RESEARCH ARTICLE

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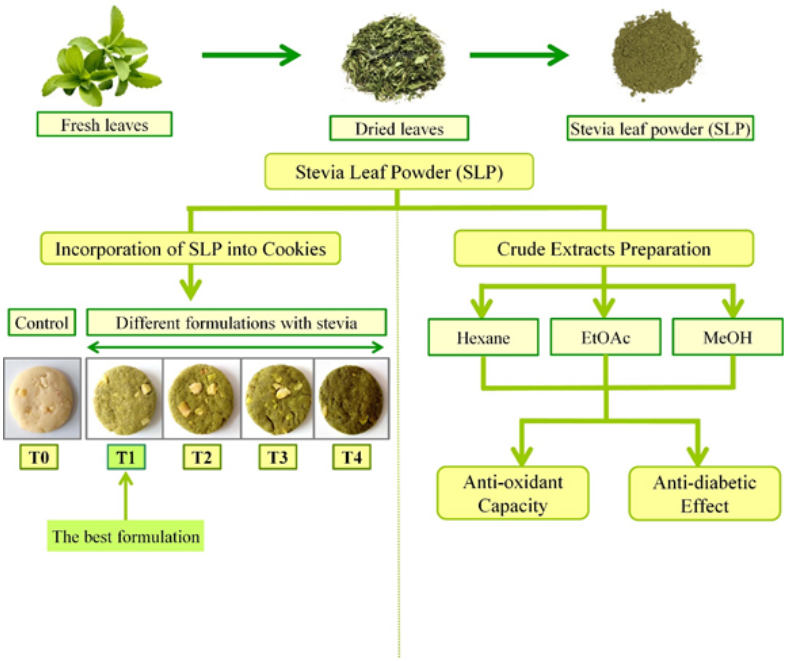

Highlights

- *Stevia rebaudiana* leaf powder contains beneficial phyto-nutrients.
- *Stevia rebaudiana* leaf powder has demonstrated strong antioxidant capacity.
- *Stevia rebaudiana* leaf powder displayed a notable anti-hyperglycemic effect.
- *Stevia rebaudiana* leaf powder has been found to be an effective substitute for fermentable sugars

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Evaluation of Stevia Leaf (*Stevia rebaudiana* Bertoni) Powder as Sugar-Substitute in Cashew Nut Fortified Cookies

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Abstract: *Stevia rebaudiana* is an herbal supplement used as a substitute for sugar in food processing. Considering the potentials of stevia as a zero-calorie-natural sweetener, this study aimed to assess the antioxidant and anti-hyperglycemic properties of stevia *(Stevia rebaudiana* Bertoni) leaf powder (SLP) to find its suitability to formulate low-calorie foods. Extracts of SLP obtained with hexane, ethyl acetate, and methanol were evaluated for DPPH, $ABTS^+$, FRAP and enzyme inhibitory assays. Adopting a standard recipe, five different cookie types were prepared by incorporating varying proportions of SLP and evaluated by a 30 semi-trained panel. The nutritional composition and shelf-life stability were also evaluated. SLP had moisture (8.0 %), ash (13.5 %), crude protein (12.4 %), crude fat (6.9 %), crude fiber (18.3 %), and carbohydrate (40.9 %). Sensory results showed that the cookie formulated with the lowest amount of SLP (T_1= 2.0g) had the highest overall acceptability with the highest preference for appearance and smell. The cookie type formulated with the highest amount of SLP (T_4= 8.0g) had the highest crude protein (13.50%), crude fiber (21.76%), and lowest carbohydrate contents (40.88%). The keeping quality of the cookies remained acceptable within the storage period. These results suggest that acceptable quality cashew nut fortified cookies could be prepared by replacing sucrose with stevia leaf powder

Keywords: Anti-diabetes food; Low-calorie cookies; Natural sweetener; Sensory analysis; *Stevia rebaudiana*

INTRODUCTION

Diabetes mellitus is a degenerative disease associated with persistent hyperglycaemia, characterized by an abnormal rise in blood sugar levels. Previous reports showed that diabetes can lead to an imbalance in the metabolism of carbohydrates, fats, and proteins (Lacroix & Li-Chan, 2014; Kazeem et al., 2013). Being categorized as a chronic ailment, diabetes is commonly associated with several complications, leading to dysfunction of different organs such as eyes, kidneys, heart, nerves, and blood vessels (International Diabetes Federation, 2021). Over the years, controlling hyperglycemia has received much attention from several groups engaged in diabetes research. Restriction of the intake of fermentable sugars from regular meals is an important approach to managing diabetes (Adekola & Marikkar, 2022). Pertaining to this, the search for low-calorie sweeteners to control the blood glucose level among diabetic patients has been intensified. Fructose is one of the low-calorie sweeteners, which is reported to be 1.2 times sweeter than sucrose, allowing for reduction of sugar in sweetened foods (Wight, 2014). The flavor enhancement effect of fructose on foods is an additional advantage. Saccharin, sucralose, acesulfame, and aspartame are some of the synthetic non-nutritive sweeteners, which are tried and tested by the food industries (Shankar et al., 2013).

The search for natural sweeteners as a substitute for sugar in food applications has continued throughout history. *Stevia rebaudiana* is a herbal supplement used as a substitute for fermentable sugars such as sucrose and glucose. According to historical records, *Stevia rebaudiana* was first commercially adopted as a sweetener in Japan in the early 1970s. During the course of time, its uses have spread across many parts of the world including countries like Malaysia, Thailand, and China. Stevia leaves are found to contain phytonutrients called steviol glycosides, which is sweeter than ordinary sugar without contributing to calories (Peteliuk et al., 2021). Adding to the advantage is the fact that the sweetness of stevia leaves is preserved for an extended period of time without an appreciable decline in its taste (González et al., 2014). Some initiatives were taken recently in Sri Lanka to develop micro-propagation protocols for multiplication of *Stevia rebaudiana* targeting commercial-scale cultivation. Nonetheless, exploratory studies to evaluate the biological and functional properties of locally produced *Stevia rebaudiana* leaf powder are scanty. Also a study on the effect of SLP as a sugar substitute in cashew-nut-incorporated butter cookies has not been undertaken previously. In this study, it was aimed to evaluate the antioxidative and antihyperglycemic effects of SLP and its incorporation on the nutritional composition, calorific value, and quality attributes of low-calorie cookie formulation.

