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Original Research Article

# Suitability of the Neonatal Streptozotocin Diabetes Model and Folklore Therapeutic use of Low-Dose Neem Leaf Extract to Treat Hyperglycemia Associated with Type 2 Diabetes Mellitus

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ABSTRACT

Neem leaf has been used in folklore medicine to combat diabetes mellitus and other ailments. Difficulty finding suitable type 2 diabetes mellitus (T2DM) models led to the exploration of the neonatal streptozotocin (nSTZ) model to investigate the impact of low-dose neem leaf extract (NLE) on T2DM-associated hyperglycemia. Following ethical approval, two- and three-day-old neonatal rat pups (n=66) received 60 mg/kg STZ intraperitoneally while normal controls (n=9) received an equivalent volume of citrate buffer. Weaned animals received free access to chow and water and a constant 12 hour on/12 hour off light cycle. Tail vein blood glucose was assessed weekly following an eight-hour fast, using Accu Chek Advantage glucometer. Animals received tap water (Normal and Diabetic Control) or 0.8 % NLE (Diabetic Treated) for six weeks. The results were expressed as means ± standard error of the mean using the Origin Pro 7.0 software. The significance of the mean difference between groups was determined with the IBM SPSS for Windows software version 23 via the paired samples T-test. Statistical significance of $p<0.05$ was used. The mortality was 74% pre-STZ (32.6% post-STZ) with 40.9% or 81.8% success (of STZ-injected and STZ-survivors), respectively. Fasting blood glucose was significantly ($p<0.05$) elevated in the diabetic groups and was not alleviated by low-dose NLE. Because of the variability in mortality and success rates, further investigation is warranted for better reproducibility of the nSTZ T2DM model with minimal mortality. Furthermore, low-dose therapeutic NLE does not positively impact hyperglycemia and should not replace clinically prescribed medicines.

***Keywords*:** Neem leaf extract, neonatal rat model, streptozotocin, hyperglycemia, type 2 diabetes mellitus

## Introduction

Diabetes mellitus (DM) is classified as a group of metabolic disorders characterized by hyperglycemia, polydipsia, polyphagia, polyurea, and glycosuria, along with pruritis and slow wound healing.[1-3] It is classified into two main types (types 1 and 2). Type 1, previously called insulin-dependent or juvenile diabetes, typically arises from an autoimmune destruction of pancreatic beta cells or islets of Langerhans, resulting in a diminution of insulin secretion and an impairment in blood glucose regulation.[3] Type 2 DM (T2DM) typically arises from insensitivity of the tissues to insulin. Regardless of these types, glucose accumulation ensues and a plethora of negative effects such as cardiovascular, brain, and renal complications, and neuropathy (tingling, ocular problems) are consequential, primarily if the condition is chronic or untreated.[1] Non-fasting glucose readings greater than 11.0 mmol/L (200 mg/dL) or fasting glucose equivalent to greater than 7.0 mmol/L (126 mg/dL) on at least two occasions confirms the presence of diabetes.[1,3-4] Various doses of STZ can be used to induce T2DM in neonatal rat pups and this model allegedly closely simulates T2DM characteristics.[3,4]

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Neem (*Azadirachta indica* A Juss [*Melia azadirachta* Linn]) leaves have been traditionally boiled to make teas (neem leaf tea) or placed in water overnight (neem leaf water) for early morning consumption to maintain good health or alleviate the effects of ailments such as diabetes mellitus, inflammation, and malarial infections.[5-7] Neem leaves have been used as a folklore remedy to treat diabetes in Jamaica; however, conflicting reports point to a lack of antidiabetic properties. It seems likely that this is a dose-dependent matter, given that most researchers report antidiabetic properties; however, a low concentration of 0.8% reportedly did not alleviate diabetes.[5-8] This current study was, therefore, designed to assess the suitability of the T2DM models and the validity of the use of a low-dose extract of neem leaves and associated stems (NLE) as an antidiabetic agent and to determine whether it can alleviate hyperglycemia associated with T2DM.

