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Comparison of two non-caloric sweeteners in experimental study: Diabetic wound healing perspective

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Stevia, known for its rich phenolic content, has potential benefits in wound healing. This study aimed to evaluate the effects of two different non-caloric sweeteners (NCS), Stevia and Saccharin, on diabetic wound healing and determine the optimal dosage. A total of 36 Wistar albino rats were induced with diabetes using streptozotocin (STZ) and randomly divided into six groups: (A) diabetes control, (B) diabetes with wound control, (C) diabetes + wound + 250 mg/kg Stevia, (D) diabetes + wound + 500 mg/kg Stevia, (E) diabetes + wound + 250 mg/kg Saccharin, and (F) diabetes + wound + 500 mg/kg Saccharin. Dorsal wounds were created using a punch biopsy, and NCSs were administered via oral gavage for seven days. Biochemical analyses were performed to assess oxidative stress markers, inflammation, protein carbonyl (PC) levels, matrix metalloproteinases (MMPs), and lipid profiles in serum and wound tissues. Histological evaluations were conducted using hematoxylin-eosin and Masson′s trichrome staining. Both NCSs contributed to wound healing; however, 250 mg/kg Stevia showed better results. This group exhibited increased wound closure rates and collagen accumulation while oxidative stress, inflammation, PC, and MMP levels were significantly reduced in wound tissue. Stevia at 250 mg/kg effectively enhances diabetic wound healing by reducing oxidative stress and inflammation while promoting extracellular matrix remodeling. It was more efficient than 500 mg/kg Stevia and Saccharin at both doses, likely due to its rich phenolic composition. Stevia may serve as a potential adjunct in diabetic wound management.

Keywords: *Stevia rebaudiana*, Saccharin, Inflammation, Diabetic wound, Oxidative stress

Diabetes mellitus (DM) is one of the most serious global health problems. The World Health Organization estimates that 643 million people will have diabetes by 2030[1]. Permanent hyperglycemia in DM initiates the progression of correlated macro- and microvascular complications of the disorder[2]. These radicals target biological macromolecules, causing oxidative stress through lipid peroxidation and decreased levels of non-enzymatic (endogenous) and enzymatic antioxidants. As a result, oxidative stress plays a crucial role in the development of secondary complications of diabetes and the impairment of insulin sensitivity and pancreatic β-cell function[3].

Some lifestyle changes, including eating healthy food, avoiding obesity, and increasing physical activity have been identified as effective strategies for managing and preventing Type 2 DM. Therefore, replacing sucrose or sugars with low-energy sweeteners has gained popularity as a dietary improvement[4]. Non-caloric sweeteners (NCS) have little to no caloric value compared to natural sugars, effectively reducing energy intake[5].

The Stevia plant is a perennial herbaceous plant belonging to the Asteraceae family. In addition to its sweetening properties, Stevia has been reported to have antihyperglycemic, anticancer and antimicrobial properties and to protect blood glucose levels by increasing glucose tolerance in diabetic patients. The sweetness of this plant, which has been found to have no mutagenic, teratogenic and genotoxic effects, is due to the steviol glycosides it contains such as stevioside, rebaudioside A, B, C, D and E, glucoside A and steviolbioside[6]. Stevia leaves are 100-300 times sweeter than sugar[7]. Stevia plant, which offers various advantages in terms of functional properties and effects, is a heat-resistant and calorie-free natural herbal sweetener that can be used by diabetic patients and people with calorie restrictions[8].

Saccharin, on the other hand, is widely used non-caloric sweetener and has an essential place in the

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lives of diabetes patients[9]. Its chemical properties and biochemical activities are being investigated on suspicion of being carcinogenic[10]. Some studies have shown that Saccharin, increases incidence of bladder tumors in male rats[11]. In another study, saccharin was found to have harmful effects on liver by increasing effect on liver enzymes[12]. Saccharin is also thought to be one of the causes behind inflammatory bowel disease[13]. In addition, it has been reported that aspartame and saccharin induce apoptosis and cell death in intestinal epithelial cells, resulting in overproduction of reactive oxygen species (ROS). It is revealed that sweeteners such as sucralose, aspartame, and saccharin have detrimental effects on the intestinal epithelium[14].

For a long time, the effects of plant extracts on wound healing have been investigated with various experiments and clinical applications. However, although the Stevia plant is known to have positive impacts on wound healing, its relationship with oxidative events in diabetic wound healing is not clear. Saccharin and stevia sweeteners have been identified as components of the dietary regime of individuals with diabetes mellitus. However, there is a paucity of comparative research on the effect of these sweeteners on wound healing. When the relevant studies in the literature are examined, there is no study which compares the effects of NCS during diabetic wound healing. Accordingly, we have devised a study to explore the effects of saccharin and stevia, two different NCS, on diabetic wound healing at two separate doses for each. In addition, this is the first study to address this issue.

Materials and Methods

Animal groups and experimental procedures

Stevia was purchased from Fibrelle (Fibrelle Inc., Istanbul, Turkey). Saccharin (≥ 99 %) was purchased from Sigma-Aldrich (Interlab Inc., Istanbul, Turkey). Stevia and saccharin were diluted with distilled water in separate tubes and vortexed. Solutions were prepared fresh each morning. The solutions were administered orally to rats in the morning. The Animal Experimentation Ethics Committee of Gazi University, Ankara, Turkey, granted institutional ethical approval for this experiment. The Local Ethics Committee approved the study protocol for Animal Experiments (Ref. no. G.Ü.ET-22.008). Rats were provided by Gazi University Laboratory Animal Breeding and Experimental Research Center and were kept in the animal house at standard temperature (28±2 °C) and relative humidity (46±6 %) conditions with a 12h light-dark cycle and adequate ventilation. They were provided with food and water *ad libitum* for the entire duration of the experiment. In the experiments, 36 adult male Wistar albino rats (250-300 g) were used.

Rats were subjected to a 24h fast, after which their fasting blood glucose levels were measured using a glucometer. The experiment included subjects whose fasting blood glucose levels were within the healthy range. A single dose of 60mg/kg streptozotocin (Sigma-Aldrich, Interlab Inc., Istanbul, Turkey) was injected intraperitoneally to the rats to cause diabetes[15]. Blood glucose levels were measured 72h after injection using a glucometer. Blood sugar above 250 mg/dL was considered diabetic.

