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Efficacy of *Stevia rebaudiana* pure and crude leaf powder on the larval crawling ability of *Drosophila melanogaster*

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Abstract:

Energy is necessary for our body to perform various functions. Sugars, or sweeteners, are some of the primary energy-producing components in our diet. Sweeteners have long been used in a variety of foods and drinks. Stevia has a more complex and enduring flavor than other sweeteners. It has been discovered that stevia extract, or rebaudioside-A, is 300 times sweeter than regular sweetener. This plant contains steviol glycoside, an energy component that mostly produces stevioside and rebaudioside, which is 30-50 times sweeter than normal sugar. As a dietary supplement for humans, stevioside has many benefits: it is stable, low in calories, helps preserve oral health by lowering sugar intake, and can be used by individuals with diabetes, phenylketoneuria, and obesity. Stevia also has side effects or adverse effects. While stevia is thought to be safe for diabetics, products that include maltodextrin or dextrose need to be used with caution. Sturdy growth characterized by non-nutritious sweetness, which may be enhanced by palatable but unappealing extensions. As an alternative to using animal models, researchers tried to assess the effects of stevia dry leaf and stevia extract on the growth and survival of *Drosophila melanogaster*. An active third instar larve is used to assess the crawling behavior using agar media and the movement is traced on the graph sheet. The data is analyzed and discussed.

Key words: *D.melanogaster*, diet, crawling, larvae, stevia, stevia dried leaf.

Introduction

Energy is necessary for our body to perform various functions. Sugars, or sweeteners, are some of the primary energy-producing components in our diet. Most people enjoy sweet snacks. But the empty calories can mount up if you frequently consume foods and beverages with a lot of added sugar. Weight gain may occur as a result of added sweets. Additionally, it can increase your chance of developing major health issues like diabetes and heart disease. You might consider using less processed sweeteners, like honey and molasses, to avoid consuming table sugar. However, additional sugars also come in various forms. Sweeteners have long been used in a variety of foods and drinks. The growing public concerns about diabetes, obesity, and health in the past few decades has led many to consider non-nutritive sugar substitutes as a way to curb food cravings.

Stevia has a more complex and enduring flavor than other sweeteners. A lot of powdered sugar have a bitter or licorice-like taste when they absorb too much. 95% of the freshness in stevia plants comes from their steviol glycosides. The little, processed stevia leaves have a sweetness that is between thirty and forty times that of regular sugar. It tastes a little bit bitter as well (Mahendra , Supreet , 2019). It can be added to other hot and cold beverages, as well as tea, coffee, and milk. It has been discovered that stevia extract, or rebaudioside-A, is 300 times sweeter than regular sweetener. It is less bitter compared to stevia dried leaves. Although rebaudioside-A and stevioside are the most prevalent and thoroughly studied steviol glycosides, over thirty other steviol glycosides have been reported in the scientific literature to date. Some distinctive effects in stevia powder have long ledge life and high inversion sufferance (Mahendra, Supreet, 2019).

The Stevia plant's leaves and extracts have been used as flavorings for many years. A class of naturally occurring sugar has been extracted from the stevia plant, called diterpene glycosides. While all units of rebaudioside and stevioside are among the eight glycosides found in stevia plant leaves. Among these components, stevioside and rebaudioside-A are the most prevalent. A glycoside ingredient that is not a carbohydrate is stevioside. This plant contains steviol glycoside, an energy component that mostly produces stevioside and rebaudioside, which is 30-50 times sweeter than normal sugar (Goyal, 2010). Heat and pH are firmly fixed and it is not fermentable (Mahendra, Supreet, 2019). Because the glycosides in stevia are not broken down by the human digestive system, it has no calories like other sugars that are created by humans. In addition, the Stevia plant contains certain sterols as well as antioxidant compounds including tannins, triterpenes, and flavonoids.

