



Nano-Stevia reduces the liver injury caused by streptozotocin (STZ)-induced diabetes in rats by targeting PEPCK/GCK genes, INSR pathway and apoptosis

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Abstract

Objectives Extensive application of stevia in the treatment of type 2 diabetes mellitus (DM) has been proven by a large number of previous studies. We prepared stevia loaded in nanoniosomes (nanostevia) to improve its bioavailability, functionality, and stability and explore its protective effects and underlying mechanisms in the liver of STZ-induced diabetic rats.

Methods Single-dose intraperitoneal injection of STZ (50 mg/kg body weight) was used to establish diabetic model. The mRNA levels of PEPCK and GCK genes and the protein level of INSR were evaluated by Real time-PCR and Western blot assays, respectively. TUNEL assay was used to detect apoptotic cell death in the liver tissue.

Results Diabetic rats exhibited significantly reduced levels of INSR (** $P < 0.001$) as well as elevated levels of PEPCK (** $P < 0.001$). Both stevia and nano-stevia were capable of increasing levels of GCK and INSR and reducing levels of PEPCK (## $P < 0.01$ and ### $P < 0.001$, respectively). In addition, significantly increased number of apoptotic cell death was seen in the liver tissue of diabetic rats (** $P < 0.001$) which was markedly mitigated by treatment with both Stevia and nano-Stevia (# $P < 0.05$ and ## $P < 0.01$, respectively).

Conclusion Both stevia and nano-stevia demonstrates potent anti-apoptotic activity in the liver tissue of diabetic rats by targeting PEPCK/GCK genes and INSR pathway. These finding show that nano-stevia has more potential to reduce the liver injury caused by STZ-induced diabetes in rats and hence can be considered a valid agent and alternative therapy for attenuating complications of type 2 DM.

Keywords Diabetes mellitus · Streptozotocin · Liver injury · PEPCK/GCK genes · Apoptosis

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Introduction

Diabetes mellitus (DM) is a lifelong progressive disease that is characterized by elevated blood glucose levels [1]. High blood glucose level in DM patients can be attributed to body's inability for producing or using insulin [2]. The prevalence of DM around the world has been estimated to be 4.4% which in turn give rise to increasing the number of DM patients to 439–552 million by 2030 [3, 4]. The most prevalent form of DM is type 2 DM which includes 90–95% of cases [5]. The main symptoms of type 2 DM are loss of pancreatic β -cell mass, insulin resistance, derangements in lipid and carbohydrate metabolism, and accumulating human islet amyloid peptide deposits [6, 7]. Elevated blood glucose levels within cellular vicinity enhance generation of free radicals and weaken natural antioxidant defenses which in turn leads to triggering oxidative stress and diabetes complications such as liver damage, blindness [8], renal failure [9], and neurological and cerebrovascular disorders [10]. Owing to the key role of liver tissue in glycogen synthesis, gluconeogenesis, and the homeostasis of insulin and plasma glucose, measuring serum liver enzymes like gamma-glutamyltransferase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) can be considered a valid method to predict type 2 DM [11]. Hosphoenolpyruvate carboxylase (PEPCK) was known to be one of the key enzymes in the liver regulating the conversion of non-sugar substances into glucose during gluconeogenesis process [12]. Increasing the expression of PEPCK in the liver of type 2 DM rats and subsequently enhancing gluconeogenesis has been reported by previous studies [13]. Glucokinase (GCK) or hexokinase IV (EC 2.7.1.2) is another important enzyme which plays an important role in enhancing glucose uptake for glycogenesis and energy storage in hepatocytes and promoting the insulin release by pancreatic β cells [14, 15]. It has been reported that gene variations in GCK increase the risk of type 2 DM [16]. In addition, a previous study has shown that therapeutic agents could ameliorate T2D in high-fat diet (HFD) + streptozotocin (STZ) treated mice by reducing apoptotic markers and targeting PEPCK and GCK [17]. Furthermore, the insulin receptor (INSR) is highly expressed in the liver tissue and displays a powerful role in normalizing in vivo response to insulin [18, 19]. Unwanted side effects of available drugs for type 2 DM has limited their extensive use and hence medicinal plants can be considered alternative agents for them [20]. Stevia (*Stevia rebaudiana* Bertoni), sweet herb of Paraguay, is a natural and the non-caloric sugar compound belonging to the family Asteraceae [21]. Stevia was known to be a potent antioxidant and anti-inflammatory agent against various diseases such as cancer [22], and diabetes [23]. It has been well-known that nanocarriers have ability

of improving stability, functionality, and bioavailability of phenolics in the treatment of different diseases [24]. Here, the aim of the study was to reduce the dose of stevia and keep its prolonged activity by loading into nanoniosomes. We have investigated the protective effects of stevia loaded in nanoniosomes (nanostevia) in STZ treated rats. In order to assess the effects of stevia on molecular mechanisms, we evaluated the mRNA levels of PEPCK and GCK and apoptotic cell death.

