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Research Article

Quality Assessment of Stevioside-Supplemented Biscuit by Analysing Its Effects on Liver Functions and Histomorphology of Rabbit

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ABSTRACT

Stevia (*Stevia rebaudiana*) is a small plant. Its leaves contain stevioside, a sweet compound that is 250–300 times sweeter than sucrose. Stevioside is a natural sweetener that is used as a sugar alternative and, hence, is a suitable choice for the diabetic patient. Some artificial sweeteners are used as sugar alternatives, which have more side effects than natural sweeteners. The present study was conducted to evaluate the effects of stevioside-supplemented biscuits on liver function in rabbits and to compare these effects with those of sugar- and saccharin-supplemented biscuits. For this purpose, three types of biscuits were prepared: stevioside-supplemented, sugar-supplemented, and saccharin-supplemented biscuits. Then, these biscuits were fed to rabbits to check their effects on the liver functions of rabbits. Therefore, four experimental groups, namely control (T1), stevioside (T2), sugar (T3), and saccharin (T4), were established, each containing three rabbits. Blood samples were collected on days 0, 7, 14, and 21 to determine the haematobiochemical profiles, including SGPT (serum glutamate pyruvate transaminase), SGOT (serum glutamate oxaloacetate transaminase), ALP (alkaline phosphatase), and RBS (random blood sugar). The results showed that the SGPT (22.35 U/L), SGOT (25.50 U/L), ALP (145.34 U/L), and RBS (4.8 mmol/L) levels in rabbits were lower in response to stevioside-supplemented biscuits. In contrast, the rabbits fed with sugar-supplemented biscuits had higher values (SGPT = 36.18 U/L, SGOT = 41.0 U/L, ALP = 292.22 U/L, and RBS = 6.31 mmol/L). Similarly, saccharin-supplemented biscuits also resulted in higher levels of SGPT = 50.08 U/L, SGOT = 56.0 U/L, ALP = 264.65 U/L, and RBS = 6.52 mmol/L) compared to stevioside-supplemented biscuits. Moreover, histological observations of the rabbits' livers revealed that cytoplasmic glycogen storage in hepatic cells was higher in the stevioside-treated group than in the sugar- and saccharin-supplemented groups. Altogether, these results suggest that stevioside reduces SGPT, SGOT, ALP, and RBS levels than those of sugar and saccharin. It may serve as a suitable alternative to sugar.

Introduction

Stevioside is a popular natural sweetener and used as a dietary supplement. It has both economic and medicinal importance. Recently, it has been introduced as a crop in a number of countries including Bangladesh (Ahmad *et al.,* 2011). The increasing demand for low-sugar food alternatives has brought Stevia into the spotlight. Therefore, it is an attractive source of natural sweetener for diabetic patients. Many countries *i.e.,* Japan, China, and India are using stevioside-supplemented foods such as jam, jelly, yogurt, sauces, ice cream, etc (Mali *et al.,* 2015). In Bangladesh, some artificial sweeteners such as saccharin, aspartame, cyclamate, etc. are being used in diabetic foods though these have serious side effects. The potential toxic effects for saccharin are bladder carcinoma, hepatotoxicity, low birth weight; for aspartame are migraine, lymphoma, leukemia, chromosomal aberration (Mandal *et al.*, 2018).

Stevioside influences insulin secretion, lowering blood pressure, and decreasing blood sugar (Gregersen and

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Jeppesen, 2004; Wang *et al.,* 2012). Stevia is abundant in beta carotene, ascorbic acid, protein, calcium, iron, magnesium, phosphorus, and various other phytochemicals (Lemus-Mondaca *et al.,* 2012). Its versatile applications include serving as an anti-inflammatory, anti-mutagenic, antitumor, diuretic, digestive aid, food additive, immune-modulator, and a remedy for obesity (Chatsudthipong, 2009). In Japan, stevia and its derivatives are employed in the production of baked goods, processed foods, beverages, fruit juices, pastries, chewing gum, and sherbets (Sairkar *et al.,* 2009). Unlike most artificial sweeteners, stevia can withstand high temperatures and low temperatures. Therefore, it remains unchanged in cooking and freezing.