According to Crammer and Ikan (1986), the glycosides in stevia are high intensity sweeteners that are 50–300 times sweeter than regular sugar while also having a high melting point and limited water solubility (Crammer et al. 1986). The plant is known to contain eight glycosides (Kennelly 2008, Starrat et al. 2002). As a dietary supplement for humans, stevioside has many benefits: it is stable, low in calories, helps preserve oral health by lowering sugar intake, and can be used by individuals with diabetes, phenylketoneuria, and obesity (Geuns, 2003). The leaves of the South American herb *Stevia rebaudiana* contain a number of sweet-tasting diterpene glycosides. Purified glycoside is produced by further processing once the leaves are dried and stored (Kalpana & Khan, 2008). The application of these glycosides as sugar substitutes accounts for the commercial significance of stevia. For diabetics, cutting off sugar from food is advantageous. Since steviol glycosides do not include aromatic amino acids, they are likewise safe for those with phenylketonuria. Because stevia extracts or stevioside replace excess sugar in food, obese people may experience weight loss. Indian sweet dishes from the past contain a lot of sugar in them. Additionally, there is proof that attempts have been made to use sugar replacements in place of all or some of this sugar (Chetana et al. 2006; Arora et al. 2008).

There are many benefits of stevia. One non-nutritive sweetener is stevia. It has nearly no calories as a result. This feature can be intriguing to you if you're attempting to reduce weight. The research isn't conclusive yet, though. Nonnutritive sweetener use may have different effects on people's health depending on how much and when it is ingested. One significant drawback of this research, though, is that it was conducted in a lab rather than in an actual setting where people would naturally be found. Additionally, stevia leaf powder may aid with cholesterol management, per a 2009 study (Sharma et al.,2009). For a month, each study participant drank 20 milliliters of stevia extract every day. According to the study, stevia reduced triglycerides, LDL ("bad") cholesterol, and total cholesterol without having any unfavorable side effects. Additionally, it raised HDL, or "good," cholesterol. It's uncertain if consuming smaller doses of stevia on occasion would have the same effect. Stevia may help control blood sugar levels if you have diabetes. In a 2010 study, (Anton et al. 2010) stevia was found to dramatically reduce insulin and glucose levels in 19 healthy, lean subjects and 12 obese participants. Despite consuming less calories, it also made study participants feel content and full after eating.

Stevia also has side effects or adverse effects. Due to a lack of safety data, they have not authorized the use of crude or whole-leaf stevia extract in processed foods and beverages. Raw stevia herb is thought to be harmful to the kidneys, reproductive system, and cardiovascular system. While stevia is thought to be safe for diabetics, products that include maltodextrin or dextrose need to be used with caution. Maltodextrin is a starch, whereas dextrose is glucose. These items provide a minor boost to calories and carbohydrates. Additionally, sugar alcohols may somewhat alter the carbohydrate count. The occasional use of stevia could not have a significant effect on your blood sugar levels. However, if you consume it all day, the carbohydrates mount up. Additionally, non nutritive sweeteners may cause metabolic problems and glucose intolerance, according to the same study. Some people may experience digestive issues including bloating and diarrhea after consuming stevia products formulated with sugar alcohols. According to a 2012 study (Paul et al. 2012), a glycoside called stevioside found in stevia plants helps boost cancer cell death in a human breast cancer line.

Taste is the first degree of food and drink preference for animals. Seeing sweetness is a sign that sugar is there and that calorie intake is feasible. Certain sugars cannot be essential to life because their sweet taste may not always indicate that they are nourishing or nutritious. The same sensory neurons that distinguish distinct sugar tastes individually also notice unique sugars in the gustatory systems of mammals and insects. A recent study (Mahendra, Supreet, 2019) examines the article on the appealing and relatively healthy usefulness of sugars to memory development in the case of *Drosophila*. Sturdy growth characterized by non-nutritious sweetness, which may be enhanced by palatable but unappealing extensions. A nutritional fact is transmitted to the brain in a matter of minutes, at which point it can be utilized to control the development of a sugar inclination memory. By using the post-ingestive reward assessment method, flies are able to quickly identify and learn about the many types of sugars. Demir et al. (2014) used *Drosophila* to test the genotoxicity of several sweeteners using comet and wing spot assays, including aspartame, sucharin, acesulfame, and sucralose. It was employed in two tests during the procedure, demonstrating that the sweeteners were not genotoxic. Trans heterozygous flies react negatively to aspartame, yet it was crucial that they grow larger. Because somatic recombination in the genesis of mutant clones is responsible for the important results produced by aspartame, negative results were obtained in the case of heterozygous flies. Aspartame has genotoxic effects in comet assay. As an alternative to using animal models, researchers tried to assess the effects of stevia dry leaf and stevia extract on the growth and survival of *Drosophila melanogaster* (Mahendra, Supreet, 2019).