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MATERIALS AND METHODS

Materials

Sampling of *Stevia rebaudiana* leaves was done from plants propagated vegetatively in the greenhouses at NIFS, Kandy during the period 2nd December 2021 to 10th January, 2022. Chemicals and reagents used in the study were either analytical or HPLC grade. Food ingredients such as wheat flour, sucrose, butter, and salt were purchased from popular commercial establishments in Sri Lanka.

Preparation of Stevia Leaf Powder (SLP)

Three thousand grams of fresh leaf samples of stevia were cleansed by running tap water. They were dried subsequently at ambient conditions (27 °C, relative humidity 60%) for 4-5 h to remove surface moisture. The leaves were dried again in a forced-draft convection oven at 55 °C for 24 h. The dried leaves were ground into powder form by using an electric grinder. SLP (15 g) was sifted through 425, 297, 180, 105, 88 and 63 µm sifters, manually. The particle size range was 88-180 µm. The powdered leaf (SLP) samples were finally kept under refrigerated conditions until further analysis.

Crude Extract Preparation

A sample of 200 g SLP was taken into a 1000 mL conical flask and added with hexane so that the height of the sample to become double. The mixture was sonicated for 30 min at 30-40 °C until the filtrate was recovered. The resulting residue was then extracted with ethyl acetate using the same procedure. Then the residue resulting from the extraction was subjected to the same procedure using methanol as solvent. All three extracts were concentrated under reduced pressure using a rotary evaporator. The concentrated extracts were collected into clean glass vials separately and stored under freezing conditions for further analysis.

Phytochemical Profiling by LC-MS

Phytochemical profiling was conducted by following the LC-MS method reported by Gunarathne et al. (2022). A five g of SLP was extracted with a 25 mL portion of 70 % aqueous methanol (HPLC grade methanol, >99.99 % in ultrapure water) using ultra-sonication for 30 min. The extract was subjected to filtration via a syringe filter (25 mm, 0.45 um). The filtrate was then taken for the LC-MS analysis. The LC-MS analysis was performed using an ultra-high pressure liquid chromatograph (UHPLC) (UltiMate™ 3000, Thermo Scientific, Germany) equipped with an auto-sampler (ACC-3000), quaternary pump (LPG-3400SD) and diode array detector (DAD - 3000), which was able to take signals at 224 nm, 254 nm, 280 nm and 360 nm wavelengths. In order to obtain fragment ion m/z, an iron trap mass spectrometer (LCQ FLEET, Thermo Scientific, USA) equipped with an electrospray ionization (ESI) source operating in full scan, auto MS^n mode was fitted to the system. A 10 µL portion of the sample was injected to the system equipped with an Ascentis RP – Amide column (5 µm) (Supelco Analytical 15 cm x 4.6 mm, Merck, Germany). The mobile phase was a mixture of methanol (A) and acidic water comprising 0.01 % formic acid (B). The solvents were flowed to the system at a flow rate of 0.400 mL/min in a gradient elution. Where the gradient process was arranged as; 90 % of solvent B from 0-5min, 90-2 % of solvent B from 5-65 min, 2 % of solvent B from 65-70 min, 2-90 % of solvent B from 70-75 min, 90 % of solvent B from 75-80 min. The MS spectra were taken in negative iron mode and the parameters were adjusted as; sheath N_2 gas flow rate, 36 arbitrary units; aux N_2 gas flow rate, 9 arbitrary units; heat temperature, 350 ºC; spray voltage, 4.50 kV; capillary voltage, -40.00 V; capillary temperature, 320 ºC; tube lens, -95.00 V. The mass chromatogram was obtained from 110-1500 m/z. In this study, ten phenolic compounds were used as authentic standards while calibrating the same setting source parameters for qualitative detection and quantitative analysis.

Determination of DPPH Radical Scavenging Activity

This assay was carried out by following the method reported by Gunarathna et al. (2022). Briefly, a concentration series of crude extracts was prepared by reconstituting crude extract in MeOH and added with 150 µL aliquots of each concentration solution into 60 µL of 0.3 mM DPPH solution in 96 well micro-plate, allowed for 30 min in dark at RT. The absorbance values were recorded against the control at 517 nm. Both ascorbic acid and butylated hydroxyl anisole (BHA) were used as positive controls. The calculation of the percentage inhibition was obtained as shown below and the determination of IC_{50} value was taken graphically.

$$RSA\% = \frac{\delta A_{control} - \delta A_{sample}}{\delta A_{control}} \times 100$$

Where; $\delta A_{control}$ = Absorbance $_{control}$ – Absorbance $_{control\ blank}$, δA_{sample} = Absorbance $_{sample}$ – Absorbance $_{sample\ blank}$; RSA %, Percentage of radical scavenging activity.

Determination of Ferric Reducing Antioxidant Power

This assay was carried out by following the method reported by Gunarathna et al. (2022). A fifty µL aliquot of sample solution (reconstituted crude extract with distilled water) was added to a 96-well microplate and mixed with 150 µL of FRAP solution and allowed to incubate for 4 min at RT. The absorbance values were recorded at 593 nm and the values were expressed as µmol $FeSO_4$ per g of crude extract. In this experiment, both ascorbic acid and BHA were used as positive controls. FRAP solution was prepared by mixing 10 mM TPTZ solution (in 40 mM HCl) and 10 mM $FeCl_3.6H_2O$ solution with 40 mL of 300 mM, pH 3.6 acetic buffer in the ratio of 1:1:10 and was subjected to heating up to 37 °C just prior to use.

Determination of $ABTS^+$ Radical Scavenging Activity

The ABTS assay was performed according to the method described by Marikkar et al. (2016) with slight modifications. The stock solution of $ABTS^+$ radical cation was prepared by mixing equal proportions of ABTS (7.8 mM) and potassium persulfate (2.45 mM) at room temperature for 16 hours in the dark. As the next step, the prepared ABTS working solution was diluted with PBS (pH 7.4) to an absorbance of 0.7 ± 0.0 at 743 nm. In the sample preparation, a 150 ppm sample solution was

prepared by mixing crude extracts in 3% DMSO. A fifty µL portion of sample solution was then mixed with 150 µL portion of ABTS working solution. Thereafter, the mixture was incubated for 10 min at room temperature and the absorbance was measured at 734 nm. Ascorbic acid was used as the positive control and the results were expressed as µmole of Trolox per gram of crude extract.