Serum Glutamic Pyruvic Transaminase (SGPT) is an enzyme primarily found in the liver, but also present in smaller amounts in other tissues like the kidneys and heart. SGPT is a test that measures the level of the enzyme Alanine Aminotransferase in the blood. Elevated SGPT levels may indicate liver damage, such as hepatitis, cirrhosis, or fatty liver disease. Serum Glutamic-Oxaloacetic Transaminase (SGOT) is also a blood test used to assess liver function. It measures the levels of the enzyme Aspartate Aminotransferase (AST) in the blood, which is found in the liver, heart, and other tissues. Elevated SGOT levels can indicate liver damage or injury, as well as damage to other organs. Alkaline phosphatase (ALP) is produced mainly by the liver and bones, but also by the intestines and kidneys. ALP test is often used to help monitor conditions affecting the liver, bones, or bile ducts. Abnormal ALP levels can also be associated with conditions like hyperparathyroidism, vitamin D deficiency, and certain cancers. Stevioside plays a preventive role in acute inflammation of the liver induced by CCl_4 and prevents necrosis by blocking NF-κB and proinflammatory cytokines (Ramos-Tovar *et al.,* 2018). Stevioside modulates oxidative damage in the liver and kidney of high fat streptozocin-diabetic rats (Rotimi *et al.,* 2018).

Sugar-free products are very important nowadays (Cross, 2007). Some bakery products, such as cake (Schirmer et al., 2012), yogurt (Abdel-Salam et al., 2009), and bread (Parimalavalli, 2007), have been prepared using stevioside; however, biscuits have not yet been prepared with it. Many people are looking for biscuits that exert fewer effects on blood glucose levels. Although some types of biscuits containing artificial sweeteners are available in Bangladesh, biscuits made with natural sweeteners such as stevioside are not available in the country. Therefore, the aim of this study was to prepare biscuits using stevioside as a natural sweetener and to compare them with biscuits prepared using sugar and saccharin. To select the best biscuits, we fed these biscuits to rabbits and investigated the effects of these biscuits on some biochemical parameters e.g., liver functions and blood sugar. The stevioside contained biscuits will create a new option for the diabetic patient and they can consume this biscuit as a sugar alternative instead of artificial sweetener.

Materials and Methods

Preparation of biscuits

There were three types of biscuits such as stevioside, sugar and saccharin supplemented biscuits were prepared. Each kilogram of biscuits was prepared using 550 g of wheat flour, 3 g of salt, 30 g of milk powder, a small amount of vanilla, 10 g of baking powder, and 220 g of food-grade oil. First, the dry ingredients were thoroughly mixed, after which the liquid ingredients were added and blended well. The dough was then shaped into biscuits by cutting and stamping. Finally, the shaped biscuits were baked at a controlled temperature, cooled, and packaged.

The stevioside was collected from Morita Kagaku Kogyo Co., Ltd, Osaka 577-0002, Japan. In our studied location, many local bakeries prepared different biscuits which were available in the markets. Generally, biochemical compositions of those biscuits were not mentioned in such types of local biscuits but people buy these biscuits. In this study, we selected one bakery that prepared biscuits for many years. We used stevioside, saccharin, and sugar into those commercially available biscuits. Therefore, biochemical compositions of those biscuits were not studied as our target was to compare the effects of those biscuits while their other ingredients remain the same. The representative samples of the biscuits are shown in figure 1. The concentration of sugar in biscuits was 250 g per kg of biscuit. Saccharin and stevioside are significantly sweeter than table sugar (sucrose). It was reported that stevioside is 250 to 300 times sweeter (Brandle 2004 and Ashwell 2015) and saccharin is 300 to 500 times sweeter (Chattopadhyay, 2014; and PubChem, 2023) than sucrose. Therefore, we needed a much smaller amount of saccharin and stevioside to achieve the same level of sweetness as sugar. We used 1.0 g stevioside and 0.9 g saccharin per Kg of biscuit preparation. The control group was fed biscuits without any added sweetener. These control biscuits contained approximately half the amount of sucrose present in the sugar-supplemented biscuits, as a minimal amount of sugar was necessary to facilitate the coagulation of wheat flour during biscuit preparation.

(a) (b) (c)

Figure 1: Representative images of different types of biscuits. Stevia biscuit (a), sugar biscuit (b), and saccharin biscuit (c).