Hence the present study is an attempt to understand the comparative study on stevia extract and dries stevia leaves for their beneficial or adverse effects on *Drosophila melanogaster* being potential model to study food toxicity and its long term effects can be tested and recorded. An organism's general growth, development, and reproduction are impacted by both internal and external factors elements that are known to impact every biological, physiological, and developmental shift (Sterner et al. 1998, Taylor 2005)

Crawling assay

Every single task that an organism engages in to ensure its existence and procreation requires energy. e.g., movement, courting, and locomotion. Locating food, finding a partner, escaping from predators, defending one's territory, and reacting to stress all depend on locomotion, which is an essential activity. As a result, it is essential to the majority of animal behavior (Jordan et al., 2007). Food is the source of energy for animals, and diets can be categorized as quantitative (food availability) or qualitative (composition). Since animals get their energy and other nutritional needs from food, the qualitative impacts are obvious. For this reason, animals' ability to survive, move, and reproduce depends on maintaining a balance between their energy intake and expenditure (Pough, 1989; Sibly, 1991). The interaction of matter intake, digestion, and the distribution of newly acquired energy among different processes, including growth, reproduction, and locomotion, determines the balance (Karasov, 1986). These days, there is a growing tendency for fitness, appearance, and health. Energy imbalance resulting from sedentary lifestyles, urbanization, overindulgence in sugary foods, and increasing fat intake; calories taken on the one hand and calories utilized on the other. Because of this, there is a stronger demand for food goods that help consumers make healthier eating choices and encourage improved customer demand for a wider range of low-calorie products. A food additive known as a sugar substitute mimics the flavor of sugar while often having less energy from food. One such stable, non-calorific, non-carbohydrate natural sugar is derived from a plant known as Stevia rebaudiana. Because the glycoside in stevia is not broken down by the human digestive system, it has no calories like other sugars that are created by humans. Its impact on Drosophila's locomotor activity and health advantages, however, is unknown. Because human diseases and *Drosophila* share many metabolic mechanisms, *Drosophila* is an excellent model organism for studying human diseases. Rhythmic movements such as respiration, digestion, circulation, and locomotion are fundamental to animal life. In Drosophila larvae, many behaviors have been studied such as phototaxis, learning, and navigation (Iyengar et al.,1999; Freeman et al., 2010; Luo et al., 2010). These and other behaviors are based on regulating locomotion in response to stimuli or experi ence. Drosophila larval locomotion includes a repertoire of many different types of movements, including turns, burrowing, linear crawling, and other movements (Green et al., 1983; Wang et al., 1997; Huang et al., 2007). The relative complexity of larval locomotor behavior has precluded detailed analysis of the neuromuscular mechanism underlying any one type of movement, such as linear crawling. However, understanding the motor pattern that drives behavior is a requisite step in understanding its cellular basis (Marder and Calabrese, 1996). Thus, the goal of the current investigation is to learn more about how stevia affects *D.melanogaster's* locomotor behavior.

Materials and methodology

Establishment of stock

The experimental stock of *Drosophila melanogaster* was obtained from *Drosophila* stock center, Manasagangotri, University of Mysore. *D.melanogaster* is one of the most widely used and one of the most understood of all model organism. The flies obtained were redistributed and raised in different culture bottles containing wheat cream agar media (100g of jiggery, 100g of wheat powder, 10g of agar agar was boiled in 1000ml of double distilled water. 7.5ml of propionic acid was added at last). 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 12 hours dark: 12 hours light cycle. The virgin flies were isolated in pupa stage and cultured in test media. The test media containing 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. In the same way, the test media containing 1%, 2% and 3% dried stevia leaves powder based media and the flies grown in normal wheat agar media were used. The results of both the products ie stevia extract and dried stevia leaves were compared and were discussed. Five day old flies were isolated from the culture and raised in control, stevia extract media and dried stevia leaves media. It was maintained in the conditions mentioned above. They were allowed for five days in the corresponding media, control and test and then used for studying different parameters.