The induction of wounds was conducted in rats following a period of meticulous observation spanning ten days. Iodine solution was applied to the dorsal areas of the animals before the wound is created to prevent infection before the experiment. An injury was created with an 8 mm punch biopsy (Acu-Punch, Acuderm, USA) in the dorsal region of all animals, parallel to each other on both sides of the spine[16]. Wounds were made under general anesthesia by injecting ketamine (Alfamanine 50mg/kg) and xylazine (Alfazyne 5mg/kg) intramuscularly. Groups were formed as given below. Group A: Diabetes + no administration, no wounds. (n=6) (Control); Group B: Diabetes + no administration for 7 days after the wound is made. (n=6); Group C: Diabetes + it was administrated by oral gavage with 250 mg/kg Stevia extract for 7 days after the wound was made; Group D: Diabetes + it was administrated by oral gavage with 500 mg/kg Stevia extract for 7 days after the wound was made. (n=6); Group E: Diabetes + it was administrated by oral gavage with 250 mg/kg Saccharin for 7 days after the wound was made; Group F: Diabetes + it was administrated by oral gavage with 500 mg/kg Saccharin for 7 days after the wound was made. (n=6). 250 mg/kg and 500 mg/kg stevia extract were given by oral gavage to a group of rats to be treated with stevia extract. Saccharin was applied in the same way and in doses. After the applications were completed, blood was taken from the heart of the rats under anaesthesia and the wound tissues were removed. Blood was centrifuged at 5000

rpm for 10 min. The serum was stored in a -80°C until analysis. The wound tissues were sampled immediately. The tissues were placed in liquid nitrogen and stored in a deep freezer at -80°C until investigation.

Fasting blood glucose (FBG) levels were measured by a glucometer (Accu-Chek® Instant S) with a drop of blood taken from the tail veins of rats on the 0^{th} and 7^{th} days. The blood samples were analyzed to estimate serum markers using commercial kits. Tumor necrosis factor-α (TNF-α) (Rat TNF-α ELISA kit, Biotechnology Assay Laboratory), interleukin 1-α (IL-1α) (Rat IL-1α ELISA kit, Biotechnology Assay Laboratory), interleukin-1β (IL-1β) (Rat IL-1β ELISA kit, Biotechnology Assay Laboratory), aspartate transaminase (AST) (Aspartate Transaminase Assay Kit, Otto Scientific), alanine transaminase (ALT) (Alanine Transaminase Assay Kit, Otto Scientific), high-density lipoprotein (HDL) (High-Density Lipoprotein Assay Kit, Otto Scientific), low-density lipoprotein (LDL) (Low-Density Lipoprotein Assay Kit, Otto Scientific), cholesterol (CHOL) (Total Choresterol Assay Kit, Otto Scientific), triglyceride (TG) (Triglyceride Assay Kit, Otto Scientific), TAS (Total Antioxidant Assay Kit, Rel Assay) and total oxidant status (TOS) (Total Oxidant Assay Kit, Rel Assay) were determined using mentioned commercial kits according to the manufacturer's instructions.

Wound tissues were homogenized, and centrifuged, and the supernatants were used for biochemical analyses. The supernatants were analyzed for estimation of malondialdehyde (MDA) (Rat Malondialdehyde ELISA Kit, Biotechnology Assay Laboratory), nitric oxide (NOx) (Rat NOx1 ELISA kit, Biotechnology Assay Laboratory), protein carbonyl (PC) (Rat Protein Carbonyl ELISA kit, Biotechnology Assay Laboratory), glutathione (GSH) (Rat Glutathione ELISA Kit, Biotechnology Assay Laboratory), ascorbic acid (AA) (Rat Vitamin C ELISA kit, Biotechnology Assay Laboratory), collagen (Rat Collagen Type1 ELISA kit, Biotechnology Assay Laboratory), matrix metalloproteinase-2 (MMP-2) (Rat MMP-2 ELISA kit, Biotechnology Assay Laboratory) and matrix metalloproteinase-9 (MMP-9) (Rat MMP-9 ELISA kit, Biotechnology Assay Laboratory) by commercial kit.

The oxidative stress index (OSI), expressed as a percentage of the ratio of TOS value to TAS value was calculated.

Wound size measurement and wound closure rate calculation

Healing was photographed on the rats' first day of wound formation and on the seventh day of wound formation. The wound closure rates (WCR) were assessed and measured using the ImageJ software (NIH, USA).

WCR was calculated as follows:

WCR (%) = [(wound size day 0–wound size on day 7)/wound size day 0] × 100)[17]

Results were expressed as arithmetic mean ± standard error and ANOVA Analysis of Variance was performed.

Histological analyses of wound tissues

Hematoxylin and Eosin (HE) staining and Masson′s trichrome (MTC) staining for histopathological analyses of wound tissues were performed[18].

Statistical analysis

The statistical analysis was carried out by one-way analysis of variance (ANOVA). P values <0.05 were considered significant.

Results

Effects of stevia and saccharin administration on FBG diabetic rats

In particular, the fasting blood glucose (FBG) value of Group C after treatment showed a significant decrease of 51.8% compared to before treatment of same group (P<0.05). When Group C was compared to Group B, FBG levels in diabetic rats treated with 250 mg/kg stevia (Group C) were decreased than those in the untreated control group (Group B). Post-treatment FBG values of Group C, Group D, Group E, Group F decreased by 51.8%, 13.2%, 20.8% and 0.48%, respectively, compared to before treatment. Post-treatment FBGs of rats in Group C were found to be significantly lower than those in Group F (P<0.05), Table 1. The post-application FBG value in rats in Group D and Group F was higher than in Group A and Group B. FBG levels of the groups are given in Table 1.