Methods and materials

Haigen Bio-tech Company (China) was producer of *Stevia rebaudiana* Bertoni powder. According to the manufacturer's instructions, it has been extracted and crystalized as TSG (total steviol glycosides). The powder includes 97% Rebaudioside A and 3% of other glycosides like stevioside. More information about ingredients of *stevia rebaudiana* Bertoni powder can be observed in COA [<https://www.steviahaign.com/steviol-glycosides/stevia-extract-powder-natural-sweeteners.html#>]. Solarbio Science & Technology Co. (Beijing, china CAT No. S8050) supplied Streptozotocin. Other chemicals such as chloroform, cholesterol, sorbitan monostearate (Span 60), and polyoxyethylene sorbitan monostearate (Tween 60) were obtained from Merck, Germany.

Animals and Ethics statement

Male Wistar rats (total number: 40, weight: 200–270 g) were procured from the experimental and comparative studies center of the Iran University of Medical Sciences (Tehran, Iran). Wistar rats were kept at 22 ± 2 °C with a 12-h light/dark schedule. The animals had free access to standard chow and water. Ethical approval was obtained from Islamic Azad University ethical committee for animal research.

Preparation of nano- niosomes

Nano- niosomes were prepared according to thin layer hydration method [25]. In brief, after dissolving cholesterol and surfactants (span60 and tween 60) with 1:1 molar ratio in 10 ml chloroform, the rotary evaporator was used for 1 hat 60 °C and 120 rpm to evaporate chloroform. Afterwards, the dried thin films were hydrated using stevia solution in PBS (10 mL, 60 mg/mL, at 30 °C for 1 h and 120 rpm). Eventually, the resulting solution was sonicated for 5 min and stored at 4 °C in a refrigerator.

Characterization of Nano-niosomes

The hydrodynamic size of nano-niosomes containing drug or without drug was measured using dynamic light scattering (DLS) using He–Ne laser at a wavelength of 632.8 nm (Malvern, Zetasizer Nano ZSE, Worcestershire, UK).

Entrapment efficiency

Entrapment efficiency was measured according to a previous study [26]. Briefly, the formulations were ultra-filtered at 4000×g for 30 min. D using an exerting an Amicon.

Ultra-15-membrane (MWCO 30,000 Da). Filtration resulted in passing free drugs through a filter membrane while the Stevia carrying niosomes remained in the top chamber. In order to evaluate drug concentration, UV–visible spectroscopy (JASCO, V-530, Tokyo, Japan) at a wavelength in the range 420 nm was utilized. For each formulation, the drug concentration was measured to its standard curve. Finally, we used the following formula to calculate encapsulation efficiency:

$$\text{Encapsulation Efficiency (\%)} = [(A - B) / A] \times 100.$$

A is defined as the initial proportion of drug-loaded niosomal formulations, and B is defined as the amount of free drug transmitted through the membrane.

Drug release study

In order to evaluate drug release, a dialysis bag (MWCO=12 kDa) was used to compare drug release of free drug and drug-loaded niosomal formulations. The bag was put in PBS solution (50 mL, 1X, pH=3, 5, 7.4) under gradual stirring condition (50 rpm) at 37 °C. The aliquots were taken and replaced by fresh PBS solution at particular time intervals [26].

Physical stability assessment

In order to determine physical stability, we measured nanoparticle size and EE of niosomal solutions during the refrigerated storage at interval times of 0, 14, 30, and 60 days based on a previous study [27].

Induction of diabetes Mellitus

The animals have fasted overnight and type 2 DM was induced by intraperitoneal injection of a single dose of STZ (50 mg/kg body weight i.p.) dissolved in PBS buffer solution. The rats had a limited access to sucrose solution for one night (20% sucrose solution) to prevent hypoglycemic mortality by STZ. A glucometer (Accu-Chek, Roche Diagnostics, Penzberg, Germany) was used to measure fasting

blood glucose after 72 h of STZ injection and evaluate the progression of DM in the animals. The animals with a 200 mg/dl glucose level or more on the third day after STZ injection were accepted to be diabetic. The normal control animals only received distilled water and standard diet.

Experimental design

This study includes five groups and eight rats were designated in each group and categorized as explained below:

- (1) Normal control group. This group includes normal healthy rats (control, n=8).
- (2) Diabetic group: All the rats received only a single dose of STZ (50 mg/kg) dissolved in PBS buffer solution (diabetic, n=8).
- (3) Nano- niosome group. STZ-induced diabetic rats were intra-gastrically treated with 1ml of only nano-niosome for 30 days daily (Nano- niosomes, n=8).
- (4) Stevia group. STZ-induced diabetic rats were intra-gastrically treated with 1ml of stevia (20 mg/dl) for 30 days daily (Stevia, n=8).
- (5) Nano-stevia group: STZ-induced diabetic rats were intra-gastrically treated with 1ml of nano-stevia (20 mg/dl) for 30 days daily.