Determination of Alpha- amylase Inhibitory Activity

α- amylase inhibitory activity of crude extracts of SLP was performed according to the methods described by Gunarathne et al. (2022) with slight modifications. Firstly, a concentration series of plant extracts (6000 - 42000 ppm) was prepared by dissolving the crude extracts in distilled water with 5% DMSO. A portion of 50 µL of each sample solution was mixed with an equal amount of α- amylase enzyme solution (20 mg/mL) in a semi-centrifuge tube. After 30 min incubating the mixture at room temperature, a 100 µL portion of 1% starch solution was added to it. The mixture was further incubated for another 10 min at room temperature. In the next step, a 100 µL portion of DNSA (3,5-dinitrosalicylic acid) reagent was added to the mixture and incubated for 15 min at 85 °C in a water bath. Thereafter, the final mixture was cooled down to room temperature and diluted with 900 µL of distilled water. A 200 µL portion of the final mixture was transferred into the 96 wells microplate and the absorbance was measured at 540 nm. Acarbose (Glucobay tablet) was used as the positive control of this study. The percentage enzyme inhibition was calculated using the following equation and the IC_{50} values were obtained graphically by plotting the percentage α- amylase inhibition against sample concentrations of each extract.

$$Percentage\ \alpha - amylase\ inhibition = \frac{\delta A_{control} - \delta A_{sample}}{\delta A_{control}} \times 100$$

Where; $\delta A_{control}$ = Absorbance $_{control}$ – Absorbance $_{control\ blank}$; δA_{sample} = Absorbance $_{sample}$ – Absorbance $_{sample\ blank}$

Determination of Alpha-glucosidase Inhibitory Activity

α-glucosidase inhibitory activity of crude extracts of SLP was determined according to the method described by Gunarathne et al. (2022). Initially, a concentration series (3.91- 1000 ppm) of plant extracts was prepared by dissolving the crude extracts in distilled water with 3% DMSO. A hundred µL of 30 mM phosphate buffer (pH 6.5) was then added into 96 wells of micro-plate, followed by mixing with 25 µL of sample solution. As the next step, 25 µL α-glucosidase enzyme solution (12.5 µL/ mL) was added into it to incubate for 5 min at 37 °C. After that, a 50 µL portion of pNPG (*p*-nitrophenyl-α-D-glucopyranoside) solution (0.8mg /mL) was added and followed by incubating for another 30 min at 37 °C. Acarbose (Glucobay tablet) was used as the positive control of this study. The absorbance value was measured at 410 nm and the percentage α - glucosidase inhibitory activity was calculated using the following equation. The IC_{50} values were calculated graphically by plotting percentage α- glucosidase inhibition against the sample concentration of each extract.

$$Percentage\ \alpha - glucosidase\ inhibition = \frac{\delta A_{control} - \delta A_{sample}}{\delta A_{control}} \times 100$$

Where; $\delta A_{control}$ = Absorbance $_{control}$ – Absorbance $_{control\ blank}$; δA_{sample} = Absorbance $_{sample}$ – Absorbance $_{sample\ blank}$

Formulation of Low-calorie Cookie

In an effort to completely replace sugar, SLP was incorporated into cookies based on the sweetness equivalence value of SLP for sugar. As shown in Table 1, a 40 g portion of sugar was added to 100 g of flour for the preparation of the control of cookies (T_0). T_0 was formulated with 100% sugar (40 g) without incorporating SLP (0 % SLP). To replace sugar with SLP, four treatments were designed (T_1, T_2, T_3 & T_4). Based on the report of Savita et al. (2004), crude SLP was 10-15 times sweeter than sucrose. In a separate study, Singh and Rao (2005) reported that SLP is 15-20 times sweeter than sugar. Hence, sweetness equivalence calculation was adopted considering stevia is sweeter than sugar at 5 times (T_4),10 times (T_3), 15 times (T_2) and 20 times (T_1). Hence, the required amount of SLP for T_1, T_2, T_3 and T_4 were calculated based on the sweetness equivalence of SLP to sugar as T_1 (SLP =40/20=2 g), T_2 (SLP =40/15=2.67 g), T_3 (SLP =40/10=4 g) and T_4 (SLP =40/5=8 g), respectively. Five different samples of cookies were formulated by incorporating varying proportions of dehydrated SLP as shown in Table 1. T_0 was the control, which was formulated with 100% sucrose without SLP (0% SLP). Other four treatments namely, T_1, T_2, T_3 and T_4 were formulated by replacing sucrose with SLP. In this study, cookies were prepared in accordance with the method previously described by Marikkar et al. (2020). Briefly, salted butter was creamed with a powdered mixture of sugar/ dehydrated SLP and baking powder after mixing in a mixer for 3 min at low speed. Varying proportions of water were added in order to gain the required dough consistency. Mixing was continued using a mixer for the next 2 min at a high speed. After adding flour, mixing was continued for next 2 min at low speed scraping the bowl at every 30 sec. The dough was finally allowed to rest for 10 min followed by cutting into pieces using cookie mold (5 cm diameter and 1.5 cm thickness). The cut dough pieces were then baked for 25 min in an oven pre-heated at 150 °C. The cookies were kept at 25 °C for 30 min to cool down and then packed in sealed low-density polyethylene (LDPE) bags until further analysis.

Proximate Compositional Analysis

For chemical analysis, dried stevia leaf samples and cookies were ground into powder. The estimate of the moisture, crude protein, crude fat, total ash and crude fiber contents of the formulation was obtained according to standard methods (AOAC 2000). The following formula was used to calculate the carbohydrate content of the samples.

Total carbohydrate (%) = 100- (crude protein + crude fat + ash + moisture+ crude fiber content)

Table 1: Proportional distribution of cookie ingredients (wheat flour, salted butter, sucrose, *Stevia rebaudiana* Bertoni leaf powder (SLP), roasted cashew and baking powder) across different treatments, T_0 to T_4[1].