Crawling assay

Active third instar larvae from control and treated media were obtained and collected by washing them in 1% 10X PBS. They were then transferred to a petridish containing 1% agar + 1% sucrose media [previously heated and allowed to become hard]. A thin layer of yeast paste was spread on the agar media to trace the movement of the larvae. The petridish was placed over a graph sheet and the larvae were placed on the media. The number of grid lines per cm^3 crossed per larva in 2 minutes was counted. A total of 3 replicates with 10 larvae (30 larvae total) in each group were used in each respective media.

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 for crawling assay. A graph of media v/s mean crawling assay was plotted for both stevia pure extract and stevia dried leaf extract. The graph of the two were compared.

Results

The larval crawling assay was conducted using 1% non nutritive agar media (Delcour procedure, 1969)., a graph sheet and third instar larvae grown in control and treated media were used to study the effect of stevia on the crawling behavior of the larva. The area of cubic squares covered by the larvae was measured in every 2 minutes on agar media containing yeast paste. The graph of the larval movement was drawn and the area was counted.

Fig.1 shows the larval crawling assay setup with agar media and graph sheet. Fig.2 is a graphs that depicts the movement of the larva in different directions covering different area on the graph sheet. The effect of stevia (pure) on larval crawling behavior is provided in the fig.3 It was noted that larvae obtained from stevia (2%) had shown greater movement than larvae from control media. It is non significant with $p > 0.05$, $df = 3$ and $F = 0.540$. The effect of dried stevia leaf on larval crawling behavior is provided in the fig.4. It was noted that larvae obtained from dried stevia leaf (3%)hadshown greater crawling behavior than larvae from control media. It is non significant with $p > 0.05$, $df = 3$ and $F = 0.730$. The comparison of stevia and dried stevia leaf is shown in fig.5. It is found that larvae obtained from treated media had shown more crawling behavior than those from control media. Tukey's Post Hoc test revealed that there is a significant difference in the larval crawling between different concentrations compared to control.