Sample collection

A mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) was used to anesthetize the animals which were fasted overnight at the end of the experiment. Liver tissue samples were collected from all the rats from different groups and washed in an ice-cold saline solution. Some liver tissues were stored at -80 °C for assessing expression of genes and some of them were fixed in 10% formalin solution and processed for histological evaluations using paraffin technique.

Tunnel assay

Tunnel assay was performed based on Roche kit protocol. To This end, the samples were deparaffied and placed in xylene solutions. Afterwards, the sections were rehydrated using a graded series of ethanol (90%, 80%, and 70%) and rinsed with PBS buffer. After incubation with proteinase K for 20 min at 37 °C, 50 µL Tunnel dye was added to each sample and samples were incubated at 37 °C for 1 h again. Finally, samples were rinsed with PBS buffer and detecting TUNEL-positive cells (green) was carried out using Zeiss LSM 5 fluorescent microscope. Only TUNNEL label solution was utilized for negative control samples whereas both TUNEL-enzyme solution and TUNEL-label solution (1:9) were used for positive control samples. The nuclei were

stained by incubating the tissue by wit DAPI dye (1 mg/ml) (blue dots).

Western blot assay

The RIPA lysis buffer was used to obtain tissue lysis. The BCA Protein Assay kit (The Thermo Scientific Pierce BCA Protein Assay Kit, ThermoFisher Scientific, US) was used to detect protein concentrations. In order to separate soluble protein, 4–20% gradient SDS/PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used. Afterwards, separate soluble protein were transferred to the nitrocellulose membrane and nonspecific reaction sites of PVDF (Immobilon®-FL PVDF membrane pore size 0.45 µm-Sigma-Aldrich) membranes were blocked with TBS buffer. The membranes were incubated with primary antibody (Dilution 1:1000) at 4 °C overnight and then the appropriate horseradish peroxidase-conjugated secondary antibody (Dilution 1:3000) at room temperature for 2 h. The antibody incubations were followed by rinsing using TBS buffer (three times). Enhanced chemiluminescence (ECL) was used to detect the protein bands. Finally, a film camera was used to visualize the bands. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as loading control and for normalization. Ratio of expression of each protein to the expression of GAPDH in the same sample was measured using Alpha Ease ® FC Imaging System according to their optical densities.

Real-time PCR assay

A mortar and pestle was utilized to powder frozen liver tissues in liquid nitrogen. Total RNA was extracted by homogenization of samples in TRIzol (Invitrogen). A cDNA synthesis kit Fermentas was used to reverse RNA into cDNA based on manufacturer's instructions. Using Rotor-Gene Q 5plex System using SYBR® Premix Ex Taq™ II (TliRNaseH Plus, RR820Q), a triplicate run of quantitative PCR was performed. The internal control in this study was GAPDH. Calculating relative changes in gene expression was performed using the $2^{-\Delta\Delta CT}$ method. Primers used in this study are listed in Table 1.

Statistical analysis

Results were presented as means ± standard error (S.E.M) for all statistical tests using GraphPad Prism software

version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey post hoc test was used to evaluate multiple group differences. In order to determine normal distribution of all data, Shapiro–Wilk and Kolmogorov–Smirnov tests were used. Differences were considered to be statistically significant at $p < 0.05$.

Results

Characterization of nano-niosomes containing Nano-Stevia

As shown in Fig. 1A, DLS results revealed that the hydrodynamic size of only nano-niosomes was about 143.4 while a shift to 175.1 nm was seen in nano-niosomes containing stevia. Compared to placebo niosomes, increased hydrodynamic size of nano-niosomes was observed after loading of nano-stevia. As shown in Fig. 1B and according to TEM image, only nano-niosomes were spherical in shape and had good stability. In addition, TEM image showed that size of only nano-niosomes was about 140 ± 3.35 .

Optimization of nano-niosomes containing Nano-Stevia

Entrapment efficiency (EE) and release rate were measured to evaluate the optimization process. The outcomes of the Box–Behnken trials revealed that EE% in nanoniosomes containing drug was about 68.89. A direct association between EE% and increasing drug content, as well as the surfactant was seen (Fig. 2).

As depicted in Figs. 3, 91% of drug released in 6 h and a constant release process was seen after 6 h.

Physical stability

The particle size and EE of nano-Stevia Stevia loaded niosomes during 2 months of storage at 4 ± 2 °C and 25 ± 2 °C showed that there were no significant differences in the particle size and EE of particles at 4 ± 2 °C and 25 ± 2 °C on Day 0 and 14 (Fig 4). There was no sediment from the water-insoluble contents of the layers of niosomes over this time.