Rearing and treatments of rabbits

A total of 12 apparently healthy mature male rabbits were brought from the market with an average weight of 850 g and then categorized into 4 experimental groups (T_1, Control; T_2, Stevioside; T_3, Sugar, and T_4, Saccharin) with three replications. The cages for rearing rabbits were constructed using 5 mm diameter rods. Each rearing chamber, designed for a single rabbit, was measured 1.5 square feet. The experiment was conducted following a Randomized Complete Block Design (RCBD), with each chamber considered as a block. The research methodology was reviewed and approved by the Institutional Ethical Committee (IEC) of Hajee Mohammad Danesh Science and Technology University, Dinajpur (IEC approval number: BS/2024/04). Each rabbit was served with 20 grams of biscuit per day besides the usual foods except the control group (Figure 2).

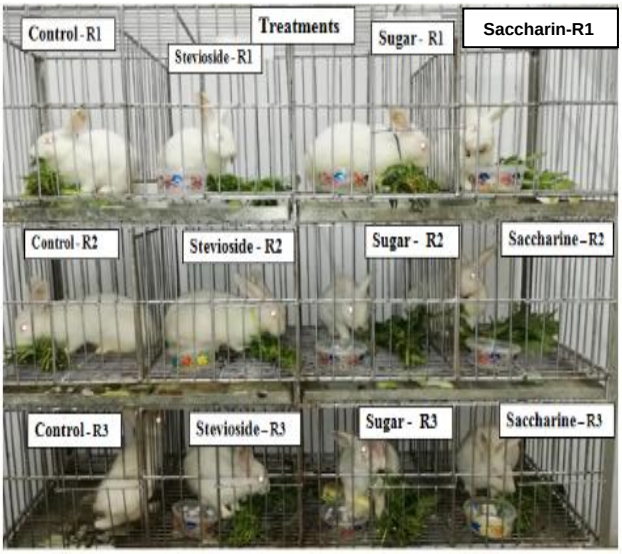

Figure 2: Rearing of rabbits.

Measurement of biochemical parameters

The Serum Glutamate Pyruvate Transaminase (SGPT), Serum Glutamate Oxaloacetate Transaminase (SGOT), Alkaline Phosphatase (ALP), and Random Blood Sugar (RBS) of rabbits were estimated. Blood samples were collected from rabbits on day 0 (the day before starting the treatment), day 7, day 14, and day 21. The samples were collected from rabbits two hours after the meal. Then the tests were performed following the Reitman-Frankel Colorimetric method (manufacturer: Linear Chemicals-Chromatest; Spain) using blood sample. The absorbance of all biochemical parameters in serum was measured by spectrophotometer (Shimadzu, UV-1900i; Serial No.-A12536184199; Japan).

Determination of SGPT and SGOT

Serum Glutamate Pyruvate Transaminase (SGPT) and Serum Glutamate Oxaloacetate Transaminase (SGOT) are both liver enzymes that are commonly measured in blood tests to evaluate liver function. They help metabolize amino acids. The SGOT and SGPT tests are usually done together and are part of a liver function test panel. There was no difference in the procedure of the SGOT and SGPT tests done. The only difference between the two tests was the enzyme they measured. SGOT measured the levels of the enzyme in the blood, which is normally found in the liver, heart, muscle, and other tissues. SGPT measured the levels of the enzyme in the blood, which is primarily found in the liver.

The substrates, standard and colour developer were ready-to-use. By means of a funnel, the contents of the

10x concentrate 4N NaOH preparation were poured into a 2-litre volumetric flask. The bottle was rinsed with distilled water, completed to the mark and mixed. The solution was warmed up. The reagents and samples were brought at room temperature before use. L-aspartate, lactate dehydrogenase, malate dehydrogenase, a coenzyme (NADH), α-ketoglutarate were used as reagents. 1 mL of working reagent was mixed with the 100 µL sample and incubated at 37°C for 1 minute. The absorbance (A) of the sample was taken at 1 minute interval for 3 minutes. Then the average absorbance differences per minutes (ΔA/min) were calculated using following formulae:

SGPT = (ΔA /min) ×1768.