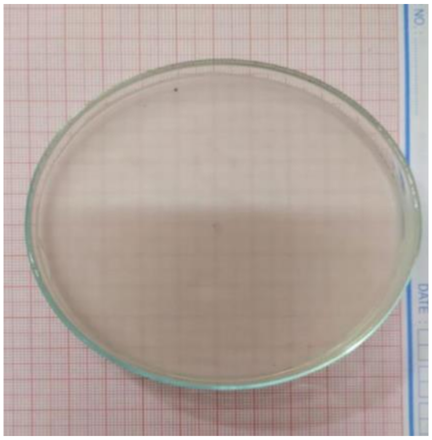

Fig.1: Experimental setup

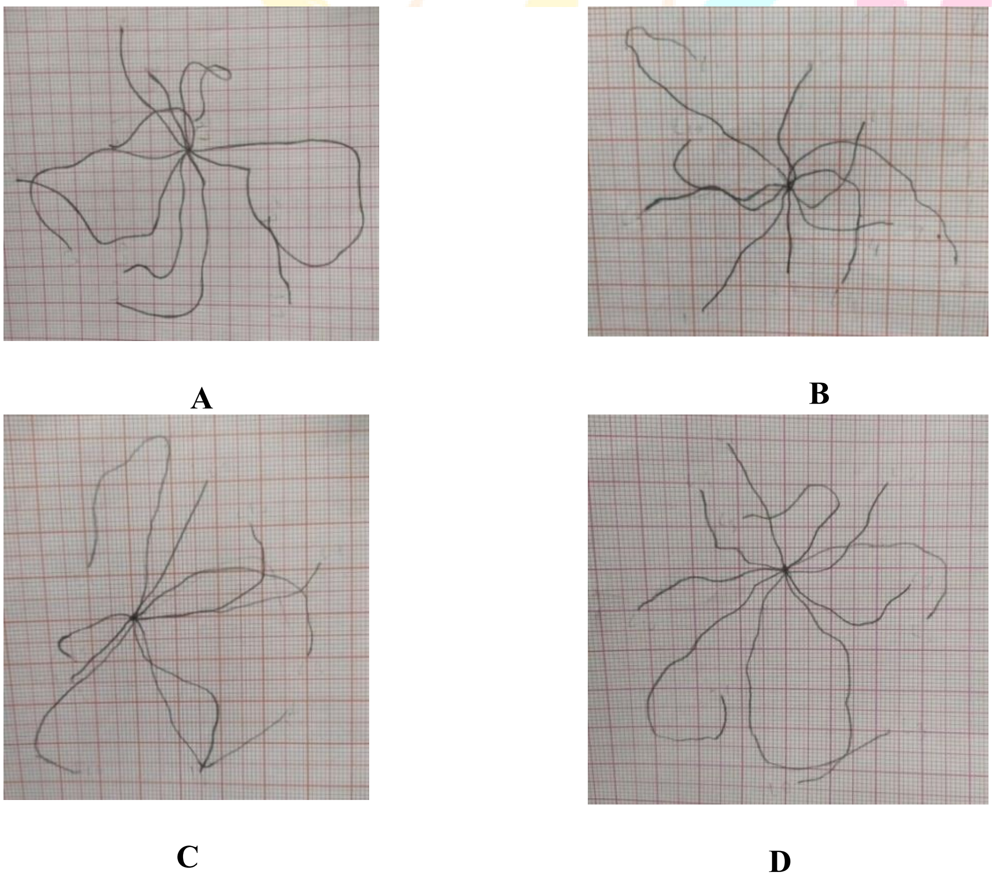

Fig. 2: Graphs that depicts the movement of the larva in different directions covering different area on the graph sheet. A- control media; B- 1% stevia; C- 2% stevia; D- 3% stevia (Total 10 replicates/ larvae)

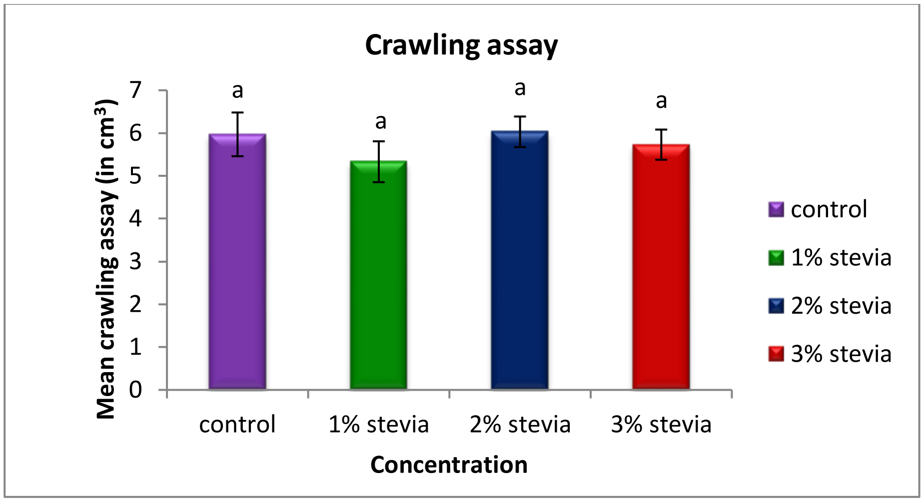

Fig. 3 Effect of stevia on larval crawling behavior of *D.melanogaster* third instar larvae.