## Materials and Method

### *Ethical Considerations*

Ethical approval was granted by the University Hospital of the West Indies/University of the West Indies/Faculty of Medical Sciences (UHWI/UWI/FMS) Ethics Committee.

### *Plant Collection and Identification*

The Ministry of Agriculture of Jamaica granted permission to harvest and use the neem plant and a sample was authenticated by the resident botanist and herbarium curator at The UWI, Mona herbarium, Mr. Patrick Lewis. The specimen was assigned voucher number 35688 and deposited in the herbarium. As previously described by McCalla *et al*.,[8] fresh, healthy neem leaves with their stems were collected from The UWI Mona Campus and Botanical Garden (coordinates: 18.0059° N,

76.7468° W) between July and August 2004 and dried for 3−5 days in a solar drier (maximum temperature 40°C).

*Plant Extraction*
Plant extraction was previously reported by McCalla *et al*.[8] Briefly, the powder from milled, dried leaves and their associated stems was weighed and boiled in distilled water (100 g/L portions) under reflux for four hours. A strainer and muslin cloth were used to filter the cooled mixture. The filtrate was measured and made up to 1 L, yielding a neem stock solution of 0.011–0.048 g/mL, then a 0.8% stock solution (0.008 g/mL)[9] was made by diluting the stock with distilled water, for oral feeding. Portions of the filtrate that were not used were freeze-dried and refrigerated at 4 ˚C for future use. The diabetic treated rats received 0.8 % freshly made NLE *ad libitum* for six weeks for assessment of the effect of the NLE on their blood glucose levels.

*Induction and Assessment of Diabetes*
The Bonner-Weir *et al*.[10] method was modified for this study and previously reported by McCalla *et al*.[8] Briefly, two- and three-day-old neonatal rat pups (n=66) were injected intraperitoneally with 60 mg/kg STZ (Sigma, France) in 0.1 M sodium citrate buffer, pH 4.5 as previously described.[8] Normal Control (NC) pups (n=9) received an equivalent volume of citrate buffer. The neonates were subsequently reunited with their mothers and weaned at four weeks. Animals were allowed free access to chow and water and kept at a constant light cycle of 12 hours on/12 hours off.
After an overnight fasting for eight hours, the animals were injected with STZ, and their tails were pricked to evaluate their blood glucose concentration using an Accu Chek Advantage glucometer (Roche Diagnostics, Germany). The glucose concentrations were subsequently evaluated at weekly intervals from week 2. The 90-minute oral glucose tolerance test was used to evaluate the type of diabetes after conducting an eight-hour fast: hyperglycemic rats received 15 mg/kg glibenclamide in 1 mL dimethylsulphoxide [DMSO] orally via a syringe with a 20-gauge ballpoint gavage needle.[8,11-13] The first glucose value was taken immediately after glibenclamide administration, then at half-hour intervals for 90-minutes. The diabetic rats had fasting blood glucose (FBG) > 6.9 mmol/L (or 10.9 mmol/ L non-fasting) and were classified as type 2 DM rats if the FBG level returned towards the initial (time zero) glucose level after peaking but remained in the diabetic range.[13] Diabetic rats were randomized into two groups: Diabetic Control (DC, n=11) and Diabetic Treated (DT, n=8). The DT group received NLE while the DC and NC (n=9) rats received tap water *ad libitum* for six weeks. Rats were sacrificed via inhalation of diethyl ether at the end of the study period.

*Evaluation of fasting blood glucose, weights, and chow and fluid consumption*
*Glucose Evaluation*
The FBG was assessed weekly.

*Weight Evaluation*
Weights were evaluated weekly using an Ohaus dial-o-gram triple-beam scale with a maximum capacity of 2610 g. Neonates (two and three days old) were weighed using an Acculab balance (L-Series LA-60, max 60 g, Germany), with a readability of 0.0001 g. Weight gain and weight gain percentages were calculated.

*Chow and fluid consumption evaluation*
Chow and water were measured weekly. A measuring cylinder was used to measure the volume of liquid remaining. An Ohaus dial-o-gram triple-beam scale with a maximum capacity of 2610 g was used for the chow. The volume of water and NLE and mass of chow consumed were calculated as the difference between the total given and the quantity remaining.

