quantitatively (availability). Since animals rely on food for energy and other nutritional needs, the qualitative impacts are significant. Thus, for animals to survive, move, and reproduce effectively, a balance between energy intake and expenditure is crucial (Sibly, 1991). The equilibrium is influenced by the intake, digestion, and allocation of newly acquired energy to various processes such as growth, reproduction, and mobility (Karasov, 1986).Here are several assays designed to quantify the effects of genetic mutations and environmental conditions on *Drosophila* climbing behavior. Most of these assays utilize the flies' natural tendency to climb, known as negative geotaxis, or the climbing assay. In 1967, Benzer proposed that the counter-current apparatus used for studying phototaxis could also be adapted to study gravitaxis. Building on this initial concept, Ganetsky and many others have refined and expanded the assay.

The basic principle involves placing a known number of flies in a vial and tapping it firmly against a hard surface, causing the flies to fall to the bottom. Due to their innate behavior, the flies will attempt to climb to the top of the vial, moving against gravity. This quantitative assay measures how many flies climb past a marked point on the vial within a given time period. Additionally, measuring the speed of the flies' climbing has proven to be a reliable parameter, revealing defects that might not be evident when only considering the total number of flies climbing (Botella JA, et al., 2004)

In contemporary times, there is an increased focus on fitness, appearance, and health. Sedentary lifestyles, urbanization, excessive consumption of sugary foods, and higher fat intake cause an energy imbalance, where calories consumed exceed calories required. This has led to a growing demand for food products that promote better health, driven by heightened health awareness among consumers. Consequently, there is an increased preference for a broader range of low-calorie products. Sugar substitutes, which mimic the taste of sugar but often with fewer calories, are one such category of products. These substitutes are typically about 200 times sweeter than sugar and can be either synthetic or natural. Synthetic substitutes are often referred to as artificial sweeteners (Kirtida, 2011).

The usage of artificial sweeteners has surged due to their lower cost, reduced calorie content, and perceived health benefits. However, how these sweeteners impact locomotor behavior is a pertinent question. Food intake directly affects an organism's physiological needs, balancing its energy requirements and enabling various activities. *Drosophila*, a model organism with a metabolic system highly conserved with humans, is ideal for studying human diseases. Thus, the current study aims to explore the impact of artificial sweeteners on *D. melanogaster's* locomotor behavior.

Stevia rebaudiana powder is the most popular natural sweetener permitted for use in various products. It is about 200 times sweeter than sucrose (Food and Drug Administration, 2018). Research has demonstrated that replacing regular sweeteners with these alternatives can positively affect blood glucose levels, insulin resistance, body weight, seeking behavior, fat accumulation, and lipogenic effects (Mathur et al., 2020). The present study examines the efficacy of stevia leaf powder and its pure extract by evaluating the climbing ability of flies.

Materials and Methods

Establishment of stock

The experimental stock of *Drosophila melanogaster* was obtained from the *Drosophila* Stock Center at Manasagangotri, University of Mysore. *D. melanogaster* is one of the most widely used and well-understood model organisms. The flies obtained were redistributed and raised in different culture bottles containing wheat cream agar media (100g of jaggery, 100g of wheat powder, and 10g of agar-agar boiled in 1000ml of double distilled water, with 7.5ml of propionic acid added at the end). Twenty flies (10 males and 10 females) were introduced into culture bottles and maintained at a temperature of

22°C ± 1°C with a relative humidity of 70% in a 12-hour dark: 12-hour light cycle. The virgin flies were isolated in the pupal stage and cultured in test media. The test media contained 1%, 2%, and 3% natural sweetener stevia powder (pure extract) based media, and the flies grown in normal wheat agar media were used as control. Similarly, test media containing 1%, 2%, and 3% dried stevia leaves powder based media were used, and the flies grown in normal wheat agar media served as controls. The results of both products, i.e., stevia extract and dried stevia leaves, were compared and discussed. Five-day-old flies were isolated from the culture and raised in control, stevia extract media, and dried stevia leaves media. They were maintained under the conditions mentioned above. The flies were allowed to grow in the corresponding media (control and test) for five days before being used for different parameters.

Negative Geotaxis (Climbing Assay)

The flies obtained from the two different stevia extract-based media were subjected to a climbing assay at 5 days and 25 days of age. In this assay, a tube is used to record the climbing ability of flies. It is a long transparent hollow tube about 25 cm in length with a diameter of 1-2 cm. One end of the tube is closed with a cap, and the other end is closed with a cotton plug. The tube is marked at two different height levels, 5 cm and 10 cm. Male and female flies were anesthetized using ether and placed in vials separately. The flies were then transferred into the assay tube and closed with a cotton plug. The tube was gently tapped to encourage the flies to climb upwards. The height reached by the flies in a set time (10 seconds) was measured. The climbing ability was quantified by measuring the distance traveled or the number of flies reaching a certain height. The heights climbed by the flies within 10 seconds were noted down.

Statistical Analysis

The data obtained were analyzed using IBM SPSS version 29.0. Mean, standard error, one-way ANOVA, and Tukey's Post-Hoc test were carried out for the data obtained from the climbing assay. A graph of concentration versus climbing assay in seconds was plotted for both stevia pure extract and stevia dried leaf extract.