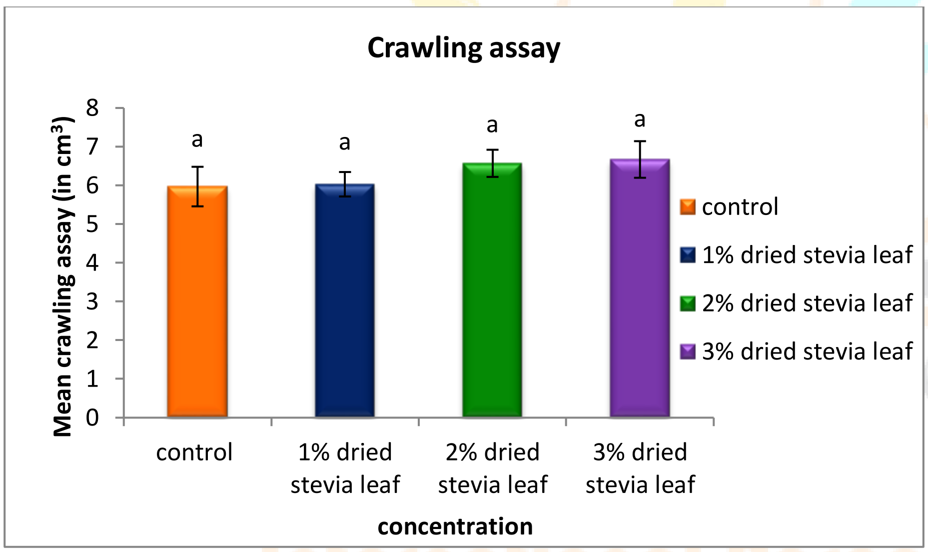

Fig. 4 Effect of stevia dried leaf on larval crawling behavior of *D.melanogaster* third instar larvae.

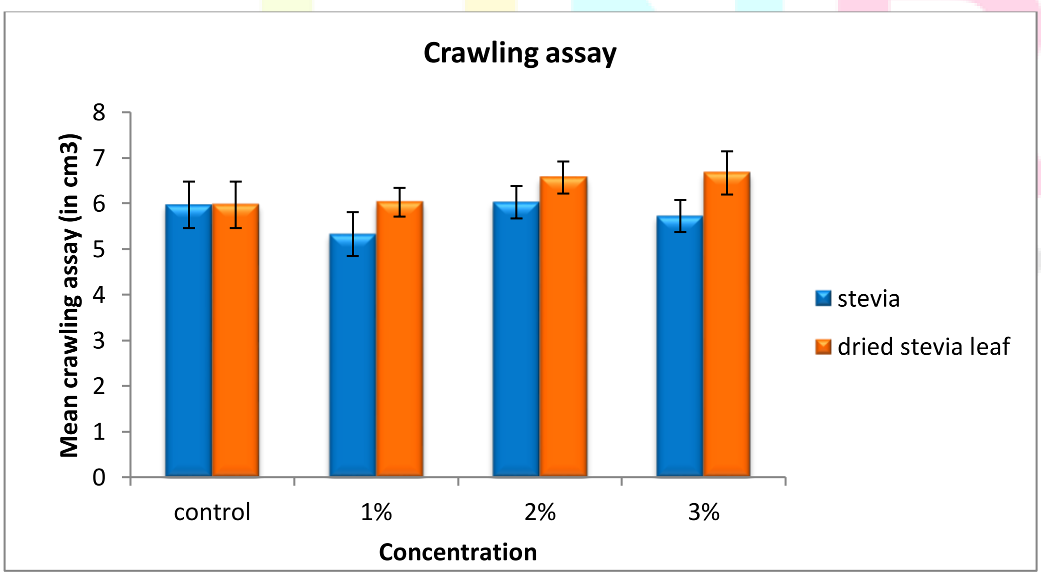

Fig.7: Effect of stevia and stevia dried leaf on the crawling behavior of *D.melanogaster* third instar larvae.