*Patient and public involvement*
There was no patient or public involvement.

*Data analysis*
The Origin Pro 7.0 software (Northampton, MA) was used to generate the statistical data and graphs used in this study. Data were expressed as means ± standard error of the mean (SEM). The IBM SPSS for Windows software version 23 (New York, USA) was used to determine the significance of the mean difference between the groups via One-Way ANOVA, Levene's test for variance, and Bonferroni or Games-Howell post-hoc test. Statistical significance was taken as $p<0.05$.

## Results and Discussion

*Description of neonates*
Neonates were previously described by McCalla[3] as follows: The appearance of the neonates on the day of birth revealed the presence of a partially intact umbilical cord or a black mark at the attachment site. These marks were no longer visible by days 6 to 9. Eye closure prevailed for 11-14 days and the ears folded downward to cover the ear orifice. Unfolding of the ears occurred between days 1 and 5, and the orifices became clearly defined on days 10-11 of birth.
The bodies of the pups were hairless at birth, with a red to reddish-pink hue. The color of their body began changing to pink by day 2 and a white hue made its emergence at 5 days old, symbolizing hair growth. Paws were not separated at birth; however, they began to separate by 5 days old with completion by day 10. At birth, rat pup gender could not be differentiated but gender became evident as early as day 9 and nipples were seen in female pups. Pups had whiskers and tails that were 1 inch long in approximation. Injection day (2-3 days old) weights were 6-13 g.

*Success of streptozotocin-diabetes induction*
Hyperglycemia (> 6.9 mmol/L, fasting) was evident in 81.8 % (27/33) of the surviving STZ-injected pups between 6 and 14 weeks of age and largely occurred at 8-10 weeks of age, as previously indicated.[8] This also represents 40.9 % of the total number of pups injected with STZ (Table 1). Fluctuation between hyperglycemic and normoglycemic glucose concentrations in STZ-injected rats was also seen between the neonatal and adult stages of diabetes development (Table 2). Stable diabetes remained for the duration of the study.
The results show that most of the animals became diabetic between eight and ten weeks old. A similar time-to-diabetes development is reported for several STZ models combined with other substances including nicotinamide and high fat[14-15] but most researchers do not indicate the time taken for diabetes development. Contrasting reports show the development of diabetes in 4 weeks in the STZ/fructose model versus 14 weeks in the STZ/sucrose model.[2,16]

**Table 3:** Mortality rate of citrate buffer- and streptozotocin-injected neonatal (nSTZ) rat pups

| Parameter | Control | nSTZ |
|---|---|---|
| Number of animals (n) | 37 | 129 |
| Total mortality | 0 | 42 |
| MORTALITY (%) | 0.0 | 32.6 |

nSTZ, Streptozotocin

**Table 1:** Success rate of the neonatal streptozotocin-induced diabetes model

| Parameter | Total | % |
|---|---|---|
| Number of animals (n) | 66 | 100 |
| Survival Rate | 33 | 50 |
| Diabetes Success (of STZ survivors) | 27 | 81.8 |
| Diabetic (of total rats injected) | 27 | 40.9 |

nSTZ, Streptozotocin

Although the success rate for induction of diabetes in the surviving rat pups in the nSTZ model is very high, there are concerns regarding the wide variability in the time taken for the animals to develop a stable form of diabetes (6-14 weeks). This also affects the starting weight of the animals for the experiments. Older rats consume less food and water than younger ones that are rapidly developing. Other parameters may also be affected. The success rate of the different diabetes models remains largely unreported, and we believe that each model could be improved or benefit from better reporting of this information.

*Mortality*

Mortality occurred in forty-six percent (46 %; n=176) of the neonates before STZ injection and in 32.6 % (range 0 to 100 %; n=66) of those receiving STZ injection, with death occurring within ten days of injection and within one day post-STZ injection in one-third of the STZ-injected rats (Figure 1 and Table 3). No mortality occurred in the control neonates (n=37) injected with citrate buffer.