Results

Effect of Pure stevia powder:

Figure 1-7 represent the old and young flies climbing abilities treated with pure stevia powder. Figure 1 illustrates the effect of pure stevia on the climbing assay of 5-day-old male *D. melanogaster*. The graph shows a significant increase in climbing ability in flies treated with 2% stevia compared to the control group ($F=24.976$, $df=3$, $p<0.05$). **Figure 2** depicts the effect of pure stevia on the climbing assay of 5-day-old female *D. melanogaster*. Here, the control group outperformed the stevia-treated group significantly ($F=32.834$, $df=3$, $p<0.05$). **Figure 3** compares the effect of pure stevia on both male and female 5-day-old flies, showing significant differences in climbing ability between media ($F=15.013$, $df=3$, $p<0.05$), between sexes ($F=21.966$, $df=1$, $p<0.05$), and between media and sex interaction ($F=45.178$, $df=3$, $p<0.05$). **Figure 4** presents the effect of pure stevia on the climbing assay of 25-day-old male *D. melanogaster*. The control group exhibited higher climbing ability compared to the stevia-treated group, but the difference was not significant ($F=1.912$, $df=3$, $p>0.05$). **Figure 5** shows the effect of pure stevia on the climbing assay of 25-day-old female *D. melanogaster*, with the control group outperforming the stevia-treated group significantly ($F=6.356$, $df=3$, $p<0.05$). **Figure 6** compares the effect of pure stevia on the climbing assay of 25-day-old male and female flies. Significant differences were observed between media ($F=5.675$, $df=3$, $p<0.05$) and between sexes ($F=62.523$, $df=1$, $p<0.05$), but not in the interaction between media and sex ($F=0.855$, $df=3$, $p>0.05$). **Figure 7** shows the impact of pure stevia on the climbing ability of both 5-day-old and 25-day-old male and female *D. melanogaster* flies. The data suggests varying effects based on age and sex. The climbing ability of young male flies is significantly higher in 2% stevia-treated media compared to the control,

whereas young female flies show higher climbing ability in the control group. Older male flies do not show significant differences between control and stevia-treated media. However, older female flies have higher climbing ability in the control group compared to stevia-treated media.

Effect of Dried Stevia Leaf:

Figures 8-13 gives an account of the 5 day and 25 day old flies and their climbing abilities treated with stevia leaf powder

Figure 8 illustrates that 5-day-old male *D. melanogaster* have significantly higher climbing ability in 2% dried stevia leaf-treated media compared to the control ($F=15.658$, $df=3$, $p<0.001$). **Figure 9** demonstrates that 5-day-old female flies have significantly higher climbing ability in the control group compared to all dried stevia leaf-treated media ($F=91.424$, $df=3$, $p<0.001$). **Figure 10** presents a comparison of 5-day-old male and female flies treated with dried stevia leaf, indicating significant differences between media types ($F=38.824$, $df=3$, $p<0.005$), sexes ($F=17.082$, $df=1$, $p<0.005$), and their interaction ($F=45.082$, $df=3$, $p<0.05$). **Figure 11** shows that 25-day-old male flies have slightly higher climbing ability in 3% dried stevia leaf-treated media compared to the control, but this difference is not significant ($p>0.05$). **Figure 12** reveals that 25-day-old female flies have higher climbing ability in 2% and 3% dried stevia leaf-treated media compared to the control, though this difference is also not significant ($p>0.05$). **Figure 13** compares the climbing ability of 25-day-old male and female flies treated with dried stevia leaf, showing insignificant differences between media types ($F=1.748$, $df=3$, $p>0.05$), but significant differences between sexes ($F=63.087$, $df=1$, $p<0.05$), with an insignificant interaction ($F=0.350$, $df=3$, $p>0.05$). **Figure 14** provides a comparative analysis of the effects of dried stevia leaf on the climbing ability of both young (5-day-old) and older (25-day-old) male and female *D. melanogaster* flies.

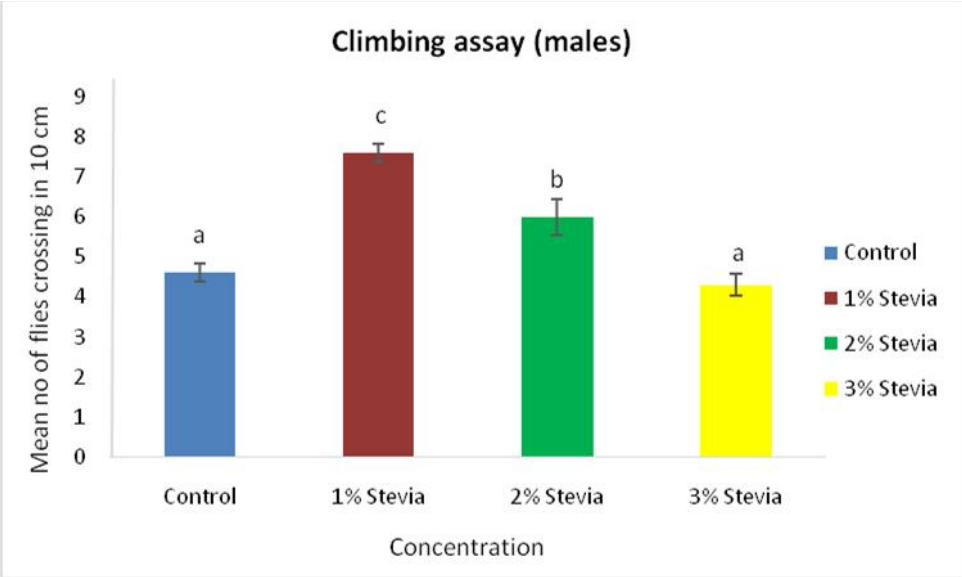

Figure 1: Effect of pure stevia on the climbing assay of 5 day old male *D. melanogaster* flies.