Discussion

In the present investigation, the larval crawling assay was conducted using 1% non-nutritive agar media (Delcour procedure, 1969). A graph sheet was used to measure the area covered by third instar larvae grown in control and treated media.This graph depicts the movement of larvae in different directions, covering various areas on the graph sheet. Larvae from different media (A- control, B- 1% stevia, C- 2% stevia, D- 3% stevia) were observed.Larvae from 2% pure stevia-treated media showed greater movement than those from the control media. However, the difference was not statistically significant ($p > 0.05$, $df = 3$, $F = 0.540$). Larvae from 3% dried stevia leaf-treated media demonstrated greater crawling behavior compared to the control media. This difference was also not statistically significant ($p > 0.05$, $df = 3$, $F = 0.730$).Larvae from both pure stevia and dried stevia leaf-treated media showed more crawling behavior than those from the control media. Tukey's Post Hoc test revealed that there is a significant difference in the larval crawling between different concentrations compared to control. Both 2% pure stevia and 3% dried stevia leaf-treated media resulted in increased larval movement compared to the control. However, neither difference was statistically significant.The results suggest a potential stimulatory effect of stevia on larval movement, with both pure and dried forms showing increased activity. The lack of statistical significance implies that the effect may not be strong enough to be conclusively different from the control under the conditions tested. Further research with larger sample sizes is necessary to determine if these trends hold true.Larvae from different concentrations of stevia (1%, 2%, 3%) exhibited varying degrees of movement, with 2% pure stevia and 3% dried stevia leaf concentrations showing the greatest movement.This indicates a potential dose-dependent response to stevia, where higher concentrations might enhance larval movement more effectively. However, the non-significant results highlight the need for further investigation to establish a clear dose-response relationship and to identify the optimal concentration for maximum effect. Larvae from both pure stevia and dried stevia leaf-treated media showed increased crawling behavior compared to control. The significant difference identified by Tukey's Post Hoc test indicates that stevia treatment has a measurable effect on larval movement.The significant findings from the Post Hoc test suggest that stevia, in both pure and dried forms, can influence larval behavior. The increased activity in treated larvae implies that stevia contains bioactive compounds that may stimulate locomotion. The exact mechanisms by which stevia affects larval movement are not yet clear and warrant further study.

Stevia, a small evergreen shrub, has long been utilized for its medicinal properties and as a natural sweetener. Unlike other sugars, stevia leaves offer both practical and sensory benefits, containing high levels of rebaudioside A and stevioside among various steviol glycosides. The United States has approved the use of Rebaudioside-A in food preparations. Research involving stevia has been conducted on various organisms, including mice, rabbits, and mosquitoes. Notably, stevia has demonstrated larvicidal properties against *Anopheles stephensi* mosquitoes and has been used as a vector control agent in combination with synthetic insecticides. Its high antioxidant enzyme activity also makes it resistant to diseases caused by free radicals.

Drosophila melanogaster is a widely used model organism in biomedical research due to its genetic tractability and short life cycle. This study explored the effects of stevia and dried stevia leaf powder on the locomotor behavior of *Drosophila melanogaster* larvae. The findings indicate that stevia positively influences larval crawling behavior, with larvae exposed to stevia powder displaying increased movement compared to those on control media. The results suggest that stevia powder is more effective in enhancing larval crawling behavior than dried stevia leaf.

Previous nutritional studies in *Drosophila* have predominantly used sucrose-based food (Skorupa et al., 2008; Musselman et al., 2011; Pasco and Leopold, 2012; Morris et al., 2012; Na et al., 2013; Musselman et al., 2013; Hirabayashi et al., 2013). However, the beneficial effects of natural sweeteners like stevia on locomotor ability had not been tested until now. The current study subjected flies raised on various sugar-based media (normal, stevia, and dried stevia leaf) to larval crawling assays to evaluate their locomotor abilities.

In *Drosophila*, larvae exhibit inhibition thresholds when encountering new or unpalatable food (Melcher et al., 2007). It is hypothesized that natural sweeteners like stevia can reduce calorie intake and potentially prevent obesity and diabetes (Kirtida, 2011). The present study showed that larvae fed with stevia-enhanced diets demonstrated improved locomotor behavior compared to those on normal diets. This suggests that incorporating stevia into a diet with lower calorie content can enhance the locomotor behavior of *Drosophila melanogaster*.

Based on the study's findings, stevia powder is more effective than dried stevia leaf in maintaining and enhancing the fitness of the organism. Therefore, it is recommended to use stevia powder over dried stevia leaf to support better locomotor behavior in *Drosophila melanogaster*. This research highlights the potential benefits of stevia as a dietary supplement, promoting increased physical activity and overall fitness.

Conclusion

The study suggests that stevia, both in pure and dried forms, can influence the crawling behavior of *Drosophila melanogaster* larvae, with trends indicating increased movement. While individual comparisons did not yield statistically significant differences, overall analysis and post hoc tests revealed significant effects, indicating that stevia may stimulate larval activity. The results highlight the potential of stevia as a factor influencing larval behavior, but further research with larger sample sizes and detailed mechanistic studies are needed to confirm these findings and understand the underlying biological effects of stevia on larval behavior.

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