A lack of mortality in the control group implies that the mortality rate (32.6 %) in the nSTZ-injected group can be partially attributed to the administration of STZ. Since almost half (46 %) of the neonates died before receiving STZ, we must also conclude that other factors are responsible for the mortality rate of the STZ-injected rats. The (unknown) pre-injection cause of death is a probable confounding factor in post-STZ mortality. Complications of diabetes or glucose dysregulation as well as possible inadequate care by the mother may also be contributory factors. Portha *et al*.[17] reported a mortality rate (30-50 %) which is like ours (32.6 %) and Li *et al*.[18] found no significant difference between the mortality in the nSTZ and normal rats but did not show the data. Barragán-Bonilla *et al*.[2] reported high mortality rates with a higher STZ dose (90 mg/kg b.w. STZ; 90.5 in male and 94.7 % in female rats) and lamented this high mortality rate which was seemingly dose-dependent. Additionally, the appearance of T2DM was accelerated with the combination of STZ and sucrose. Using 90 mg/kg b.w. STZ proved to be more effective than 70 mg/kg b.w. in inducing most of the characteristics of diabetes, but the lower dose was more successful (84.6 and 86.5 %) and produced a lower mortality rate (15.4 and 13.5 %). The mortality rate is not reported by most authors and that remains a limiting factor. The mortality occurring after injection could be due to factors including, but not limited to the use of STZ, injection technique, pup handling, as well as underlying pathological conditions in the mother or neonates before delivery.

The high mortality reported in other studies as well as ours questions the worthwhileness of continued utilization of the nSTZ model in its current form. This may be why some researchers explore a combination of STZ with other substances including nicotinamide, high fat, fructose, or sucrose, or used lower doses of STZ.[2,10,14-16] Variability in the time taken to achieve stable T2DM means that the researcher should exercise discretion in determining the most suitable model for their research. A review of multiple T2DM models by Fang *et al*.[19] (including how each stage is clinically manifested) and Arulmozhi *et al*.[20] (neonatal T2DM models) led them to conclude that the HFD/STZ and the nSTZ models are appropriate for T2DM simulation because the former mainly mimics the later stage of DM and the latter resembles the secretory characteristics of T2DM.

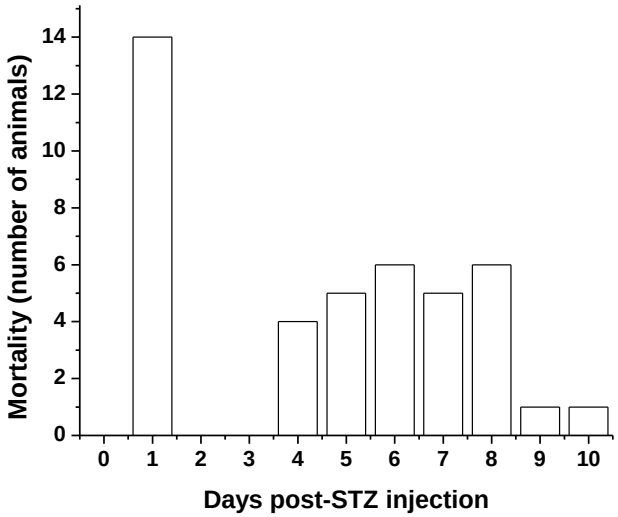


**Figure 1:** Mortality of streptozotocin-injected rats

**Table 1:** Fasting blood glucose in citrate buffer control and STZ-injected rats

| | Weeks post-buffer or STZ injection | | | |
|---|---|---|---|---|
| **Group** | **2** | **3** | **5** | **7** |
| Control (n=9) | 4.3 ± 0.2 | 5.9 ± 0.2 | 3.8 ± 0.1 | 4.4 ± 0.1 |
| STZ-injected (n=13) | 3.4 ± 0.2 * | 7.9 ± 0.3 ** | 6.0 ± 1.1 | 5.4 ± 0.4 |