Determination of ALP

Alkaline phosphatase (ALP) is an enzyme that helps break down proteins and is found in the liver. Elevated ALP levels can indicate liver disorder or other health issues. To measure ALP, magnesium chloride, diethanolamine (pH 10.4), p-nitrophenyl phosphate were used as reagents. 1 mL of working reagent was mixed with the 20 µL sample and incubated at 37°C for 1 minute. The absorbance (A) of the sample was taken at 1 minute interval for 3 minutes. Then the average absorbance difference per minutes (ΔA /min) was calculated following the formulae:

ALP = (ΔA /min) ×3300.

Determination of RBS

Random blood sugar test measures blood glucose levels at any time of day, regardless of when the last meal was eaten. It's a quick and convenient way to assess blood sugar levels. An enzymatic colorimetric assay was followed to measure the glucose concentration in the blood sample. One millilitre of working reagent was mixed with the 10 µL sample and incubated for 5 minutes at 37°C. The absorbance (A) of the sample was taken and calibrated against the blank. The colour was stable for at least 60 minutes. Calculation was done using the following formulae:

Glucose (mg/dL) = $\frac{(A)Sample}{(A)Calibrator} \times 100(calibratorconc.)$

Conversion factor. mg/dL. × 0.0555 = mmol/L.

Histomorphological Analysis of Liver

Sample collection and fixation: On day 21 of the experiment, rabbits were sacrificed, and liver samples were carefully collected during necropsy. Tissues were immediately fixed in 10% neutral buffered formalin to preserve structural integrity.

Dehydration and paraffin embedding: Fixed tissues were rinsed under tap water overnight, and then dehydrated through a graded ethanol series (50%, 70%, 80%, 90%, 95%, 100%) followed by clearing in chloroform for 90 minutes. Dehydrated tissues were infiltrated with molten paraffin wax at 60°C and allowed to solidify in cassettes to form paraffin blocks.

Sectioning: Paraffin blocks were mounted in a microtome and sectioned at 4 µm thickness. Ribbons of tissue sections were transferred to APES-coated glass slides to enhance adhesion and dried in an oven at 60°C for 10 minutes. Sections were cleared with xylene prior to staining.

Staining: Liver sections were stained with Haematoxylin and Eosin (H&E) following standard protocols. Staining steps included: xylene, ethanol series, haematoxylin, differentiation, bluing, eosin, and final dehydration before mounting with coverslips.

Microscopy and evaluation: Stained sections were examined under a light microscope at 40× magnification. Multiple fields per section were evaluated for hepatocyte organization, cytoplasmic glycogen deposition, and any signs of necrosis. Comparisons were made across the control, stevioside-, sugar-, and saccharin-treated groups to assess the effects of different biscuits on liver histology.

Statistical analysis

All data are presented as mean ± standard deviation (SD) for each group (n = 3). The data were assumed to be approximately normally distributed based on biological rationale, and no data transformations were applied. Differences between groups were analysed using one-way Analysis of Variance (ANOVA), followed by Tukey's post-hoc test to determine significant pairwise differences. A p-value ≤ 0.05 was considered statistically significant. All statistical analyses were performed using R software. A replication number of three (n = 3) was used due to practical limitations in rearing rabbits and the volume of blood samples available for biochemical assays.

Results and Discussion

The levels of Serum Glutamate Pyruvate Transaminase (SGPT), Serum Glutamate Oxaloacetate Transaminase (SGOT), and Alkaline Phosphatase (ALP) in blood samples were measured to evaluate the effects of stevioside-, sugar- and saccharin-supplemented biscuits on the liver functions of Rabbits. Blood collection was performed on day 0, day 7, day 14 and day 21 for each group to determine the haematobiochemical profiles.

Figure 3 shows that the SGPT level of the stevioside-treated group was the same as the control group on day 7 but lower than saccharin-treated group. At day 14 and day 21, the SGPT levels of stevioside-treated groups were significantly lower than control, sugar and saccharin-treated groups. This figure also shows that on day 7, day 14 and day 21, the SGPT levels of saccharin treated group were increased compared to control, stevioside and sugar-treated groups.