Table 1 — Effects of stevia and saccharin administration on FBG levels of diabetic rats

	FBG Before Application	FBG After Application
Group A	359±125.08	278.2±150
Group B	334±127.47	378±136.11
Group C	407±110.02 [a,b]	196±114.73 [a,b]
Group D	439,12±91.42	381±163.50
Group E	408,5±58.11	323.5±62.98
Group F	446± 48.34	443.83±94.15

[Values are presented as means ± Standard error of measurement (SEM) a : When the first blood glucose and last blood glucose of the rats in their group are compared (P <0.05) b: Compared to Group F (P <0.05)]

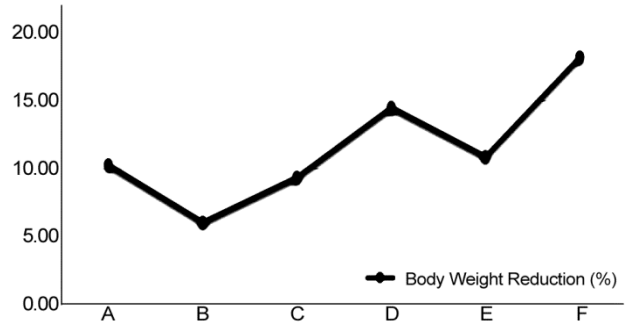

Fig. 1 — Effects of stevia or saccharin administration on body weight of diabetic rats. Group A (diabetes), Group B (diabetes+wound), Group C (diabetes+wound+250 mg/kg Stevia), Group D (diabetes+wound+500 mg/kg Stevia), Group E (diabetes+wound+250 mg/kg Saccharin), Group F (diabetes+wound+500 mg/kg Saccharin).

Effects of stevia and saccharin administration on body weight of diabetic rats

The body weight value of group D after treatment showed a significant decrease of 14.5% compared to before treatment (P<0.05). The post-treatment body weight value of Group F showed a significant decrease of 18.1% compared to the pre-treatment value (P<0.05). The post-treatment body weight values of Group A, Group B, Group C, and Group E decreased by 10.3%, 6.1%, 9.4%, and 10.9%, respectively, compared to before treatment. The percentage values of body weight reduction for the groups are shown in Fig. 1.

Effects of stevia and saccharin administration on MDA, NO_X and PC of diabetic rats

When Group A was compared with all groups, a statistically significant increase in MDA levels was observed in all groups except Group C (P<0.05). Comparing Group B and application groups, a significant difference was observed between Groups B and C (P<0.05). When Group B was compared with the application groups, a significant difference was found between Group B and Group D (P<0.05). Group C MDA levels were found to be statistically low when comparing the both of stevia administered groups (P <0.05). In comparison with the diabetic wound group (Group B), only the group that received a treatment dose of 250 mg/kg stevia (Group C) exhibited a statistically significant decrease in MDA levels (P<0.05). No significant differences were found among the saccharin consumption groups (P>0.05).

Statistically significant differences in NOx levels were observed between Group A and all other groups (P<0.05). The NOx level of Group B was higher than the other groups (P<0.05). When the application groups were compared, a statistical decrease in NOx levels was observed when Group C was compared with the other groups (P<0.05). There was a statistical increase in NOx levels in Group F compared to Group E (p<0.05) when comparing the saccharin application groups.

In the comparison between Group B and the application groups, it was found that the PC values of Group C were statistically lower (P<0.05). It was observed that the PC value of Group C decreased compared to the other application groups (P<0.05) when comparing the application groups among themselves. The MDA, NOx and PC values of the groups are shown in Table 2.

Effects of stevia and saccharin administration on GSH and ascorbic acid of diabetic rats

In the evaluation of ascorbic acid (AA) levels, a significant difference was observed between Group A and all groups (P<0.05). A statistical difference was observed between Group B and the application groups (P<0.05) when comparing Group B and the application groups. When comparing the application groups, a statistical increase in wound tissue ascorbate levels in Group C versus the other application groups could be observed (P<0.05). When Group A was compared with all groups, a statistically significant decrease in GSH levels was observed in all groups except group C (P<0.05). A significant increase in wound tissue GSH levels was observed in Group C when comparing the application groups (P<0.05). The GSH and ascorbic acid levels of the groups are shown in Table 3.

Table 2 — Effects of stevia and saccharin administration on MDA, NOx and PC of diabetic rats

	MDA (nmol/mL)	NO_x (ng/mL)	PC (ng/mL)
Group A	3.60±1.09	790.54±5.38	166.90±8.57
Group B	7.81±1.04*	1212.37±7.29*	188.45±7.16*
Group C	4.90±1.23^{a}	389.49±7.21$^{*, a}$	133.14±6.67$^{*, a}$
Group D	11.03±1.45$^{*, a, b}$	900.68±4.13$^{*, a, b}$	196.60±8.77$^{*, b}$
Group E	7.79±1.49$^{*, b, c}$	902.14±5.05$^{*, a, b}$	166.90±3.83$^{a, b, c}$
Group F	6.80±1.54$^{*, c}$	918.71±4.16$^{*, a, b, c, d}$	187.79±8.66$^{*, b, d}$

[Values are presented as means ± Standard error of measurement (SEM). When the MDA, NOx and PC levels of the rats in their group are compared (P <0.05) *: Compared to Group A (P <0.05) ^{a} : Compared to Group B (P <0.05) ^{b}: Compared to Group C (P<0.05) ^{c}: Compared to Group D (P <0.05) ^{d}: Compared to Group E (P <0.05)]

Table 3 — Effects of stevia and saccharin administration on GSH and AA of diabetic rats

	GSH (mg/L)	AA (nmol/mL)
Group A	1581.44±9.97	240.41±6.65
Group B	1186.59±6.13*	223.74±6.01*
Group C	1586.10±6.35^{a}	265.98±4.42^{*,a}
Group D	1229.90±8.90$^{*, a, b}$	133.48±8.35$^{*,a, b}$
Group E	1178.85±8.66$^{*, b, c}$	121.74±10.53$^{*,a, b}$
Group F	1337.30±8.78$^{*, a, b, c, d}$	112.88±9.12$^{*,a, b, c}$

[Values are presented as means ± Standard error of measurement (SEM) When the GSH and AA levels of the rats in their group are compared (P<0.05) *: Compared to Group A (p <0.05) ^{a}: Compared to Group B (P<0.05) ^{b}: Compared to Group C (P<0.05) ^{c}: Compared to Group D (P<0.05) ^{d}: Compared to Group E (P<0.05) *: Compared to Group A (P<0.05)]

Effects of stevia and saccharin administration on AST, ALT, HDL, LDL, TG and CHOL of diabetic rats

To investigate the effect of stevia and saccharin on liver function, serum levels of AST and ALT were measured. In wound-induced rats, liver enzymes were significantly higher than in control group (Group A). A statistical difference was found between Group B, Group C, and Group E where serum AST levels were evaluated (P<0.05). When the application groups were compared, a significant decrease in AST levels was observed in Group C. When serum ALT levels were evaluated, a statistical difference was found between Group B, Group C and Group E when compared with application groups (P<0.05). When the application groups were compared, a significant decrease in ALT levels was observed in Group C. Stevia extract at a dose of 250 mg/kg significantly improved liver enzyme activities.