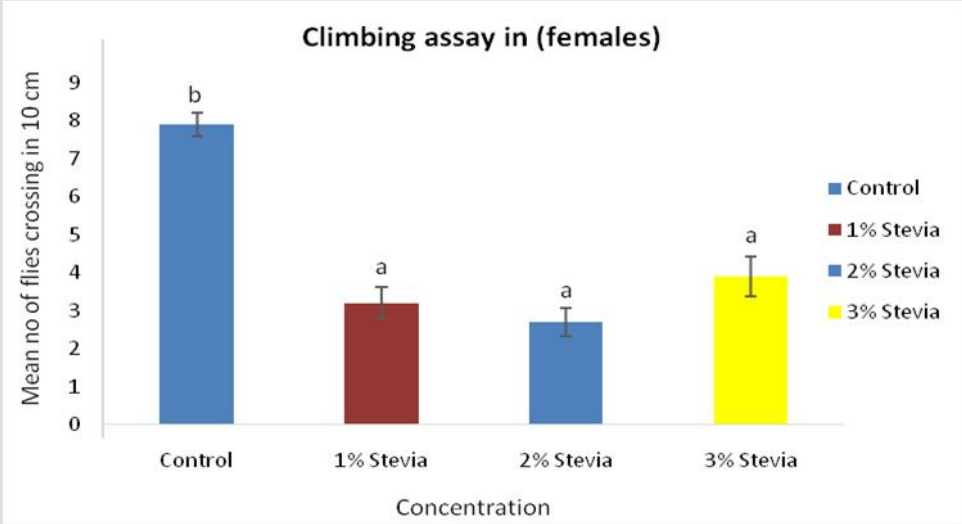

Figure 2: Effect of pure stevia on the climbing assay of 5 day old female *D. melanogaster* flies.

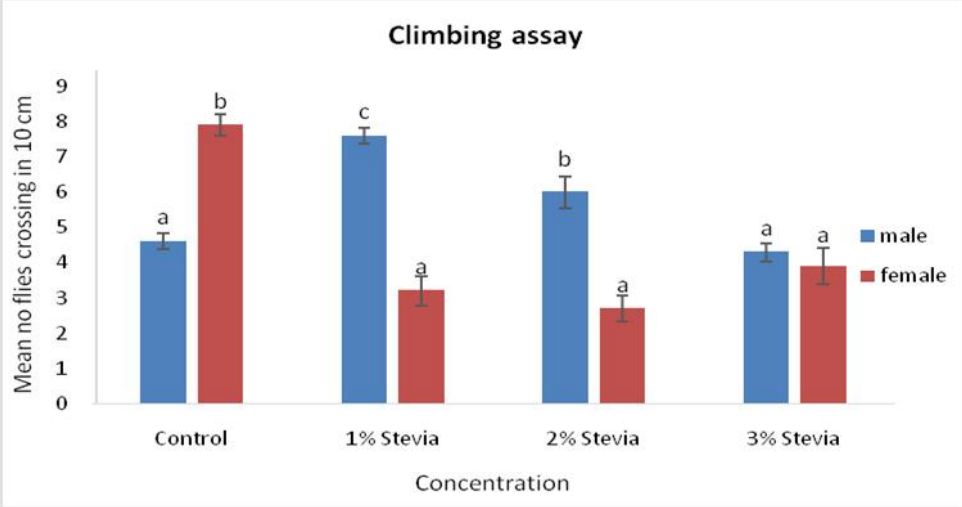

Figure 3: Effect of pure stevia on the climbing assay of 5 day old male and female *D. melanogaster* flies.

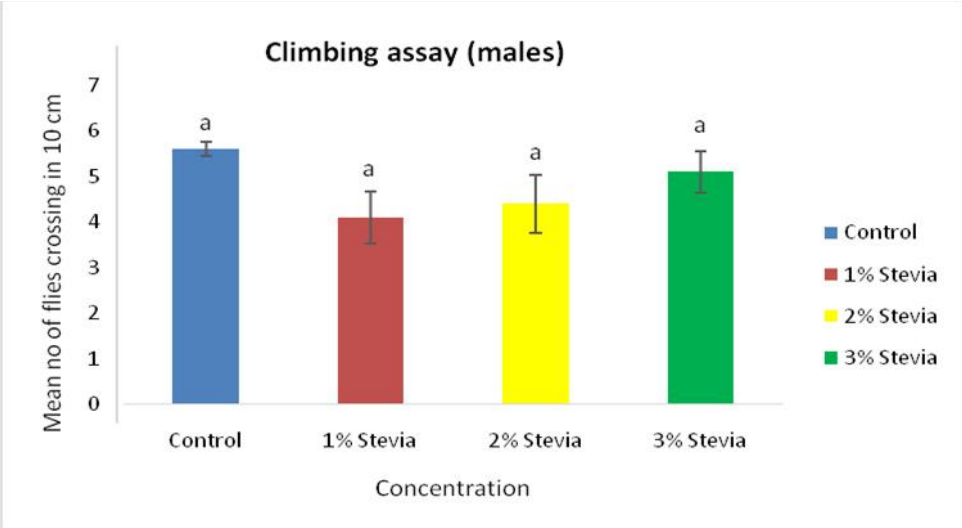

Figure 4: Effect of pure stevia on the climbing assay of 25 day old male *D. melanogaster* flies.