Treatment	Weight of ingredient (g)					
	Wheat flour	Salted butter	Sucrose	SLP	Roasted cashew	Baking powder
T_0 (356)	100.00	30.00	40.00	0.00	40.00	2.00
T_1 (361)	100.00	30.00	0.00	2.00	40.00	2.00
T_2 (345)	100.00	30.00	0.00	2.67	40.00	2.00
T_3 (325)	100.00	30.00	0.00	4.00	40.00	2.00
T_4 (338)	100.00	30.00	0.00	8.00	40.00	2.00

[1]Abbreviations: SLP, Stevia Leaf Powder; T_0, control cookie formulation by 40.00 g sugar per 100.00 g wheat flour; T_1, cookie formulation by totally replacing sugar with 2.00 g SLP per 100.00 g wheat flour; T_2, cookie formulation by totally replacing sugar with 2.67 g SLP per 100.00 g wheat flour; T_3, cookie formulation by totally replacing sugar with 4.00 g SLP per 100.00 g wheat flour; T_4, cookie formulation by totally replacing sugar with 8.00 g SLP per 100.00 g wheat flour.

Determination of Calorific Value

Calorific values of the samples were calculated using the following formula:

Calorific value = (Fat content × 9 kcal) + (Protein content × 4 kcal) + (Carbohydrate content × 4 kcal)

Sensory Evaluation

The sensory evaluation was performed in accordance with the method previously described by Marikkar et al. (2020). Accordingly, a preference ranking test was performed using thirty semi-trained panelists to rank samples according to their preference on a scale from 1 to 5, where the highest preference rank was 1 and the lowest preference rank was 5. Five coded samples were given to the individual panelist to rank them according to their preference for individual sensory attributes namely appearance, color, smell, taste, texture, and overall acceptability of cookies. Obtaining ethical clearance is waived off for this study as it is not involved with either human clinical trials or *in vivo* animal model studies.

Shelf-life Studies

Samples containing 5 cookies of each type were packed under hygienic conditions in clean packages. Samples were sealed immediately with low-density polyethylene (LDPE) bags to preserve the freshness. During the shelf-life evaluation, sample bags were kept at room temperature (27 ^{o}C) until all analyses were completed. The whole shelf-life study was conducted for a period of one month by determining the moisture content and the microbial count (total plate count and yeast and mold count) of the samples at once in every two weeks' interval, continuously. For microbiological analysis, enumeration of aerobic colony count was done by incubating micro-organisms in nutrient agar (NA) medium under 37 ^{o}C for 48 hours. The yeast and mold count was done by incubating in potato dextrose agar (PDA) medium with 0.01% Chloramphenicol held at room temperature (AOAC Official Method 2000; 990.12; AOAC Official Method 2000; 997:02).

Statistical Analysis

All experimental measurements were done in triplicate (n=3). All results were presented in the form of mean ± standard deviation (SD). Data were statistically analyzed by one-way analysis of variance (ANOVA) using Tukey's Test of MINITAB (version 20.3) statistical package at a 0.05 probability level. Non- parametric Friedman rank sum test was employed to evaluate the sensorial data using the Minitab software package version 19 (Minitab inc., State College, PA, USA).

RESULTS AND DISCUSSION

Phenolic Profile of *Stevia rebaudiana* Leaves

The distribution of selected phenolic compounds present in the MeOH extract of SLP is shown in Table 2. Out of the ten selected phenolic standards, caffeic acid, caffeine, catechin, chlorogenic acid, ferulic acid, gallic acid, p-coumaric acid, rutin, sinapic and vanillin were present in SLP. There were significant (p<0.05) differences with regard to the contents of phenolics detected in the MeOH extract. Rutin (34.6 ± 0.0 mg/ 100 g of leaf powder) emerged as the predominant among the phenolics while gallic acid was not detected at all. The results further indicated that SLP had higher contents of caffeic acid, catechin, and caffeine, but lower content of p-coumaric acid (0.2 ± 0.0 mg/ 100 g of SLP). These bioactive constituents are already known to contribute various biological properties displayed by plants.

Functional Properties of SLP

Although stevia leaf is well-known as a non-caloric sweetener, investigation of its biological activities could be beneficial to its suitability in the prevention of complications related to diabetes. For instance, biological activities such as the anti-oxidant and anti-hyperglycemic effects of SLP are very much pertinent for investigation. According to Table 3, IC_{50} values of DPPH radical scavenging activity of different extracts of SLP tended to follow the order of MeOH>EtOAc. Hexane extract, on the other hand, showed only a weak radical scavenging activity. Ascorbic acid, which was the positive control showed significantly (p<0.05) higher DPPH radical scavenging activity with an IC_{50} value of 8.4 ± 0.0 µg/mL. When considering the FRAP antioxidant power of SLP, the highest value (567.5 ± 7.5 µmole of $FeSO_4$ / g of crude extract) was observed for MeOH extract and the values were aligned in the decreasing order of MeOH>EtOAc>Hexane. Nonetheless, the reducing power of each crude extracts of SLP was

Table 2: Distribution of selected phenolics in MeOH extract of stevia leaf powder[#].

Phenolic compound	Content (mg / 100 g of leaf powder)
Caffeic acid	$27.9^{h} \pm 0.0$
Caffein	$10.6^{f} \pm 0.0$
Catechin	$16.3^{g} \pm 0.0$
Chlorogenic acid	$0.4^{b} \pm 0.0$
Ferulic acid	$0.9^{e} \pm 0.0$
Gallic acid	ND
p-coumaric acid	$0.2^{a} \pm 0.0$
Rutin	$34.6^{i} \pm 0.0$
Sinapic acid	$0.7^{d} \pm 0.0$
Vanillin	$0.49^{c} \pm 0.0$

[#]Each value in the table represents the mean ± SD of three replicates. Within the column, means that do not share a similar superscription letter is significantly different at 95% confidence (α=0.05) Abbreviations: ND, Not detected.

significantly ($p< 0.05$) lower than that of ascorbic acid (10.32 ± 0.02 mmole of $FeSO_4$ / g). Among all crude extracts of SLP, only MeOH extract displayed $ABTS^+$ radical scavenging activity (269.6 ± 6.2 µmole of trolox/ g), but the value displayed by ascorbic acid (1783.0 ± 3.0 µmole of trolox/ g) was significantly ($p<0.05$) higher.