When serum HDL levels were evaluated, a statistical difference was found between Group A and Group C, and an increase in HDL levels was observed compared to Group A (P<0.05). Comparing Group B and application groups, a significant difference was observed between Group B and F (P<0.05). No statistical difference was observed when serum LDL levels were evaluated (P>0.05) when all groups were compared.

A statistically significant difference was observed between the control group and all groups when serum TG levels were evaluated (P<0.05). When comparing Group B and application groups, it was found that there was a statistical decrease in serum TG levels in Group E compared to Group B (P<0.05). In evaluating serum CHOL levels, comparing Group A and all groups, serum CHOL levels were significantly increased in Group C, D, and F compared to Group A (P<0.05). When comparing Group B and application groups, there is no significant difference (P>0.05). The AST, ALT, HDL, LDL, TG, and CHOL levels of the groups are shown in Table 4.

Effects of stevia and saccharin administration on MMP-2, MMP-9 and collagen of diabetic rats

The MMP-2 levels were found to be 12.30 ± 2.8 ng/mL wound tissue for Group A, 35.16 ± 4.6 ng/mL wound tissue for Group B, 11.84 ± 3.9 ng/mL wound tissue for Group C, 11.74 ± 1.9 ng/mL wound tissue for Group D, 11.25± 1.8 ng/mL wound tissue for Group E and 13.68 ± 4.2 ng/mL wound tissue for Group F. A significant difference was detected

Table 4 — Effects of stevia and saccharin administration on AST, ALT, HDL, LDL, TG and CHOL of diabetic rats

	AST (U/L)	ALT (U/L)	HDL (mg/dl)	LDL (mg/dl)	TG (mg/dl)	CHOL (mg/dl)
Group A	96.1±6.66	50.12±4.42	20.9±4.45	4.6±1.14	17±3.53	30.8±5.11
Group B	195.21±6.78*	69.61±6.67*	24.72±4.87	5.57±2.22	36.71±4.16*	36±6.53
Group C	138.43±5.75$^{*, a}$	46.47±6.91^{a}	32.1±4.92*	7.28±1.97	39±4.43*	44.14±4.98*
Group D	186.83±7.14$^{*, b}$	89.46±5.56$^{*, a, b}$	22.82±4.49^{b}	5.5±3.07	35.25±5.89*	40.62±2.87*
Group E	140.85±6.22$^{*, a, c}$	52.51±8.45$^{a, c}$	29.41±5.45	5.5±2.07	26.33±3.88$^{*, a, b, c}$	39.33±5.85
Group F	195.1±7.98$^{*, b, c}$	142.02±6.64$^{*, a, b, c, d}$	34.64±6.20$^{*, a, c}$	6.4±2.96	39.6±5.63*	42.4±7.43*

[Values are presented as means ± Standard error of measurement (SEM).When the AST, ALT, HDL, LDL, TG and CHOL levels of the rats in their group are compared (P<0.05) *: Compared to Group A (P<0.05) ^{a}: Compared to Group B (P<0.05) ^{b}: Compared to Group C (P<0.05) ^{c}: Compared to Group D (P<0.05) ^{d}: Compared to Group E (P<0.05)]

between the Group B and all application groups (P<0.05) (Fig. 2).

The MMP-9 levels were found to be 5.22 ± 1.9 ng/mL wound tissue for Group A, 7.82 ± 1.6 ng/mL wound tissue for Group B, 5.71 ± 1.1 ng/mL wound tissue for Group C, 8.5 ± 0.9 ng/mL wound tissue for Group D, 7.75± 1.9 ng/mL wound tissue for Group E and 8.81 ± 1.0 ng/mL wound tissue for Group F. When Group A was compared with all other groups, a significant difference was recorded between Group A and Group B (P<0.05). A significant difference was found between Group A and Group D (P<0.05) , between Group A and Group F (P<0.05). There was no significant difference between Group B and the application groups (P<0.05). There was significant difference between Group C and Group F in the comparison of application groups (P<0.05).

The collagen levels were found to be 47.35 ± 3.8 ng/mL wound tissue for Group A, 55.16 ± 3.8 ng/mL wound tissue for Group B, 48.00 ± 4.0 ng/mL wound tissue for Group C, 41.61 ± 5.9 ng/mL wound tissue for Group D, 33.34± 5.1 ng/mL wound tissue for Group E and 51.75 ± 5.1 ng/mL wound tissue for Group F. When the application groups were compared among themselves, it was observed that the collagen level of Group C was significantly higher than that of Group D (P<0.05). Similarly, when the application groups were compared among themselves, it was observed that the collagen level of Group C was significantly higher than that of Group E (P<0.05) (Fig. 3).

Effects of stevia and saccharin administration on TAS, TOS and OSI of diabetic rats

A significant difference was found between Group A, Group D and Group F when compared serum TAS levels (P<0.05). When Group B was compared with the application groups, there was no significant difference (P>0.05). No significant difference (P>0.05) was observed between the application groups. Serum TOS levels were evaluated when all groups were compared; no statistical difference could be detected (P>0.05). In evaluating serum OSI levels, no statistical difference was observed between all groups (P>0.05). The TAS, TOS and OSI values of the groups are shown in Table 5.