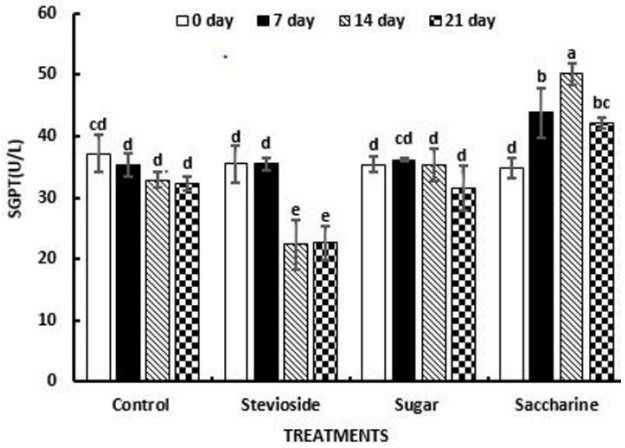

Figure 3: Effects of stevioside-, sugar- and saccharin-supplemented biscuits on Serum Glutamate Pyruvate Transaminase (SGPT) levels in rabbit's blood. Error bars represent standard errors. Values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey test
.

The SGPT levels were found to be 22.39 U/L on day 14 and 22.59 U/L on day 21 in response to the stevioside-treated group and these values were minimum compared to the control and other groups. These results suggest that stevioside-supplemented biscuits showed the best SGPT level compared to sugar and saccharin-supplemented biscuits. It is reported that stevia lowered SGPT by 62% in diabetic rats (AbdElwahab *et al.*, 2017) and the co-treatment of stevia with amlodipine and losartan led to a reduction in SGPT in rats (Yesmine *et al.*, 2013), which is consisted with our decreasing pattern of SGPT level.

Figure 4 displays that the SGOT level of the stevioside treated group was significantly lower than other groups on day 7. On day 14 and day 21, the SGOT levels of the stevioside-treated group also displayed similar results compared to the control. The minimum level of SGOT (25.50 U/L) was found on day 21 in the stevioside treated group and the maximum level of SGOT (56.0 U/L) was found on day 14 in the saccharin group.

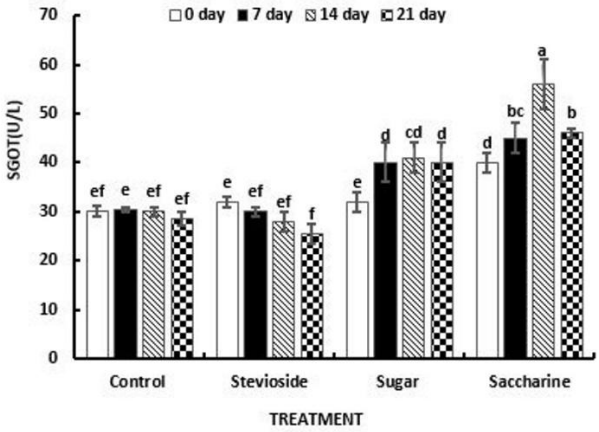

Figure 4: Effect of stevioside-, sugar- and saccharin-supplemented biscuits on Serum Glutamate Oxaloacetate Transaminase (SGOT) levels in rabbit's blood. Error bars represent standard errors. Values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey test.

The results suggested that stevioside-supplemented biscuits showed the best SGOT level as compared to sugar and saccharin-supplemented biscuits. It has been reported that when stevia was applied with amlodipine and losartan then this co-treatment led to a decrease in SGOT levels in rats (Yesmine *et al.,* 2013). AbdElwahab *et al.,* (2017) also reported that stevia lowered SGOT by 57% in diabetic rats.

Figure 5 represents that at 7, 14, and 21th days, the ALP level of stevioside-treated groups was significantly lower than control, sugar, and saccharin-treated groups. At 7 and 21 days, the ALP levels showed similar results in response to stevioside. The maximum level of ALP (292.22 U/L) was found in sugar-treated rabbits at 14 days and the minimum level of ALP (145.34 U/L) was found in stevioside-treated rabbits at 7 days. It has been reported that stevia reduced ALP levels by 41% in diabetic rats. (AbdElwahab *et al.,* 2017).