Streptozotocin (STZ) significantly ($p<0.01$) altered glucose concentration during the first two weeks post-injection. * $p<0.01$; ** $p<0.001$

**Table 4:** Effect of NLE on fasting blood glucose

| **Time** | **Mean Fasting Blood Glucose ± SEM (mmol/L)** | | |
|---|---|---|---|
| **(Weeks Post-Treatment)** | **NC (n=9)** | **DC (n=6-10)** | **DT (n=5-8)** |
| -1 | 4.6 ± 0.1 | 4.4 ± 0.3 | 4.2 ± 0.3 |
| 0 | 4.5 ± 0.1 | 11.5 ± 1.5 * | 15.4 ± 1.6 * |
| 1 | 4.2 ± 0.1 | 8.7 ± 1.7 | 15.1 ± 1.6 *+ |
| 2 | 4.4 ± 0.1 | 8.4 ± 1.4 | 12.2 ± 1.7 ** |
| 3 | 4.5 ± 0.1 | 10.8 ± 1.3 * | 12.8 ± 1.1 * |
| 4 | 4.5 ± 0.1 | 10.2 ± 1.8 * | 14.0 ± 2.0 * |
| 5 | 4.4 ± 0.2 | 14.7 ± 3.1 ** | 14.4 ± 2.2 * |
| 6 | 4.3 ± 0.2 | 7.9 ± 0.8 ** | 14.8 ± 2.8 * |

Diabetes significantly ($p<0.05$) elevated fasting blood glucose which was not ameliorated by NLE. NLE, Neem leaf extract; * $p<0.01$ vs NC; ** $p<0.05$ vs NC; + $p<0.05$ vs DC

Given these outcomes, there is a need for standardization for inter-study comparison. Whether the nSTZ model is worthwhile or not, rests with the researcher, seeing that each model has its pros and cons; therefore, careful consideration of the study objectives, desired diabetes characteristics, and the time limit of the study should be done before choosing the most appropriate model for the study.

*Effect of STZ Induction on FBG concentration*

The use of STZ successfully induced diabetes, with FBG being significantly ($p<0.05$) elevated versus normoglycemic controls (Table 4). There was no significant difference among the pre-diabetic FBG, and DC glucose was also not significantly different for the first two weeks versus normal controls. As previously reported, the mean FBG concentration was 16.3 ± 1.4 mmol/L (n=11) in the STZ-diabetic group when diabetes developed in the adults and this was significantly higher ($p<0.001$) than in the normal control, NC, group (4.6 ± 0.1 mmol/L, n=9).[3] In grouping these diabetic animals, mean FBG values were significantly ($p<0.01$) higher than NCs at 11.5 ± 1.5 (DC) and 15.4 ± 1.6 mmol/L (DT) vs 4.5 ± 0.1 mmol/L (NC). At the end of the assessment period, FBG levels were 7.9 ± 0.8 (DC) and 14.8 ± 2.8 (DT) vs 4.3 ± 0.2 mmol/L (NC).

The low dose of NLE used in this study (0.8% containing 0.008 g/mL or 8 mg/kg b.w.) did not alleviate hyperglycemia associated with T2DM. This dose which is close to that used in neem leaf teas and water, was too low to achieve the success reported for higher doses. Despite this lack of alleviation of hyperglycemia, our previous report pointed to beta cell regenerating potential over a longer assessment period, along with a three-fold increase ($p<0.05$) in the serum insulin of the DC group.[9] This shows promising scope for the use of the extract. Higher doses, such as 2 or 5% and 100 to 800 mg/kg b.w. used by researchers, have achieved the desired hypoglycemic effects as well as a positive impact on liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in serum without causing toxicity,[21] as well as positive effects on the metabolic syndrome and the antioxidant system,[22] without affecting food intake or body weight.[23] Similar doses in humans (125, 250, and 500 mg) twice daily have also successfully significantly reduced postprandial blood sugar, oxidative stress, and systemic inflammation and improved endothelial function versus placebo in a randomised, double-blind, placebo-controlled clinical study.[24] While El-Hawary and Kholief[25] observed reduced blood glucose in diabetic rats, the diabetic state was not alleviated. Hypoglycemic effects in normal rabbits have also been reported, using 500 mg/kg b.w. leaf extract and 5 ml/kg b.w. seed oil [26,27] and using 0.01 - 1.4 mg in human blood cells in a normoglycemic medium.[28]