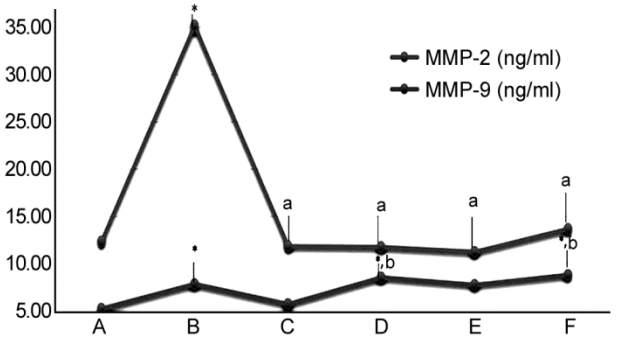

Fig. 2 — Levels of MMP-2 in wound tissue. *: Compared to Group A (P<0.05) ^{a} : Compared to Group B (P<0.05) ^{b}: Compared to Group C (P<0.05).

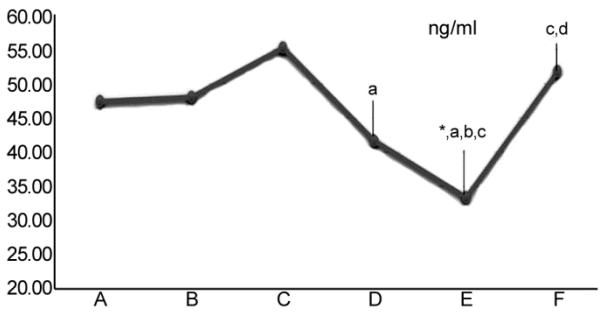

Fig. 3 — Levels of collagen in wound tissue. *: Compared to Group A (P <0.05) ^{a} : Compared to Group B (P <0.05) ^{b}: Compared to Group C (P <0.05) ^{c}: Compared to Group D (P <0.05) ^{d}: Compared to Group E (P <0.05).

Effects of stevia and saccharin administration on IL-1α, IL-1β and TNF-α of diabetic rats

The IL-1α levels were found to be 64.60 ± 2.0 ng/mL serum for Group A, 58.90 ± 1.7 ng/mL serum for Group B, 23.37 ± 4.1 ng/mL serum for Group C, 49.74 ± 3.9 ng/mL serum for Group D, 44.48 ± 3.8 ng/mL

Table 5 — Effects of stevia or saccharin administration on TAS, TOS and OSI of diabetic rats

	TAS (mmol/L)	TOS (μmol/L)	OSI
Group A	1.18±0.26	5.11±2.06	0.44±0.20
Group B	0.98±0.08	4.64±1.66	0.47±0.18
Group C	0.97±0.08	6.71±3.79	0.68±0.36
Group D	0.85±0.28*	5.13±2.91	0.66±0.37
Group E	0.94±0.16	2.57±0.96	0.27±0.09
Group F	0.81±0.15*	4.52±2.32	0.47±0.12

[Values are presented as means ± Standard error of measurement (SEM).When the TAS, TOS and OSI levels of the rats in their group are compared (P <0.05) *: Compared to Group A (P <0.05)]

Table 6 — Levels of IL-1alpha, IL-1beta and TNF-alpha in wound tissue

	IL-1α (ng/L)	IL-1β (ng/mL)	TNF-α (ng/L)
Group A	64,06±2,05	14,47±2,10	72,83±4,09
Group B	58,90±1,72	15,54±0,90	98,06±4,01*
Group C	23,37±4,10*, a	13,63±2,41	80,10±5,33a
Group D	49,74±3,95*, a, b	16,80±2,66	78,39±4,53a
Group E	44,48±3,82*, a, b	15,56±3,67	86,38±6,06*, a, b
Group F	36,87±4,53*, a, b, c, d	14,29±0,95	82,10±3,54*, a

[Values are presented as means ± Standard error of measurement (SEM).When the of IL-1alpha, IL-1beta and TNF-alpha levels of the rats in their group are compared (P<0.05)*: Compared to Group A (P <0.05) a: Compared to Group B (P<0.05) b: Compared to Group C (P<0.05) c: Compared to Group D (P<0.05) d: Compared to Group E (P<0.05)]

serum for Group E and 36.87 ± 4.5 ng/mL serum for Group F.

The IL-1β levels were found to be 14.47 ± 2.1 ng/mL serum for Group A, 15.54 ± 0.9 ng/mL serum for Group B, 13.63 ± 2.4 ng/mL serum for Group C, 16.80 ± 2.6 ng/mL serum for Group D, 15.56 ± 3.6 ng/mL serum for Group E and 14.29 ± 0.9 ng/mL serum for Group F.

The TNF-α levels were found to be 72.83 ± 4.0 ng/mL serum for Group A, 98.06 ± 4.0 ng/mL serum for Group B, 80.10 ± 5.3 ng/mL serum for Group C, 78.39 ± 4.5 ng/mL serum for Group D, 86.38 ± 6.0 ng/mL serum for Group E and 82.10 ± 3.5 ng/mL serum for Group F.

In serum IL-1α levels, a significant difference was observed between Group A and the application groups, and a decrease in IL-1α levels was observed in all application groups (P<0.05). When serum IL-1β levels were compared, there was no significant difference between groups (P>0.05) (Table 6). There is no significant difference (P>0.05) between the control group and the groups treated with stevia (250 mg/kg and 500 mg/kg) when serum TNF-α levels are examined. When Group B was compared with the application groups, it was found that there was a significant difference between all application groups (P<0.05) (Table 6).

Table 7 — Wound sizes and WCRs during the diabetic wound healing process

	Wound size (mm^2) and WCR (%)	
	Day 0	**Day 7**
Group B	30.19±2.16	16.23±1.61 (46 %)
Group C	32.88±1.07	4.14±0.34 (87 %)
Group D	30.55±2.86	7.16±0.47 (76 %)
Group E	30.17±1.66	7.00±0.40 (76 %)
Group F	32.80±1.29	8.03±0.31 (75 %)

[Values are presented as means ± Standard error of measurement (SEM). *P<0.001 compared to control group on same day]

Wound size and wound closure rate (WCR)

A statistically significant decrease in wound size was measured after treatment in Group C (P<0,001). Group C displayed a higher WCR on day 7 compared to other groups. Wound sizes and WCRs of groups are given in Table 7.