When considering the anti-hyperglycemic potential of SLP, only MeOH and EtOAc extracts exhibited inhibitory activity against α- amylase. In this case, the highest inhibitory activity was recorded for EtOAc extract (IC_{50} = 4434.8 ± 86.9 µg/mL) (Table 3). When compared to these two extracts, Acarbose displayed significantly ($p<0.05$) higher inhibitory effect against α- amylase (IC_{50} value of 0.2 ± 0.0 µg/mL). Moreover, the α- glucosidase inhibitory activity of the extracts of SLP was found to be aligned in the ascending order of MeOH<EtOAc<Hexane. Among different extracts of SLP, the highest inhibitory activity was noticed for hexane extract (IC_{50} = 21.8 ± 0.8 µg/mL). When compared to Acarbose, all crude extracts of SLP showed significantly ($p<0.05$) strong inhibitory activity against α- glucosidase (Table 3). These results provide sufficient ground for our initiative to utilize SLP as a sugar replacer in food formulations intended for diabetes.

Nutrient Composition

The nutrient composition of different cookies formulated in this study is given in Table 4. Significant ($p<0.05$) differences were noticed between the samples and the control with regard to fat, protein, crude fiber, total ash, and carbohydrate contents (Table 4). With increasing SLP in the formulation, the moisture content of the cookie samples increased only marginally. Particularly, significant ($p<0.05$) differences in moisture content were observed between the treatments and the control, except for T_1. According to Cauvain & Young (2008), the maximum limit of moisture content permitted for freshly produced cookies should be below 5 %. As mentioned in food standards, biscuits should have a moisture content below 6 % during their shelf-life period (SLS 251:1991; BIS 1011: 2002). Thus, the formulated cookies in this study were found to comply with the upper limit set by standardization bodies.

The ash content determination in food samples is generally considered important since the quantity and the type of minerals present in foods might influence their quality attributes including the taste, appearance, texture and stability (Ho & Pulsawat, 2020). As shown in Table 4, the ash content of the control was significantly ($p<0.05$) lower than those of cookies incorporated with SLP. T_4 with the highest amount of SLP, had the highest ash content of 1.4 %. Although significant differences ($p<0.05$) were noticed between the control and the treatments with regard to ash contents, no significant ($p>0.05$) difference was observed within the different treatments. As shown in Table 4, the ash content of the dehydrated SLP was 13.5 % (w/w, db). As a result, the total ash contents of the different treatments were tended to increase gradually with increasing amounts of SLP incorporation (Table 4).

Table 3: Anti-oxidative and anti-hyperglycemic potentials of different extracts of stevia leaf powder (SLP)[#].

Assay	Type of extract		
	Hexane	EtOAc	MeOH
DPPH radical scavenging activity [IC_{50} value (µg/mL)]	WK	547.4^{b} ±0.3	1696.4^{a} ±78.8
Ferric reducing antioxidant power (µmole of $FeSO_4$ / g of crude extract)	84.5^{c} ± 7.8	240.0^{b} ± 21.1	567.5^{a} ± 7.5
$ABTS^+$ radical scavenging activity (µmole of trolox / g of crude extract)	ND	ND	269.6 ± 6.2
α- Amylase inhibitory activity [IC_{50} value (µg/mL)]	ND	4434.8^{b} ± 86.9	5438.5^{a} ± 85.2
α- Glucosidase inhibitory activity [IC_{50} value (µg/mL)]	21.8^{c} ± 0.8	130.9^{b} ± 10.3	225.6^{a} ± 8.8

[#]Each value in the table represents the mean ± SD (n=3). Means within each column sharing a different superscript are significantly (α=0.05) different at 95% confidence. The inhibitory effect of the different crude extracts of *S. rebaudiana* was measured at the concentration of 2000 mg/ml. Abbreviations: SLP, Stevia Leaf Powder; EtOAc, Ethyl Acetate; MeOH, Methanol; ND, Not Detected; WK, Weak Activity

Table 4: Proximate composition and calorific value of different cookie formulations (g/100 g dry matter basis)[#].

Treatment	Moisture (%)	Total ash (%)	Crude fat (%)	Crude protein (%)	Crude fiber (%)	Carbohydrate (by difference)	Calorific value (Kcal)
T_0 (356)	3.2^{d} ±0.1	1.1^{b}±0.2	19.5^{a}±0.1	09.7^{d}±0.0	15.4^{b}±0.5	51.1^{a}±0.4	418.9^{a}±1.7
T_1 (361)	$3.4^{c,d}$ ±0.0	1.4^{a}±0.0	18.1^{a}±3.7	12.2^{c}±0.1	$19.2^{a,b}$±0.8	$45.7^{a,b}$±3.7	$394.8^{a,b}$±19.2
T_2 (345)	3.5^{c} ±0.0	1.4^{a}±0.0	18.2^{a}±1.2	12.9^{b}±0.3	$19.3^{a,b}$±0.9	$44.7^{a,b}$±2.2	$394.0^{a,b}$±2.5
T_3 (325)	3.8^{b} ±0.03	1.4^{a}±0.0	18.2^{a}±2.4	$13.2^{a,b}$±0.2	$20.8^{a,b}$±4.9	$42.6^{a,b}$±7.0	387.3^{b}±13.9
T_4 (338)	4.0^{a} ±0.04	1.4^{a}±0.0	18.5^{a}±1.0	13.5^{a}±0.2	21.8^{a}±1.5	40.9^{b}±1.8	383.7^{b}±5.8
SLP	8.0 ±0.4	13.5 ±0.7	6.9 ±0.2	12.4 ±0.5	18.3 ±1.5	40.9±0.6	-

[#]Each value in the table represents the mean ± SD (n=3). Means within each column sharing a different superscript are significantly (α=0.05) different at 95% confidence

Abbreviations: SLP, stevia leaf powder; T_0, control cookie formulation by 40.00 g sugar per 100.00 g wheat flour; T_1, cookie formulation by totally replacing sugar with 2.00 g SLP per 100.00 g wheat flour; T_2, cookie formulation by totally replacing sugar with 2.67 g SLP per 100.00 g wheat flour; T_3, cookie formulation by totally replacing sugar with 4.00 g SLP per 100.00 g wheat flour; T_4, cookie formulation by totally replacing sugar with 8.00 g SLP per 100.00 g wheat flour

As shown in Table 4, the crude fat contents of different treatments were ranged between 18.1 and 19.6 (w/w, db); the highest fat content was found with the control, but no significant (p>0.05) difference was noticed between the treatments and the control. As the crude fat content of dehydrated SLP used in this study was 6.90 % (w/w, db), its impact on the crude fat content of SLP to the formulation of cookies was negligibly small. Based on the data in Table 4, the crude protein contents of the SLP-incorporated cookies were significantly (p<0.05) higher than that of the control. According to Table 4, the protein content of dehydrated SLP used in this study was 12.4 % (w/w, db) so that the incorporation of SLP could have a significant impact on the crude protein contents of the formulated cookies, indicating the benefit of protein enrichment. According to Table 4, the crude protein content of T_1 was significantly (p<0.05) lower than those of T_2, T_3, and T_4 with no significant (p>0.05) difference between T_3 and T_4.