*Effect of T2DM and NLE on mean weekly body weights and fluid and chow consumption*

Diabetic control rat weights were significantly ($p<0.05$) negatively affected by diabetes. Weights in the treated group were significantly ($p<0.05$) higher before the genesis of diabetes and NLE treatment did not affect weights thereafter (Table 5). Diabetes significantly ($p<0.05$) hampered weight gain in the DC group, while NLE seems to have offered minimal protection against diminishing weight gain. Although lower than normal controls, there was no significant difference in weight gain; never-the-less, the weight gain percent in both diabetic groups was significantly ($p<0.05$) reduced (Figure 2 and 3). Diabetes resulted in a significant ($p<0.001$) increase in mean fluid (at least two-fold) and chow consumption during the treatment period and this was further increased by NLE treatment (Figure 4 and 5).

The low dose of NLE used in this study (0.8% containing 0.008 g/mL or 8 mg/kg b.w.) did not improve weight gain or fluid and chow consumption in the diabetic condition for the short duration of the study. We, however, previously reported improved weight gain over a longer period of assessment.[9] This shows promising scope for the use of the extract in improving weight gain. Higher doses (2 or 5% and 100 to 800 mg/kg b.w.) reportedly caused hypoglycemia and positive effects on liver enzymes in the serum without affecting food intake or body weight or causing toxicity.[21,23]

**Table 5:** Mean weekly weights (g) from birth

| Age (Weeks) | Mean Weekly Weight ± SEM (g) | | |
|---|---|---|---|
| | NC (n=9) | DC (n=5-11) | DT (n=7-8) |
| 0 | 7.1 ± 0.8 | 8.6 ± 0.3 | 10.0 ± 0.2 *# |
| 1 | 15.4 ± 1.6 | 13.4 ± 1.1 x | 17.6 ± 1.1 ** |
| 2 | 21.4 ± 2.2 | 25.7 ± 2.0 | 27.7 ± 1.9 ## |
| 3 | 35.3 ± 3.6 | 45.2 ± 2.5 | 52.1 ± 3.7 ## |
| 4 | 69.3 ± 7.3 | 71.1 ± 2.7 | 86.3 ± 3.2 * |
| 5 | 101.0 ± 10.7 | 103.4 ± 3.4 | 117.1 ± 7.0 |
| 6 | 129.4 ± 13.8 | 119.4 ± 4.3 xx | 143.7 ± 9.3 |
| 7 | 151.7 ± 16.2 | 143.5 ± 4.7 xx | 173.9 ± 7.3 ** |
| 8 | 173.0 ± 18.4 | 167.7 ± 4.3 xx | 179.1 ± 9.6 |
| 9 | 197.0 ± 21.2 | 191.5 ± 6.3 xx | 203.8 ± 10.6 |
| 10 | 217.6 ± 23.8 | 203.5 ± 9.3 | 216.8 ± 12.0 ** |
| 11 | 230.8 ± 25.2 | 208.3 ± 13.7 | 234.9 ± 13.6 ** |
| 12 | 239.6 ± 26.7 | 231.8 ± 13.6 | 234.2 ± 12.6 |
| 13 | 251.3 ± 28.1 | 243.9 ± 15.0 | 235.1 ± 14.9 |
| 14 | 253.0 ± 28.0 | 242.5 ± 12.9 | 245.3 ± 12.6 |
| 15 | 263.5 ± 29.5 | 266.8 ± 24.2 | 260.1 ± 5.8 |
| 16 | 272.5 ± 30.5 | 269.3 ± 25.9 | 270.7 ± 8.3 |
| 17 | 280.4 ± 31.8 | 270.9 ± 25.5 | 237.8 ± 9.3 |
| 18 | 288.3 ± 32.8 | 264.1 ± 24.0 | 228.0 ± 8.8 |