Histological evaluation

Histologic images from Hematoxylin and Eosin (H&E) and Masson′s trichrome (MTC) staining methods are provided in Fig. 4 & 5. For microscopic evaluation, tissues were examined on day 7 using H&E and MTC staining. Samples were assessed for re-epithelialization, angiogenesis, the presence of inflammatory cells, collagen fibrils, granulation tissue, and keratinization. The study findings indicate that a 250 mg/kg dose of stevia promotes re-epithelialization, collagen accumulation, cell proliferation, and new blood vessel formation. Notably, the 250 mg/kg stevia application yielded the lowest number of inflammatory cells. Table 8 summarizes all histological scores.

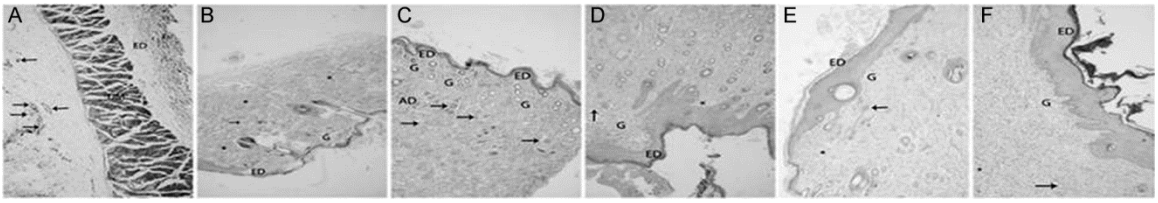

Fig. 4 — Histologic images H&E staining. [ED: Epidermis, DM: Dermis, G: Granulation, AD: Adipose tissue, C: Collagen, HF: Hair follicle, *: Inflammation, →: Blood capillaries (40×)]

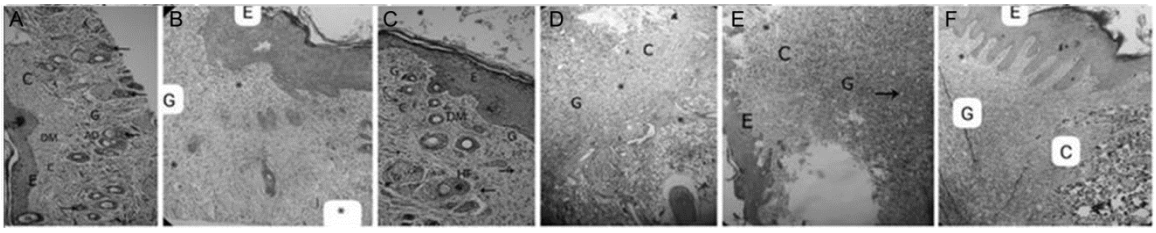

Fig. 5 — Histologic images MTC staining. [ED: Epidermis, DM: Dermis, G: Granulation, AD: Adipose tissue, C: Collagen, HF: Hair follicle, *: Inflammation, →: Blood capillaries (40×)]

Table 8 — Histological scores of tissues

	Re- epithelialization	Epidermal thickness	Granulation	Remodelling	Inflammation	Angiogenesis
Group A	++	++	++	++	-	-
Group B	+	+	+	+	++	-
Group C	++	++	+	++	-	++
Group D	+	+	+	+	+	+
Group E	+	+	+	+	+	+
Group F	+	+	+	+	+	+

Discussion

In this study, the effects of two different NCS namely, stevia and saccharin, on diabetic wound healing were compared using different doses. To our knowledge, this is the first study to compare the systemic and local effects of two non-caloric sweeteners on diabetic dermal wound healing, using both biochemical and histological parameters. Our results are noteworthy regarding dosage determination and comparison of these effects.

In diabetes, complications such as increased oxidative stress, reduced collagen and growth factor production, alterations in vascular structure, infections, and changes in cellular activity disrupt nearly every stage of the wound healing process[15]. This situation results in elevated inflammatory cytokines during the healing of diabetic wounds, leading to a prolonged inflammation phase, delayed re-epithelialization, and oxidative stress from excessive production of ROS. In diabetic cells, hyperglycemia causes increased activity in the polyol pathway, leading to higher conversion rates to fructose, better production of superoxide radicals (O_2^-), and reduced levels of nicotinamide adenine dinucleotide phosphate (NADPH). As a result, hyperglycemia can increase the risk of oxidative stress. Non-enzymatic glycation of collagen and other proteins forms advanced glycation end products (AGEs), which can worsen oxidative stress. Moreover, AGEs can reduce the solubility of the extracellular matrix and activate nuclear factor kappa B (NF-κB), a key transcription factor. All of these events can lead to delayed or impaired wound healing in diabetic patients[16].

Upon evaluation of fasting blood sugar levels, it was discovered that post-treatment FBG decreased by 51.8% solely in diabetic rats (Group C) treated with 250mg/kg stevia compared to the pre-treatment value across all groups. According to Hussein *et al*.[19], stevia extract extracted from Stevia leaf increased serum insulin levels and reduced blood glucose levels in diabetic rat groups. The secretion of insulin from the β cells of Langerhans' islets is potentiated by stevia, explaining the situation. A study by Masoumi *et al*.[20] showed that treatment using stevioside decreased glucose levels in diabetic rats compared to untreated diabetics. Stevia appears to regulate blood glucose levels. The potential mechanism behind this is that the polyphenolic compounds present in stevia regulate insulin secretion.

When body weights were evaluated, it was found that Group D and Group F had a significant decrease in post-treatment body weight of 14.5% and 18.1%, respectively, compared to their pre-treatment body weight. The stevioside in stevia extract is believed to reduce rat body weight by lowering glucose levels and increasing insulin sensitivity, thereby reducing food intake. Stevioside is believed to contribute to weight loss by reducing fat absorption and lipogenic enzymes and increasing fat excretion. Research results show a positive correlation between the dose of NCS administered to rats and body weight reduction. Similar to our study[21], found that administering stevioside to diabetic rats over a period of six weeks resulted in reduced feed intake and decreased body weight gain.