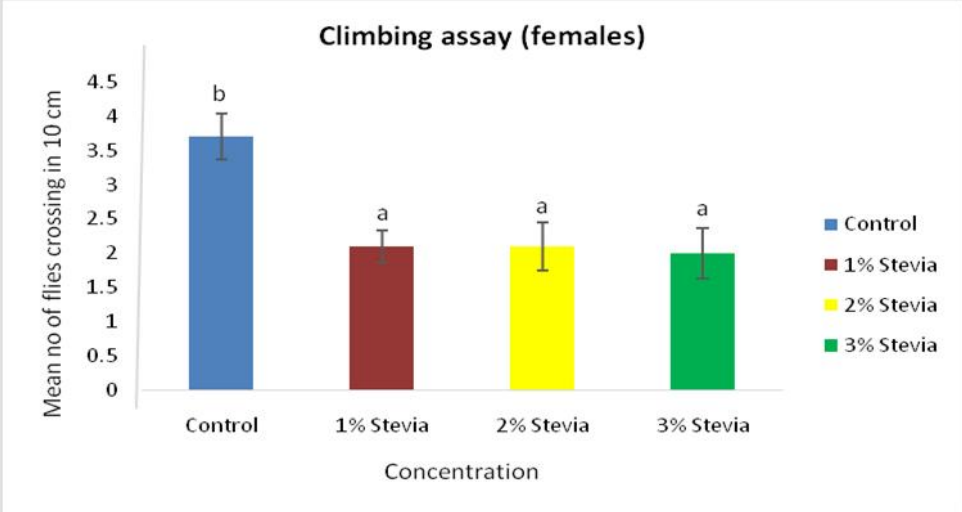

Figure 5: Effect of pure stevia on the climbing assay of 5 day old female *D. melanogaster* flies.

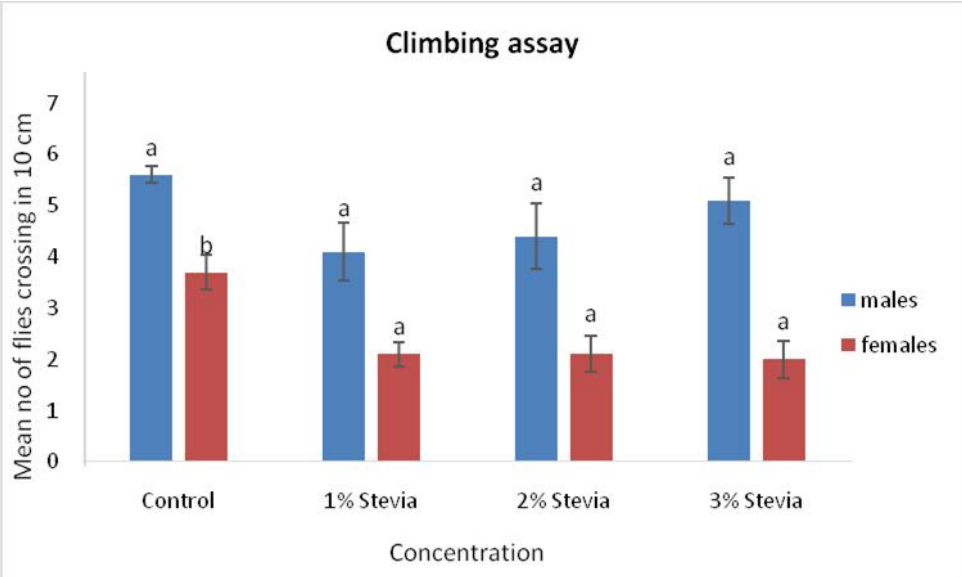

Figure 6: Effect of pure stevia on the climbing assay of 25 day old male and female *D. melanogaster* flies.