Diabetic control rat weights were significantly ($p<0.05$) negatively affected by diabetes. Weights in the treated group were significantly ($p<0.05$) higher before the genesis of diabetes and NLE treatment did not affect weights thereafter. NC, normal control; DC diabetic control; DT diabetic treated; #, ## $p<0.001$, 0.05 DT vs NC; *, ** $p<0.01$, 0.05 DC vs DT; x, xx $p<0.05$, 0.001 DC vs NC

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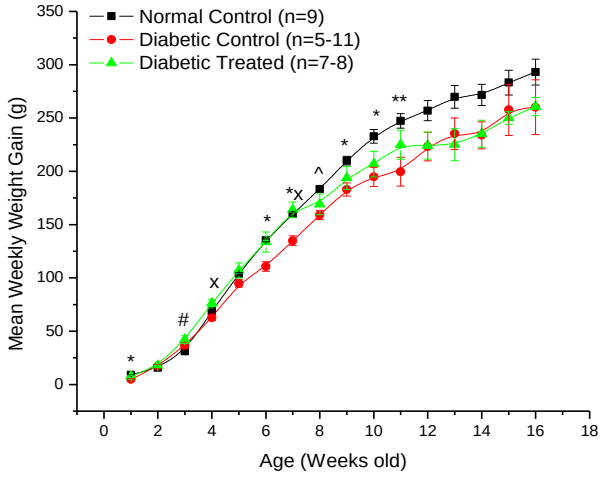


**Figure 2:** Mean weekly weight gain (g). Diabetes significantly ($p<0.05$) affected weight gain in the diabetic control group, while NLE seems to have offered minimal protection against diminishing weight gain. ^, *, ** $p<0.001$. 0.01, 0.05 DC vs NC; # $p<0.05$ DT vs NC; x $p<0.05$ DT vs DC

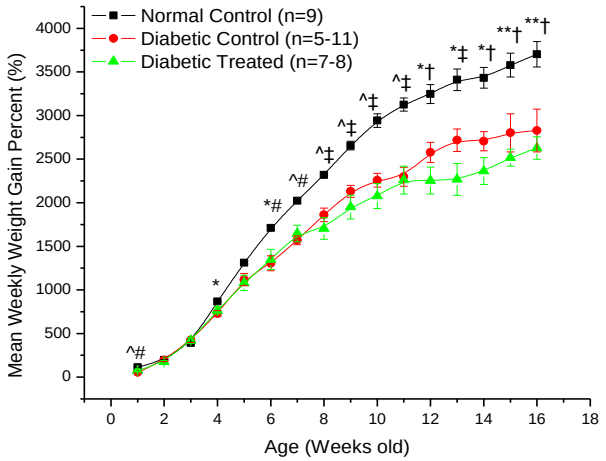


**Figure 3:** Mean weight gain percent (%). Diabetes significantly ($p<0.05$) reduced the weight gain percent which was not ameliorated by NLE. ^, *, ** $p<0.001$, 0.01, 0.05 DC vs NC; †, ‡, # $p<0.001$, 0.01, 0.05 DT vs NC

Toxicity or poisoning has been reported with the use of 20 mL neem oil in an elderly adult, with symptoms of seizures, vomiting, toxic encephalopathy, and metabolic acidosis.[29] The LD50 of the neem oil and aqueous leaf extract was found to be 31.95 g/kg b.w. or greater than 5000 mg/kg b.w. respectively, without toxicity in the liver or kidneys.[7,30,31] Furthermore, neem oil (15 mg/ml) has been found to exhibit very little or no detectable histopathological changes in mice liver and spleen with minimal toxicity to estrogen receptor-positive (MCF7; IC50, 45.7 µg/ml) and receptor-negative (MDA-MB231; IC50, 60 µg/ml) breast cancer cell lines.[32] Additionally, the ethanolic extract (1600 µg/mL at pH 8.6) was detrimental to the latter cell line.[33] These cytotoxic effects are likely attributed to the azadirachtin, nimbin, and limonoid content of neem, with nimbolide being the most potent cytotoxic limonoid and the mechanism could involve dose-dependent cellular apoptosis and the genotoxic effect of nuclear aberrations within the erythrocytes.[34-36]