The data in Table 4 show that the carbohydrate contents of the formulated cookies varied from 40.9 to 51.1 % (w/w, db); the highest value being found with the control. The total carbohydrate content of the control cookie was significantly (p<0.05) [51.0% (w/w, db)] higher than that of T_4 [40.9% (w/w, db)] while no significant (p>0.05) differences were noticed between the control and other treatments. In addition, there was a general increase in the crude fibre content of treatments with respect to that of the control (Table 4). The crude fiber component being part of the carbohydrates, they are inclusive of both soluble and insoluble polysaccharides. According to the data given in Table 4, the crude fiber contents of dried SLP was 18.3 %. Although a significant (p<0.05) difference was noticed between the crude fiber content of the control [15.37% (w/w, db)] and that of T_4 [21.76% (w/w, db)], no significant (p>0.05) difference was seen between the control and rest of the other treatments.

Calorific Value

As shown in Table 4, there was a decline in the calorific values of different cookie formulations with the increase in amounts of SLP in the formulation in the order of T_0 > T_1 > T_2 > T_3 > T_4. While the highest calorific value of 418.9±1.7 kcal was recorded for the control, the lowest value was observed for T_4 with a mean value of 383.7 ± 5.8 kcal. The total calorific value of the cookie formulated with the highest amount of SLP (T_4) was significantly lower (p<0.05) than that of any other treatment. The observed calorific reduction in SLP-incorporated cookies could be probably due to the reduction in sugar contents. The increased SLP incorporation in the formulation might indirectly help reduce the calories via reducing the available carbohydrate contents. Other than this, the increased SLP incorporation in the formulation might have an indirect impact on the total ash contents as well. For instance, there was a significant difference (p<0.05) between the calorific value of the control and T_3 mainly due to the combined effect of the above-mentioned factors. As a significant development, a 35 % drop in calorie reduction was noticed in cookies formulated with the highest level of SLP (8.0g).

Sensorial Attributes

The results of the ranking test conducted on different sensorial attributes of the formulated cookies are shown in Table 5. According to the ranking test criteria, the lowest median is an indicator of the highest level of preference. The highest preference rank level (lowest median) for appearance was recorded for T_1 while the lowest preference rank level (highest median) was recorded for T_4. There was no significant (p>0.05) difference between T_0 (control) and T_1, but significant (p<0.05) differences were noticed among the rest of the three formulations. Next to appearance, colour of the food product plays a significant role as it creates the first impression. Nonetheless, people might differ in the degree to which they are sensitive in perceiving colours. With regard to the colour attribute, the highest preference rank level was given to T_0 while the lowest preference rank level was received by T_4. The data further showed that the preference of the panelists for colour attributes seems to decline with increasing levels of SLP incorporation. As a result, it might have had a negative effect on the scores. This could be due to the fact that the greenish appearance of the cookies was increased with the increasing proportion of SLP.

Table 5: Results of Friedman test for sensorial attributes (appearance, colour, texture, taste and overall acceptability) of different cookie formulations[#]. Different superscript letters indicate significant differences between treatments.

Treatment	Appearance	Colour	Aroma	Texture	Taste	Overall acceptability
T_0 (356)	2.0^d	1.8^d	2.2^d	1.0^e	1.0^d	1.0^e
T_1 (361)	1.8^d	2.2^d	1.8^d	2.4^d	2.4^c	2.0^d
T_2 (345)	3.2^c	3.0^c	3.0^c	3.0^c	3.0^b	3.0^c
T_3 (325)	4.0^b	4.0^b	4.0^b	3.8^b	3.8^b	4.0^b
T_4 (338)	5.0^a	5.0^a	5.0^a	4.8^a	4.8^a	5.0^a

[#]Rank median within each column sharing a different superscript are significantly (α=0.05) different at 95% confidence. Abbreviations: T_0, control cookie formulation by 40.00 g sugar per 100.00 g wheat flour; T_1, cookie formulation by totally replacing sugar with 2.00 g SLP per 100.00 g wheat flour; T_2, cookie formulation by totally replacing sugar with 2.67 g SLP per 100.00 g wheat flour; T_3, cookie formulation by totally replacing sugar with 4.00 g SLP per 100.00 g wheat flour; T_4, cookie formulation by totally replacing sugar with 8.00 g SLP per 100.00 g wheat flour

Taste is the foremost important sensorial attribute in the evaluation of any novel food formulation (Jemziya & Mahendran, 2015). Sweetness played a central role in the present study in arriving at a decision with regard to the acceptability. As the aim of the evaluation was to replace sugar with SLP, a strong influence of the sweetness attribute was clearly noticed with an increasing proportion of SLP. It should be noted that there was no linear relationship between the proportion of SLP and the taste attributes of cookies. According to the data in Table 5, the panelists' preference for the taste attribute showed significant (p<0.05) differences among T_0, T_1, and T_4. This could be probably due to the fact that much of SLP in the formulation would have caused an unpleasant after-taste. The exact cause of the after-taste arising from an excessive amount of SLP is unknown, but this needs to be further investigated. Regarding after-taste sensation, there may be individual variations as panelist may differ in their sensitivity. Although some tastes were experienced as unpleasant in the first instance, people may have learned to appreciate it with repeated presentations over a period of time. Experience gained in the bitter components of the grapefruit, coffee, bitter melon etc are just a few examples of this kind.

People might also differ widely in their sensitivity to smell as it is a characteristic that depend on the smell receptors of individuals. As shown in Table 5, the highest preference rank level (lowest median) for smell attribute was recorded for T_1 while the lowest preference rank level (highest median) was recorded for T_4. Regarding this attribute, significant (p<0.05) differences were noticed between T_0 and T_2, T_3 and T_4. A similar situation has been noticed when making comparison of T_1 with rest of the other treatments such as T_2, T_3, and T_4. With regard to this sensory attribute, no significant (p>0.05) difference was noticed between T_0 and T_1.