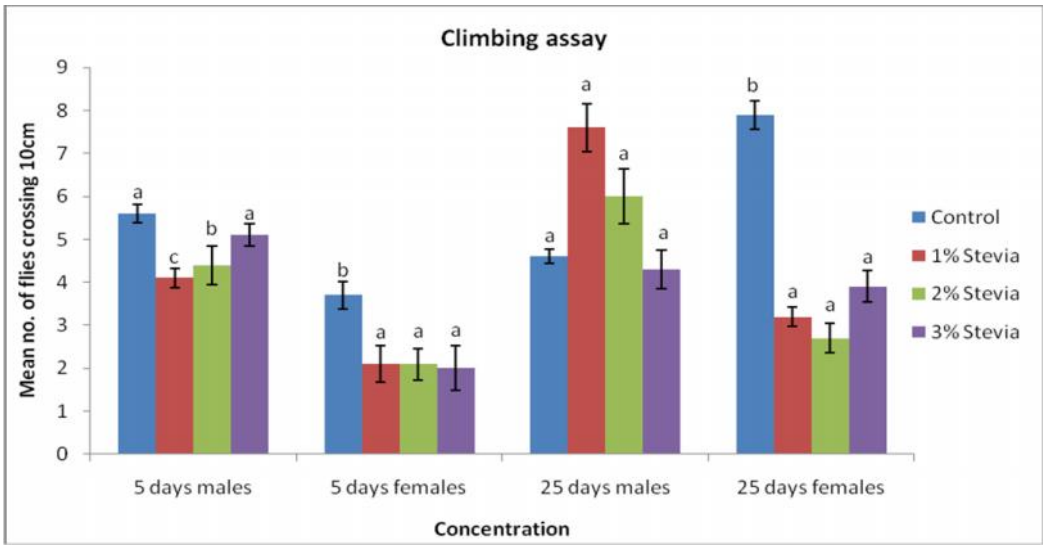

Figure 7: Effect of pure stevia on the climbing assay of 5 day old male and female and 25 day old male and female *D. melanogaster* flies.

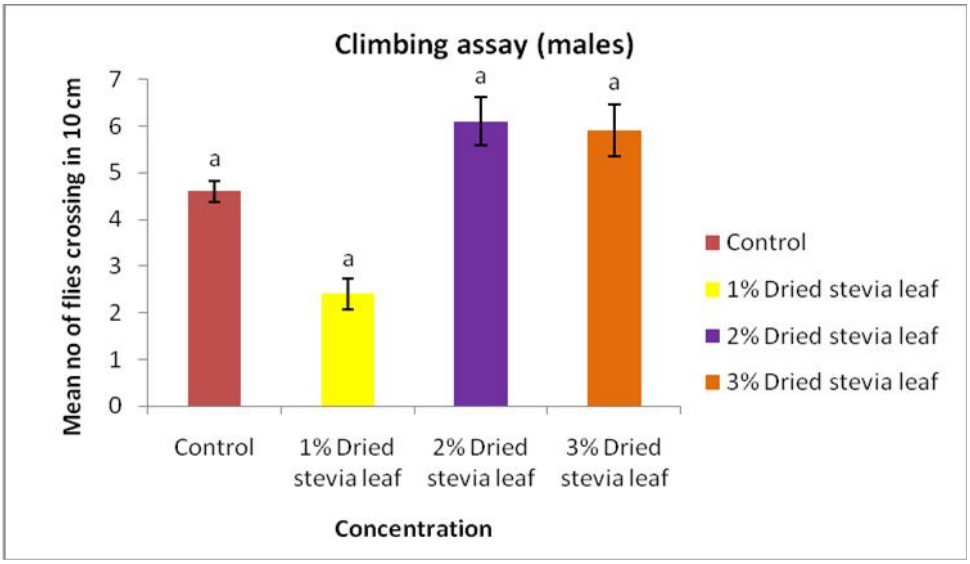

Figure 8: Effect of dried stevia leaf on the climbing assay of 5 day old male *D. melanogaster* flies.

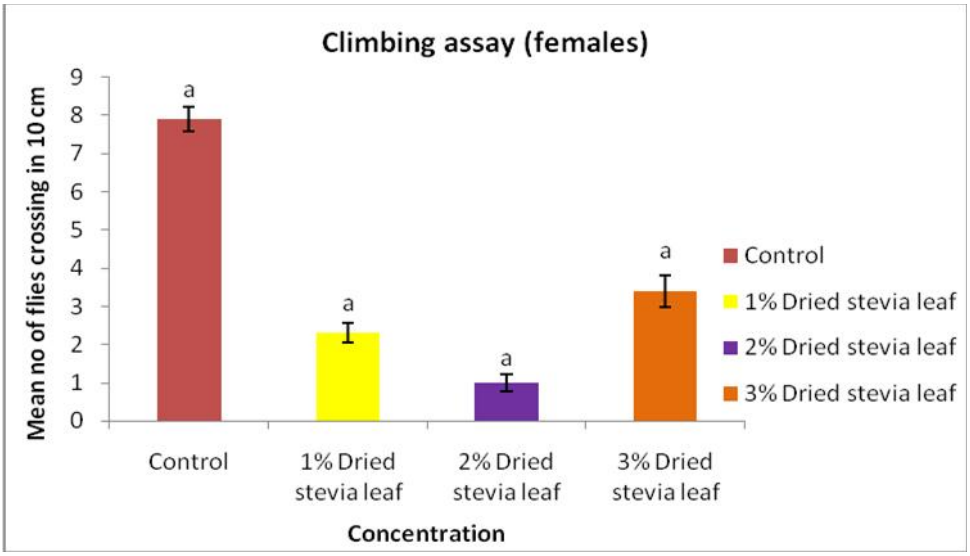

Figure 9: Effect of dried stevia leaf on the climbing assay of 5 day old female *D. melanogaster* flies.