This study has shown variability in the mortality and success rates of the nSTZ T2DM model. Due to this variability, further investigation is warranted for better reproducibility with minimal mortality. Furthermore, low-dose therapeutic NLE, as used in folklore medicine, does not positively impact hyperglycemia, and should not replace clinically prescribed medicines. The high mortality coupled with the fact that the NLE did not prove to be hypoglycemic at the concentration used allude to areas worth investigating in future studies.

*Limitations*

The current report is one of a paltry few regarding the success and mortality rates of the nSTZ rat model. It is limited by random testing of the neonates for glycemic levels rather than a weekly assessment of all animals. This led to the elimination of some adult animals from the study after 6 weeks (based on the Bonner-Weir et al.[10] model) because they were not hyperglycemic or reverted to normoglycemia. We believe that a lengthier assessment period of twenty weeks or more would have bolstered the results.

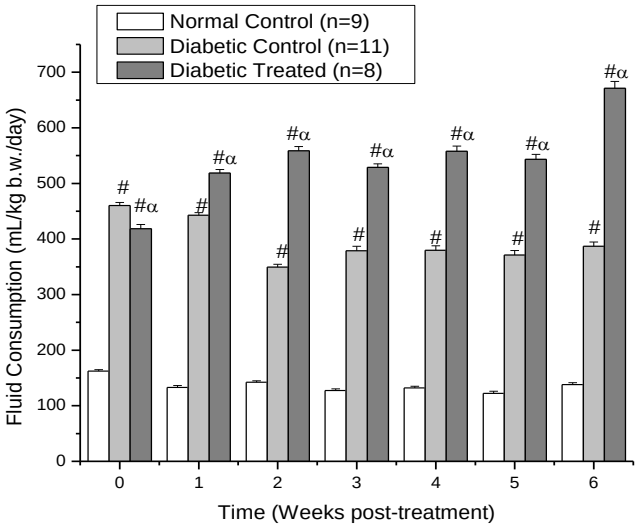


**Figure 4:** Fluid consumption post-treatment. Diabetes and NLE treatment significantly ($p<0.001$) increased both fluid and chow consumption. # $p<0.001$ vs NC; α $p<0.001$ DC vs DT

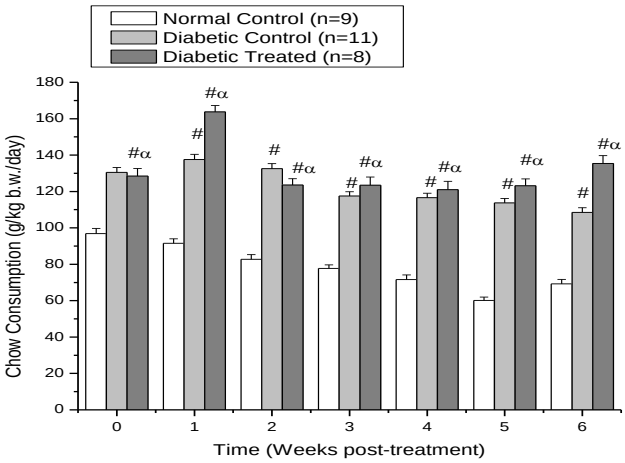


**Figure 5:** Chow consumption post-treatment. Diabetes significantly ($p<0.001$) increased chow consumption, which was further increased by NLE use. # $p<0.001$ vs NC; α $p<0.001$ DC vs DT

## Conclusion

The time taken to develop T2DM as well as the rate of mortality show much variability in the nSTZ model and, therefore, warrants further refinement for greater success. Despite the folklore therapeutic use of neem leaf extract to combat diabetes, the low dose used in this study did not positively impact hyperglycemia associated with T2DM. Since low doses may not be effective, this form of treatment should not be used as a substitute for prescribed treatments.

## Conflict of Interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

## Acknowledgments

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