As far as cookie quality is concerned, texture is one of the most important sensorial attributes that is directly linked to the food's structural and mechanical properties (Lu, 2013). Texture becomes perceivable to people when they are touched, or started to bite or masticate. Individual preference is also a matter of concern as some people prefer soft texture while others might like brittle and crunchy texture (Schifferstein et al., 2020) . As shown in Table 5, the panelists' preference for textural attribute followed the ranking order of T_0> T_1=T_2> T_3> T_4. The significant (p<0.05) difference observed in texture between T_0 and the others could be mostly due to the reduction in the amount of sucrose. As a matter of fact, sucrose is an ingredient in sweet meat formulations that remarkably influence the textural properties (Pareyt et al., 2009). According to Table 5, the panelists' preference for overall acceptability of different cookie formulations was significantly (p<0.05) different from each other. The highest preference rank was noticed for T_1 while the lowest preference rank was recorded for T_4.

The sensorial evaluation of the cookie formulations is further illustrated graphically in Fig 1. As stated before, the lowest median is an indicator of the highest level of preference in the ranking test. As such the lines appearing near zero in the radar chart indicate the highest preference levels while those that move outwards from zero indicate the low levels of preference. Based on the lines representing the medians of all sensory attributes of T_4 were confined to the area near 5, indicating the lowest preference levels of all sensory attributes. Meanwhile, the medians of all sensory attributes of T_0 had moved toward zero in the radar chart compared to those of the other four formulations except for the cases of appearance and smell attributes. With regard to appearance and smell, T_1 was confined close to the area close to 1.5 in the radar chart, indicating the highest levels of preference for both of these attributes. Among the four stevia-incorporated cookie formulations, T_1 had the lowest medians for all sensory attributes, indicating the highest preference for appearance, color, taste, texture, smell and overall acceptability. Hence, T_1 formulation was identified as the most preferred stevia-incorporated formulation for cookies.

Shelf-life Study

In this study, the microbial parameters of cookie samples were tested once every two weeks during the storage period of one month. The changes in the total plate count of the samples were compared with the initial counts of the samples as shown in Table 6.

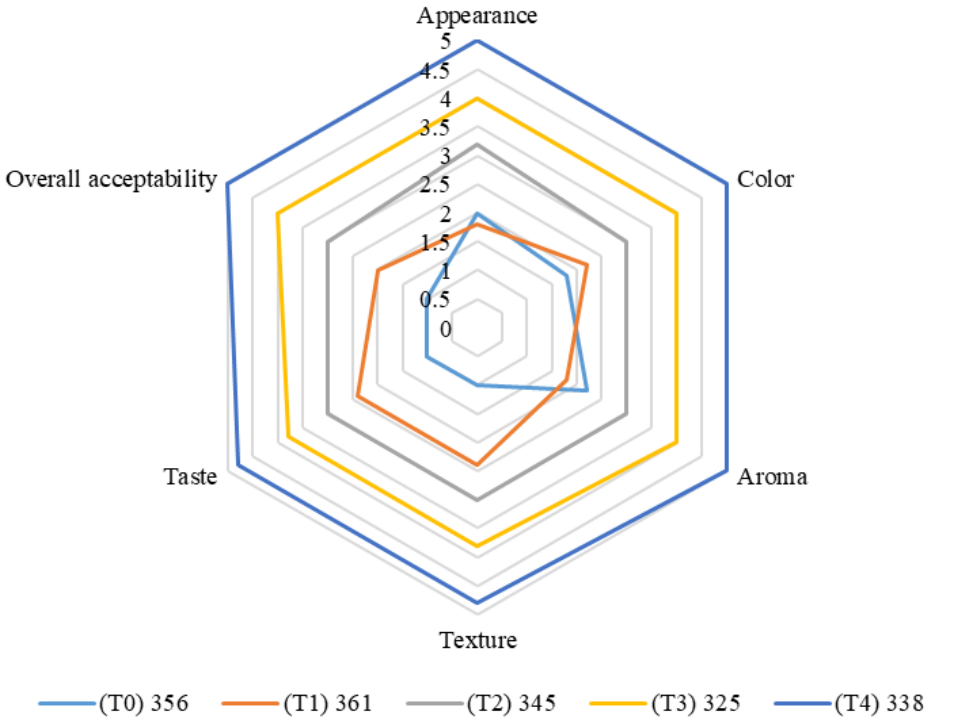

Figure 1: Radar chart of sensory attributes of different cookie formulations**.**

Abbreviations: T_0, control cookie formulation by 40.00 g sugar per 100.00 g wheat flour; T_1, cookie formulation by totally replacing sugar with 2.00 g SLP per 100.00 g wheat flour; T_2, cookie formulation by totally replacing sugar with 2.67 g SLP per 100.00 g wheat flour; T_3, cookie formulation by totally replacing sugar with 4.00 g SLP per 100.00 g wheat flour; T_4, cookie formulation by totally replacing sugar with 8.00 g SLP per 100.00 g wheat flour

Table 6: Changes in total plate count (Colony Forming Units, CFU per g) of different cookie formulations at different storage times, W0 (at the beginning), W1 (after 2 weeks of storage) and W2 (after 4 weeks of storage)[#].