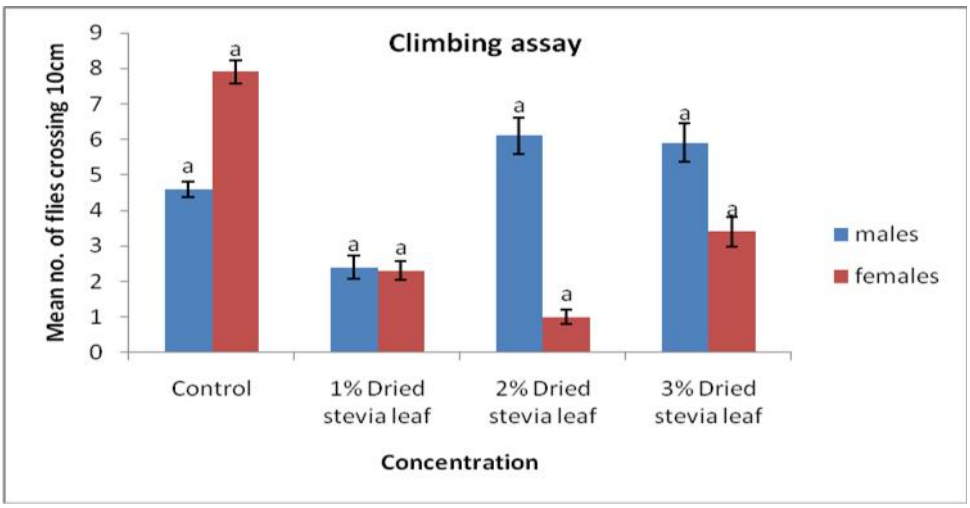

Figure 10: Effect of dried stevia leaf on the climbing assay of 5 day old male and female *D.melanogaster* flies.

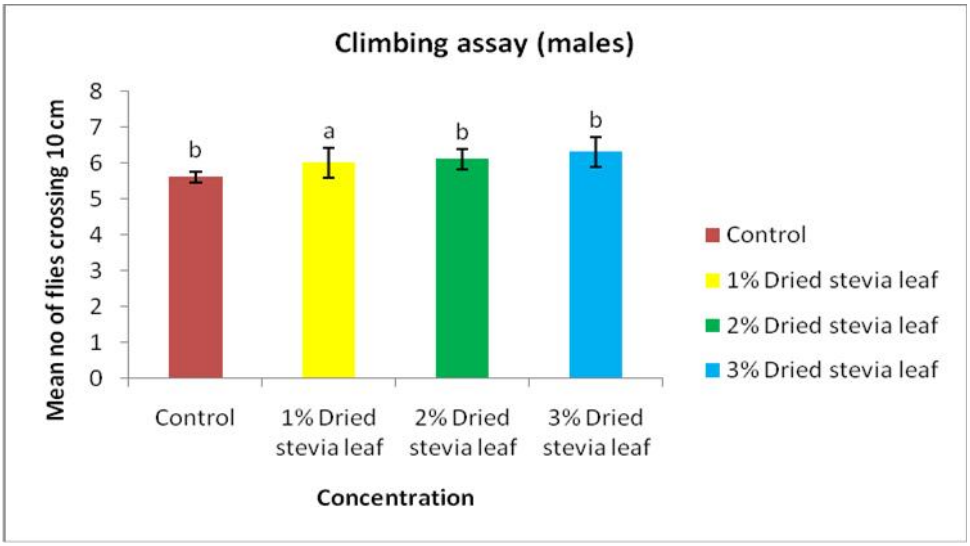

Figure 11: Effect of dried stevia leaf on the climbing assay of 25 day old male *D.melanogaster* flies.

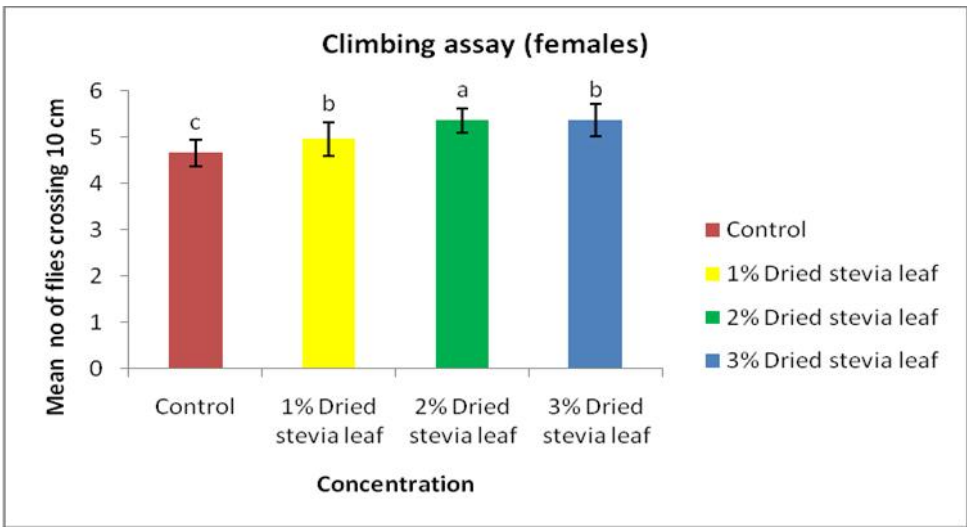

Figure 12: Effect of dried stevia leaf on the climbing assay of 25 day old female *D.melanogaster* flies.