The 250 mg/kg stevia treatment group exhibited a significant decrease in MDA values, indicating lipid peroxidation, compared to all other treatment groups. Additionally, Group C among the treated groups showed the highest level of GSH, an endogenous antioxidant. These findings may be attributed to stevia's high flavonoid content, which reduces oxidative stress during diabetic wound healing. On the other hand, an increase was observed in both MDA and GSH values in Group D, treated at a dose of 500 mg/kg. It is suggested that stevia extract, which is rich in flavonoids, may have a prooxidant effect due to high doses. This highlights the importance of using a lower dosage of 250 mg/kg for effective treatment. Additionally, the most significant decrease in PC values was seen in the same dose group (Group C) when compared to all other groups. Protein carbonyl derivatives are a widely utilized oxidative stress parameter for assessing oxidative damage to proteins. They develop from the oxidation of specific amino acids or protein breakdown via the α-amidation metabolic pathway[22]. Research studies have shown that administering antioxidants to diabetic animals reduces carbonylated proteins, corroborating our findings[23]. An increase in carbonyl content was observed in kidney and liver samples of the diabetic group[24]. A previous study conducted on C57BL/6 mice reported that stevia administration did not significantly alter protein carbonyl levels compared to the control group[25].

Ascorbic acid, also known as Vitamin C, is a significant biomolecule with antioxidant and anti-inflammatory properties. It aids cellular proliferation, migration, and collagen synthesis in wound healing[26]. The AA-GSH cycle plays a crucial role in upholding cell oxidative balance[27]. Based on the study results, AA levels were lower in the untreated diabetic group (Group B), but a significant increase was observed in the 250 mg/kg stevia treatment group (Group C). This increase correlates with GSH levels. Parallel to our results, various studies have demonstrated that antioxidant parameters in wound tissue decrease during diabetic wound healing and that treatment with antioxidants can improve these parameters[28,29]. However, both low and high-dose saccharin treatments had some impact on blood glucose levels and oxidative events in the wound tissue. Wound tissue MDA and PC levels slightly decreased in both saccharin dosage groups compared to the untreated control group. On the other hand, there was no change in GSH levels of scar tissue in both saccharin treated groups compared to the control group. Additionally, AA levels decreased in both saccharin treatment groups.

There was a reduction in FBG solely with the lower application dose as compared to the untreated group. In addition, it was found that this decrease was less effective than a 250 mg/kg stevia application. Nonetheless, the effects brought about by saccharin to these occurrences are less potent than the 250 mg/kg stevia group. The 500 mg/kg application of stevia did not elicit any favourable changes to the blood glucose level or oxidative events in the wound tissue.

Pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α), play a crucial role in regulating wound healing and the initial response of the inflammatory phase. These cytokines facilitate the

migration of neutrophils, induce a respiratory burst at the wound site, and stimulate the synthesis of metalloproteinases (MMPs) that break down and remove damaged extracellular matrix[30]. During the diabetic wound healing process, the prolonged inflammatory response damages the tissue due to the excessive production of pro-inflammatory cytokines in the wound area. This can result in delayed wound healing and the development of chronic wounds[16]. While interleukin-1β (IL-1β), an inflammatory marker, exhibited no significant change in any group, there was a substantial decrease in IL-1α and TNF-α levels in Group C.

The outcome suggests that applying the 250 mg/kg stevia extract may possess anti-inflammatory effects due to the extract's rich phenolic and flavonoid compounds. It was also determined that levels of IL-1α and TNF-α were significantly reduced in the groups treated with saccharin. Various studies have reported the NCS anti-inflammatory effects operating through the NF-κB pathway. Naşar *et al*.[31] reported that treatment with stevia leaf extract inhibited NF-κB in the CA3 hippocampal region of the brain. During the animal study, oral stevia extract administration (200 mg/kg/day, once daily) for a duration of 4 week significantly improved oxidative stress markers (catalase, MDA, and total antioxidant capacity) and downregulated IL-6, p53 and caspase-3. The difference in inflammatory cytokine (IL-6) expression level tied with the suppression of the NF-kB signaling pathway. In a recent study, researchers found that applying saccharin effectively reduced the inflammatory response in adiposity cells induced by lipopolysaccharide (LPS), by inhibiting the NF-kB signaling pathway[32]. In a similar study, Kasti *et al*.[33] showed that stevioside treatment reduced the expression of TNF-α, IL-1β and IL-6. The findings from these studies align with those presented and indicate that both sweeteners significantly decrease cytokine expression levels through the potential downregulation of NF-κB activation.

It is widely recognized that NO, a gaseous radical species, performs a diverse range of functions such as tissue repair, secretion of cytokines post-injury, fibroblast migration and differentiation, angiogenesis, collagen accumulation for tissue reconstruction, and re-epithelialization in damaged tissues[34]. Nitric oxide (NO) is produced through the action of nitric oxide synthase enzymes (endothelial nitric oxide synthase, iNOS and neuronal nitric oxide synthase) from the amino acid L-arginine. Nitric oxide has a crucial role in wound healing by regulating vascular homeostasis, inflammation, and exhibiting antimicrobial actions. Abnormal nitric oxide production in diabetes is linked to delayed wound healing and the formation of chronic wounds[16]. According to our study, induction of diabetes (Group B) dramatically increased the NO_x level in the wound tissue. However, both low and high-dose applications of non-caloric sweeteners caused a significant decrease in NO_x levels. However, administering low and high doses of stevia and saccharin caused a substantial reduction in NO_x levels. Notably, the 250 mg/kg stevia application group (Group C) showed the most significant decrease. This decrease is attributed to the suppression of iNOS expression in neutrophils and macrophages due to decreased TNF-α levels. Afzali *et al*.[35] have been reported that TNF-α is a potent inducer of iNOS expression in neutrophils and macrophages. They showed increased iNOS levels on day 14 post-injury were attributed to increase TNF-α. With the increase in iNOS levels, they also reported an increase in NO_x levels.

Consistent with our results, studies have shown that NCS impacts iNOS activation and NO production by inhibiting the NF-kB signaling pathway[32]. According to Kim *et al*.[32], in adipocytes with LPS-induced inflammation, Saccharin inhibits the NF-κB pathway, leading to suppression of iNOS mRNA and NO production. Stevia administration has been found to inhibit the release of nitric oxide in macrophages stimulated by LPS/gamma interferon (IFNγ), according to Latarissa *et al*.[36]. Kim *et al*.[32] demonstrated a reduction in iNOS and NO production in the RAW264.7 cell line induced by LPS upon treatment with Stevia leaf extract. Our and other studies support the hypothesis that steviosides in stevia can potentially impede the NF-κB pathway, leading to the suppression of iNOS messenger RNA and NO production.