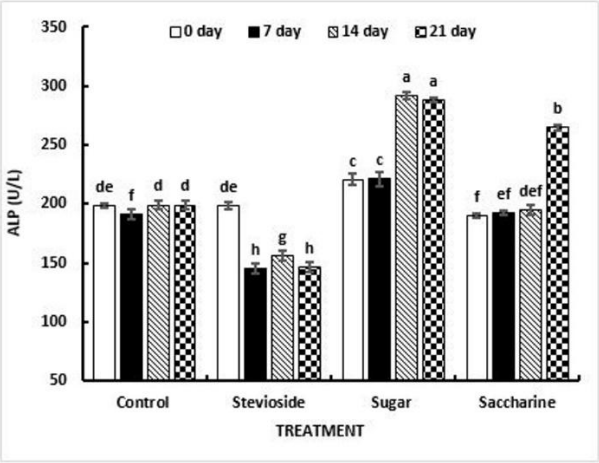

Figure 5: Effect of stevioside, sugar and saccharin supplemented biscuits on, alkaline phosphatase (ALP) levels in rabbit's blood. Error bars represent standard errors. Values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey test.

Figure 6 shows that on day 7 and day 14, the Random Blood Sugar (RBS) levels of the stevioside treated group were lower than sugar and saccharin-treated groups. However, on day 21, the results of stevioside-treated groups were significantly lower than other groups. This figure also shows that on days 7, day 14, and day 21 the RBS levels of stevioside-treated groups were gradually decreased and in the case of sugar and saccharin-treated groups they were gradually increased.

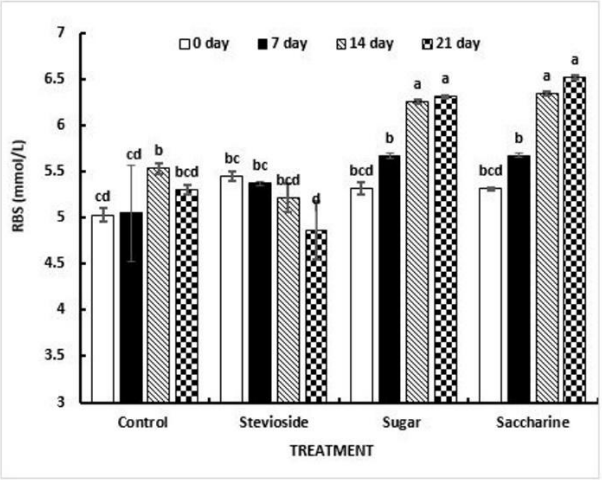

Figure 6: Effect of stevioside-, sugar- and saccharin-supplemented biscuits on Random Blood Sugar (RBS) levels in rabbit's blood. Error bars represent standard errors. Values indicated by the same letter do not differ significantly at 5% level of significance as determined by Tukey test.

The minimum level of RBS (4.86 mmol/L) was found on day 21 in the stevioside treated group and the maximum level of RBS (6.52 mmol/L) was found on day 21 in saccharin treated group. The results suggest that stevioside-supplemented biscuits can control blood glucose levels but not show any negative effects in rabbits. Oral administration of a high concentration of stevioside increased insulin sensitivity of the whole body and low concentrations of stevioside (0.01 to 0.1 mmol/L) slightly improved in vitro insulin action on skeletal muscle glucose transport (Lailerd *et al.*, 2004).

In addition to the above experiments, Histo-morphological staining was performed to observe the effects of different biscuits on the liver functions of Rabbits (Figure 7).