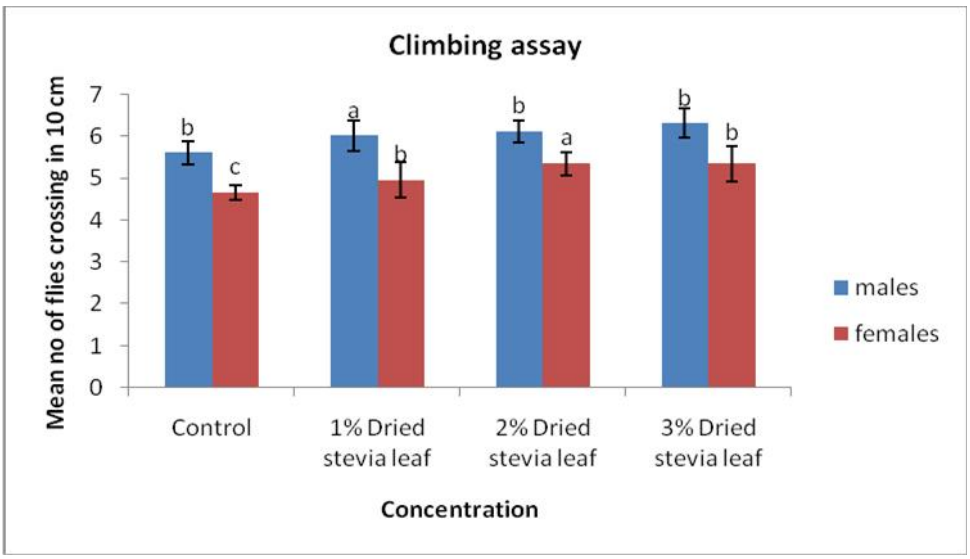

Figure 13: Effect of dried stevia leaf on the climbing assay of 25 day old male and female *D.melanogaster* flies.

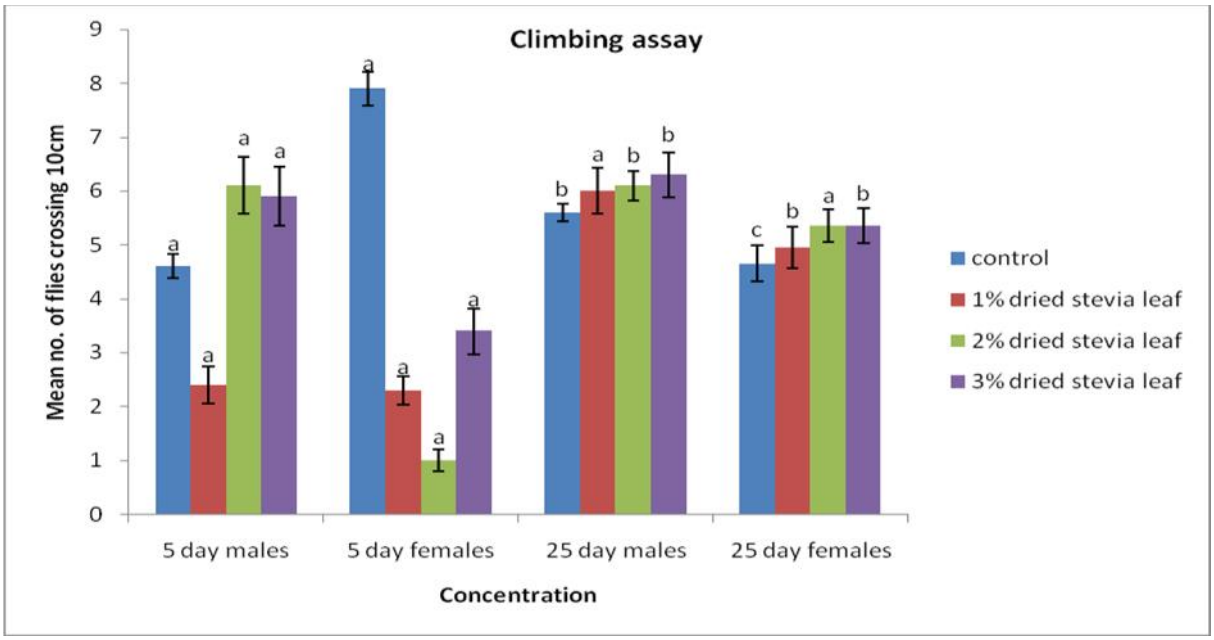

Figure 14: Effect of dried stevia leaf on the climbing assay of 5 day old and 25 day old male and female *D.melanogaster* flies.

Discussion

The rising daily consumption of artificial sweetener-containing foods has led to several health issues. Increased blood sugar levels have been associated with higher incidences of obesity, diabetes, and cardiovascular diseases, all carrying adverse side effects (Howard et al., 2002). Managing these diseases within the population requires substantial effort. Therefore, nutritional sweeteners can replace low- or no-calorie sweeteners. Over recent years, the study of sweetener alternatives has expanded significantly, as dietary assessments help determine nutritional intake levels (Liauchonak et al., 2019). Artificial sweeteners are sweetening agents that do not increase the calorie or carbohydrate content of foods (Fitch et al., 2012). In the present study, the *D. melanogaster* flies are tested for their climbing ability, motor neuron function using two different types of stevia products. The impact of pure stevia on the climbing ability of 5-day-old male *D. melanogaster*. The data indicates that the climbing ability is significantly higher in male with the 2% and 3% stevia-treated media compared to the control whereas, effect on 5-day-old female revealed that the climbing ability is significantly higher in the control group compared to the stevia-treated media., showing the reduced climbing ability in females compared to males

Effect of Pure Stevia on Climbing Assay of Older Flies (25-Day-Old)

Figure 4 depicts the climbing ability of 25-day-old male *D. melanogaster*, showing no significant difference between control and stevia-treated media ($F=1.912$, $df=3$, $p>0.05$). **Figure 5** shows the effect on 25-day-old female flies, where the control group exhibits significantly higher climbing ability compared to the stevia-treated media ($F=6.356$, $df=3$, $p<0.05$). **Figure 6** presents a comparative analysis of both male and female older flies, indicating significant differences between media ($F=5.675$, $df=3$, $p<0.05$), between sexes ($F=62.523$, $df=1$, $p<0.05$), but an insignificant interaction ($F=0.855$, $df=3$, $p>0.05$).