Collagen constitutes the major component of the extracellular matrix, and the steps involved in its remodeling, and maturation are crucial during tissue repair[37]. Flavonoids have been demonstrated to prevent cellular damage by enhancing vascularity, and promoting collagen synthesis[38]. In this study, the administration of 250 mg/kg of stevia (Group C) significantly increased the amount of collagen in diabetic wound tissue, contributing to wound healing. Our histological results confirmed that stevia

effectively promoted collagen fiber growth in diabetic wounds after 7 days of 250 mg/kg stevia application.

MMPs are endopeptidases that remodel tissue through selective proteolytic degradation processes[39]. Various cytokines and growth factors transcriptionally activate MMPs through different pathways during the wound-healing process[40]. Unregulated MMP activity can result in damage to tissues and alterations in their functionality. Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of MMPs, modulating their proteolytic and other functions and regulating ECM re-epithelialization and remodelling. These results demonstrate that maintaining the physiological equilibrium of MMP/TIMP ratio is necessary to achieve the usual wound healing process[41]. Our study revealed a marked rise in MMP-2 and MMP-9 levels in diabetic wounds. Dai *et al*.[42] had previously reported impairment of diabetic wound healing due to excessive MMP production. Applying 250 mg/kg of stevia treatment to diabetic animals led to reduced MMP levels in wound tissue. A decline in MMP-2 and MMP-9 levels may have facilitated increased re-epithelialization, consistent with our histological examinations.

Previous studies have shown that non-caloric sweeteners have either had a slight or no impact on oxidative parameters in the serum of diabetic patients[43]. There is a study showing that long-term consumption of aspartame causes reproductive damage by causing oxidative stress in male mice[44]. Aspartame has been reported to induce ROS production[14]. Similarly, our study found no effect on the serum TAS and serum TOS levels.

Considering that the liver plays a critical role in blood sugar homeostasis, it is a crucial organ in the progression of diabetes. Increased oxidative stress during diabetes can affect liver function[45]. In this study, the levels of ALT and AST enzymes that increased with diabetes induction decreased significantly, specifically with low doses of non-caloric sweeteners. The most considerable decrease in both liver enzymes was observed in Group C, where 250 mg/kg of stevia was administered. However, saccharin did not have as much effect on the AST levels as the same dose of stevia.

Moreover, ALT values measured in saccharin-treated groups were found to be higher than those of the other groups. Several studies support our findings. Sameh *et al*.[46] reported that stevia extracts reduced AST and ALT values in rats, while aspartame increased these values. The hepato-protective activity demonstrated by stevia can be attributed to the presence of flavonoids, which are rich in antioxidants that reduce oxidative.

Dyslipidemia and altered metabolism of triglyceride-rich lipoproteins are frequently observed in patients with T2DM[47]. Previous research has demonstrated decreased triglyceride and total cholesterol levels in rats following oral administration of saccharin[48]. Furthermore, it has been established that prolonged intake of NCS may hasten atherosclerosis and aging by disrupting the function and structure of apoA-I and HDL[49]. The Abdel-Aal *et al*.[50] study found that daily use of stevia aqueous extract substantially decreased total cholesterol, triglycerides, and LDL and elevated serum HDL levels. Nonetheless, in this study, there was no substantial alteration in blood lipid profiles from using either stevia or saccharin doses. This is potentially due to the short-term usage of both sweeteners.

Based on the collagen levels in the wound, wound contraction rates, and histological examinations, we hypothesized that administering 250 mg/kg of stevia could indirectly affect fibroblast contraction. Wound healing is a highly organized physiological process to restore skin integrity. Some fibroblasts differentiate into myofibroblasts that express alpha-smooth muscle actin (α-SMA), thus exhibiting contractile properties that contribute to wound contractions and enhance wound healing[51]. α-SMA plays important roles in many cellular processes, including cell division, cell motility, and generation of contractile force[52]. Transforming growth factor beta-1 (TGF-β1) is a significant regulator of fibroblast differentiation into myofibroblasts, which express α-SMA and ultimately cause wound contraction[53].

Additionally, TGF-β1 promotes collagen deposition and epithelialization during wound healing[54]. Smads are cytoplasmic signal transduction molecules that directly transfer the TGF-β1 signal from the cell membrane to the nucleus[55]. Thus, the TGF-β1/Smad pathway plays a crucial role in wound contraction. Previous research indicates that keratinocytes secrete TGF-β1[56]. Therefore, we propose that stevia may affect TGF-β1 expression, potentially impacting fibroblasts directly or indirectly.

Conclusions

In this study, it was aimed to test the usability of Stevia in diabetic wounds, to demonstrate that it

supports healing with its antioxidative effect in wound healing processes with the specified dose and to reveal its effectiveness in proliferative formations with biochemical data, and to examine the healing effect of stevia extract. Our research indicates that 250 mg/kg of stevia extract can improve wound healing in diabetic rats by regulating blood glucose levels, reducing oxidative stress and inflammation, and promoting epithelialization and collagen deposition in wound tissues. It is believed that this enhanced healing is due to the high flavonoid content of the extract. In addition, this study aimed to investigate whether there was a difference between the natural sweetener stevia and the artificial sweetener saccharin, widely used by diabetic patients, in terms of both oxidative events and wound healing responses. However, in the experiment where rats were administered two different dosages of saccharin along with 500 mg/kg of stevia extract, the same result was not observed as in rats that were given the recommended amount of Stevia extract (250 mg/kg). This study, performed for the first time in wound healing research, compared the effects of non-caloric sweeteners. The optimal dose of stevia extract was found to have no adverse effects, and it is therefore recommended for use in treating diabetic wounds.

Authors contributions

SCC: Conceptualization; data curation; formal analysis; investigation; methodology; review and editing. EGGP: Formal analysis; investigation; methodology; writing; review and editing. ENA: Investigation; methodology and writing NK: Investigation; methodology and writing. KBB: Formal analysis; investigation; methodology; review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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