Treatment	Total Plate Count (CFU/g)		
	W0	W1	W2
T_0 (356)	$(1.31^{a(B)} \pm 0.02)\times10^3$	$(1.37^{a(A,B)} \pm 0.07)\times10^3$	$(1.56^{a(A)} \pm 0.00)\times10^3$
T_1 (361)	$(1.33^{a(B)} \pm 0.02)\times10^3$	$(1.43^{a(A,B)} \pm 0.04)\times10^3$	$(1.49^{a(A)} \pm 0.02)\times10^3$
T_2 (345)	$(1.34^{a(A)} \pm 0.14)\times10^3$	$(1.44^{a(A)} \pm 0.01)\times10^3$	$(1.65^{a(A)} \pm 0.02)\times10^3$
T_3 (325)	$(1.30^{a(A)} \pm 0.02)\times10^3$	$(1.40^{a(A)} \pm 0.19)\times10^3$	$(1.59^{a(A)} \pm 0.19)\times10^3$
T_4 (338)	$(1.33^{a(C)} \pm 0.01)\times10^3$	$(1.51^{a(B)} \pm 0.04)\times10^3$	$(1.69^{a(A)} \pm 0.04)\times10^3$

[#]Each value in the table represents the mean ± SD (n=3). The means sharing a different simple superscript letter within the column and capital superscript letter within the rows are significantly (α=0.05) different at 95% confidence. Abbreviations: T_0, control cookie formulation by 40.00 g sugar per 100.00 g wheat flour; T_1, cookie formulation by totally replacing sugar with 2.00 g SLP per 100.00 g wheat flour; T_2, cookie formulation by totally replacing sugar with 2.67 g SLP per 100.00 g wheat flour; T_3, cookie formulation by totally replacing sugar with 4.00 g SLP per 100.00 g wheat flour; T_4, cookie formulation by totally replacing sugar with 8.00 g SLP per 100.00 g wheat flour.

At the initial stage of storage, mean total microbial load on nutrient agar was observed in the range of $(1.30\pm0.02)\times10^3$ CFU g^{-1} to $(1.34\pm0.14^a)\times10^3$ CFU g^{-1} (Table 6). No significant ($p>0.05$) difference was noticed among different treatments in the values recorded. After the 2nd and 4th weeks of storage, the total plate count was found to range from $(1.37\pm0.07)\times10^3$ to $(1.5\pm0.04)\times10^3$ CFU g^{-1} and $(1.49\pm0.02)\times10^3$ to $(1.69\pm0.04)\times10^3$ CFU g^{-1}, respectively. According to WHO standards (WHO, 1994),the total plate count of biscuits should not exceed 2.0×10^5 CFU g^{-1}. Although the total plate counts of all samples were found to increase with time, none of them exceeded the upper limit during the one-month storage period. During the entire period, the values of both T_2 and T_3 did not vary significantly ($p>0.05$), but the values of T_4 were different significantly ($p<0.05$). Significant ($p<0.05$) differences were noticed between T_0 and T_1 at the beginning and end of the storage period. In terms of the total plate count, there was no effect of SLP incorporation on the microbial load.

The data given in Table 7 showed that the mean yeast and mold count of the cookie samples were within the range of $(2.26\pm0.08)\times10^2$ to $(2.84\pm0.07)\times10^2$ CFU g^{-1}

at the initial stage of production. However, significant (p<0.05) differences were noticed between the control and T_1 samples with regard to yeast and mold counts (Table 7). No significant (p>0.05) differences were noticed for treatments T_2, T_3 and T_4, at the beginning or the end of the storage period. This could be partly due to the antimicrobial properties of SLP incorporation in the formulation. According to WHO standards (WHO, 1994), the yeast and mold count of biscuits should be less than 1.0×10^4 CFU g^{-1}. Even though the yeast and mold counts of all cookie samples were increased with time, none of them exceeded the above upper limit within the storage period of one-month. This could be partly due to the lower moisture content and lower water activity in cookies. In fact, water activity reduction is a limiting factor for microbial growth. This may also be partly due to the effectiveness of the packaging material such as LDPE, which served as the moisture barrier. As both of the microbial counts were less than the specified upper limits of the WHO, the microbiological quality of prepared cookies remained safe within the limit of the storage period at room temperature.

CONCLUSIONS

The effect of replacing sucrose by SLP in varying proportions in low-calorie cookie formulation has been evaluated. As extracts of SLP exhibited anti-oxidative and anti-hyperglycemic effect *in vitro*, they can be considered as functional food ingredients to incorporate in food formulations. The nutrient evaluation showed that cookie formulation with SLP incorporation would help to enhance the protein, crude fiber and total ash content of cookies. As a salient feature, a gradual decline in calorific values was noticed with the increasing replacement of sucrose with SLP. With respect to the control, both T_3 (SLP= 4.0g) and T_4 (SLP= 8.0g) formulations showed significant (p<0.05) decreases in calorific values. Among the four SLP-incorporated formulations, the highest preference of overall acceptability was recorded for T_1 (SLP= 2.0g). Shelf-life studies showed that the microbiological quality of cookies remained within the safe limit throughout the one-month storage period.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Table 7: Changes in yeast and mold counts, CFU/g, of different cookie formulations at different storage times, W0 (at the beginning), W1 (after 2 weeks of storage) and W2 (after 4 weeks of storage)[#].

Treatment	Yeast and Mold Count (CFU/g)		
	W0	W1	W2
T_0 (356)	$(2.26^{b(B)} \pm 0.08)\times10^2$	$(2.44^{a(A,B)} \pm 0.01)\times10^2$	$(2.63^{a(A)} \pm 0.05)\times10^2$
T_1 (361)	$(2.40^{b(B)} \pm 0.06)\times10^2$	$(2.43^{a(B)} \pm 0.06)\times10^2$	$(2.70^{a(A)} \pm 0.02)\times10^2$
T_2 (345)	$(2.84^{a(A)} \pm 0.07)\times10^2$	$(2.51^{a(A)} \pm 0.02)\times10^2$	$(2.72^{a(A)} \pm 0.02)\times10^2$
T_3 (325)	$(2.83^{a(A)} \pm 0.08)\times10^2$	$(2.50^{a(B)} \pm 0.02)\times10^2$	$(2.76^{a(A)} \pm 0.01)\times10^2$
T_4 (338)	$(2.74^{a(A)} \pm 0.09)\times10^2$	$(2.53^{a(A)} \pm 0.06)\times10^2$	$(2.81^{a(A)} \pm 0.03)\times10^2$

[#]Each value in the table represents the mean ± SD (n=3). The means that sharing a different simple superscript letter within the column and capital superscript letter within the rows are significantly (α=0.05) different at 95% confidence Abbreviations: T_0, control cookie formulation by 40.00 g sugar per 100.00 g wheat flour; T_1, cookie formulation by totally replacing sugar with 2.00 g SLP per 100.00 g wheat flour; T_2, cookie formulation by totally replacing sugar with 2.67 g SLP per 100.00 g wheat flour; T_3, cookie formulation by totally replacing sugar with 4.00 g SLP per 100.00 g wheat flour; T_4, cookie formulation by totally replacing sugar with 8.00 g SLP per 100.00 g wheat flour.

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