Comparative Analysis of Pure Stevia on Climbing Ability

Figure 7 provides a comparative view of the climbing abilities of both young (5-day-old) and

older (25-day-old) male and female *D. melanogaster* flies treated with pure stevia.

Effect of Dried Stevia Leaf on Climbing Assay of Young Flies (5-Day-Old)

Figure 8 demonstrates that the climbing ability of 5-day-old male *D. melanogaster* is significantly higher in 2% dried stevia leaf-treated media compared to the control ($F=15.658$, $df=3$, $p<0.001$). **Figure 9** shows that 5-day-old female flies have significantly higher climbing ability in the control group compared to all dried stevia leaf-treated media ($F=91.424$, $df=3$, $p<0.001$). **Figure 10** compares both male and female young flies, revealing significant differences between media ($F=38.824$, $df=3$, $p<0.005$), between sexes ($F=17.082$, $df=1$, $p<0.005$), and their interaction ($F=45.082$, $df=3$, $p<0.05$).

Effect of Dried Stevia Leaf on Climbing Assay of Older Flies (25-Day-Old)

Figure 11 shows that 25-day-old male *D. melanogaster* have slightly higher climbing ability in 3% dried stevia leaf-treated media compared to control, but the difference is not significant ($p>0.05$). **Figure 12** reveals that 25-day-old female flies show higher climbing ability in 2% and 3% dried stevia leaf-treated media compared to control, but this difference is also not significant ($p>0.05$). **Figure 13** compares both male and female older flies, showing insignificant differences between media ($F=1.748$, $df=3$, $p>0.05$), but significant differences between sexes ($F=63.087$, $df=1$, $p<0.05$) with an insignificant interaction ($F=0.350$, $df=3$, $p>0.05$).

Comparative Analysis of Dried Stevia Leaf on Climbing Ability

Figure 14 compares the climbing abilities of both young (5-day-old) and older (25-day-old) male and female *D. melanogaster* flies treated with dried stevia leaf.

In summary, the analysis demonstrates that both pure stevia and dried stevia leaf affect the climbing ability of *D. melanogaster*, with variations observed between different concentrations, ages, and sexes of the flies. The results indicate significant differences in several cases, underscoring the complex interaction between diet and locomotor behavior.

Important Findings

Young Flies (5-Day-Old) and Pure Stevia

The climbing ability is significantly higher in flies treated with 2% pure stevia compared to the control, indicating that a moderate concentration of stevia may enhance locomotor activity ($F=24.976$, $df=3$, $p<0.05$). Conversely, the climbing ability of femalesare significantly higher in the control group compared to stevia-treated groups, suggesting that stevia may have a negative impact on female flies' locomotor activity at this age. The comparative study indicates significant differences between media, sexes, and their interactions for both young and older flies, underscoring that both age and sex influence the impact of stevia on locomotor activity.

Older Flies (25-Day-Old) and Pure Stevia Male *D. melanogaster*: There is no significant difference in climbing ability between control and stevia-treated groups, indicating that age may mitigate the effects of stevia on locomotor activity in males ($F=1.912$, $df=3$, $p>0.05$).

Female *D. melanogaster*: The control group shows significantly higher climbing ability compared to stevia-treated groups, highlighting a potential age-related decline in locomotor function exacerbated by stevia ($F=6.356$, $df=3$, $p<0.05$).

Comparative Analysis of Pure Stevia

The comparative study indicates significant differences between media, sexes, and their interactions for both young and older flies, underscoring that both age and sex influence the impact of stevia on locomotor activity.

Young Flies (5-Day-Old) and Dried Stevia Leaf

The climbing ability of male *D. melanogaster* is significantly higher in 2% dried stevia leaf-treated media compared to the control, similar to pure stevia's effects ($F=15.658$, $df=3$, $p<0.001$). **Female *D. melanogaster*:** The control group shows significantly higher climbing ability compared to all dried stevia leaf-treated groups, indicating a negative impact of dried stevia on females ($F=91.424$, $df=3$

Young Male Flies show enhanced locomotor activity with 2% stevia concentration, whereas **Young Female Flies** Reduced locomotor activity in stevia-treated groups compared to the control. Pure stevia does not significantly impact the locomotor activity of older male flies, but it negatively affects older female flies. In **Dried Stevia Leaf,** improved climbing ability with 2% dried stevia leaf concentration in young males, but female show reduced locomotor activity in all dried stevia leaf-treated groups compared to the control. No significant improvement in locomotor activity for both males and females with dried stevia leaf treatment.

Conclusions

Age and sex significantly influence the effects of both pure stevia and dried stevia leaf on *D. melanogaster's* locomotor activity. The impact of stevia varies, showing potential benefits at moderate concentrations for young males but generally negative effects for females, particularly as they age. These findings highlight the complex interaction between diet and locomotor behavior, indicating that while stevia may have some benefits, its effects are highly variable depending on concentration, age, and sex of the organism.

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