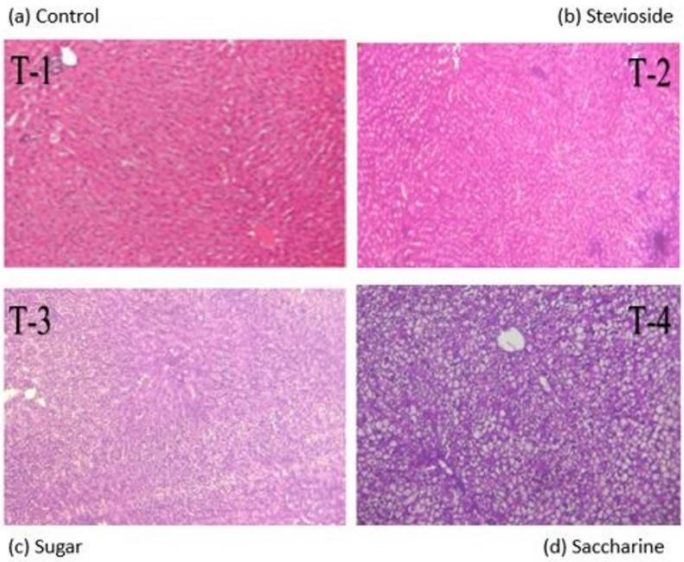

Figure 7: Effects of stevioside-, sugar- and saccharin-supplemented biscuits on the morphology of Rabbit's liver. A cross section view of liver (Hematoxylin and Eosin stain and X10). Control (7a); Stevioside (7b); Sugar (7c); Saccharin (7d).

Histomorphological observation of control rabbit's liver (Fig. 7a) showed that the cells were histologically organized and no apparent histological lesions were focused. The level of sugar deposition in cell cytoplasm was almost uniform. The hepatic cords were well organized without any reactive cell infiltration in the hepatic parenchyma. The stevioside-treated group (Fig. 7b) of rabbit's liver depicted that cells were highly organized with comparatively higher degree of glycogen deposition in their cytoplasm. No obliterations of cells or hepatic parenchyma on focus. No reactive cell infiltration in hepatic parenchyma. Stevioside possessed the capability to activate peripheral mu opioid receptors, leading to a reduction in plasma glucose levels and an increase in glycogen synthesis in the liver. The activation of peripheral mu opioid receptors is accountable for stevioside's role in regulating glucose homeostasis (Yang *et al.*, 2009). The sugar-treated group (Fig. 7c) of rabbit's liver noticed that the cytoplasmic storage of glycogen was comparatively lower as characterized by the clear or transparent cytoplasmic image. A minimal level of glycogen storage was also evident in some cells within the same focus. The saccharin-treated group (Fig. 7d) of rabbit's liver showed that the cells were well focused with or without containing nucleus. Majority of the hepatic cells did not contain any substrates in their cytoplasm. The reactive cells infiltrations were not evident.Altogether, in histological observations of rabbit's liver showed the cytoplasmic storage of glycogen in hepatic cells of stevioside-treated group was relatively similar to the control group, but distinctly higher than those treated with sugar followed by saccharin supplemented one.

The findings of the present study in rabbits are consistent with previous investigations in rats, further supporting the beneficial role of stevioside in maintaining liver health and glucose homeostasis. Similar to our results, AbdElwahab et al. (2017) reported that stevioside lowered SGPT, SGOT, and ALP levels in diabetic rats, while Yesmine et al. (2013) observed improved liver enzyme profiles in rats treated with stevioside and antihypertensive drugs. In addition, Lailerd et al. (2004) demonstrated enhanced insulin sensitivity and glucose transport, and Yang et al. (2009) confirmed stevioside's role in regulating glucose homeostasis. Stevioside has also been shown to prevent necrosis, inflammation, and oxidative damage in rat liver tissue (Ramos-Tovar et al., 2018; Rotimi et al., 2018). Altogether, evidence from both rabbit and

rat models highlights stevioside as a promising natural sweetener.

This study had some limitations. First, the sample size was small (n = 3 per group), which may limit the statistical power and generalizability of the findings. Second, the study was conducted only on a single animal species (rabbits), and therefore the results may not fully reflect the responses in other animals or humans. Third, the experimental duration was relatively short (21 days), which may not capture the long-term effects of stevioside consumption on liver function and histomorphology. Finally, only selected biochemical and histological parameters were assessed. Additional molecular or metabolic markers could provide deeper insights into the mechanisms involved. These limitations highlight the need for further studies with larger sample sizes, extended experimental durations, multiple species, and a broader range of physiological and molecular endpoints to validate and expand upon the present findings.

Conclusion

Compared to sugar- and saccharin-, stevioside-supplemented biscuit showed a decrease level of Serum Glutamate Pyruvate Transaminase (SGPT), Glutamate Oxaloacetate Transaminase (SGOT), Alkaline Phosphatase (ALP) and Random Blood Sugar (RBS) levels. Moreover, the histomorphological observation showed no adverse effects on hepatic cells. So, stevioside-supplemented biscuits may be a safer alternative than sugar and saccharin.

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Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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