Table 1 Primers used in this study

Primer	Forward	Reverse	Size
PEPCK	TTGAGATTTTGTAGAGCACA	TCTATCACAGCTCTGGAAAT	290 bp
GCK	ATATTTTTTTAGGATTTGCC	ATATCACTCTCTTCGCGAAT	350 bp
GAPDH	ATTCTCTGATTGGTCGTATT	TTTCATGGTGAATCATATT	130 bp

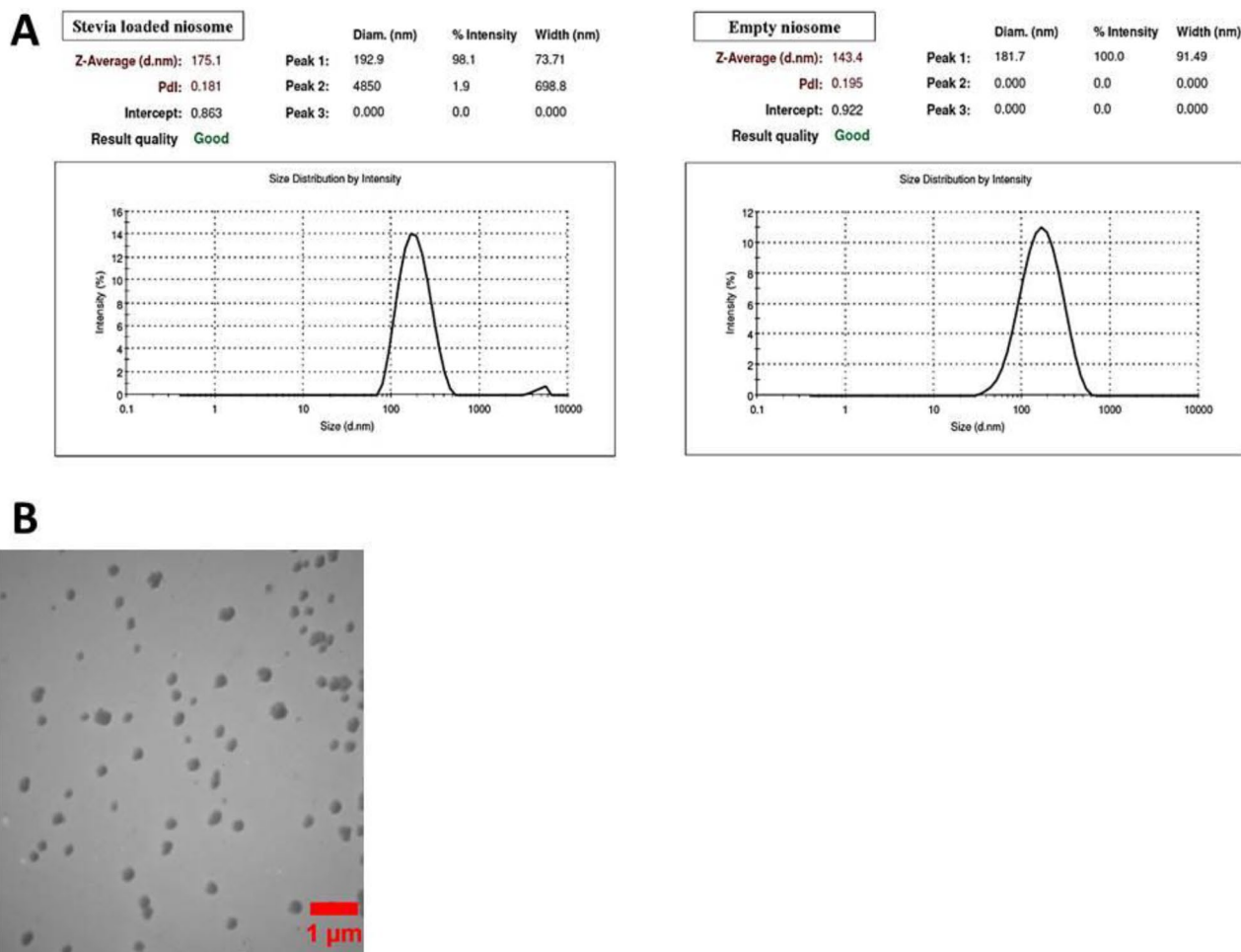


Fig. 1 Nano-niosome characterization. **(A)** DLS results show hydrodynamic size distribution for nano-niosomes and nano-niosomes containing Nano-Stevia. **(B)** Results of TEM image demonstrated that nano-niosomes were spherical in shape and had a good stability

Significant differences in the particle size and EE of particles at 4 ± 2 °C and 25 ± 2 °C were observed on Day 30 and 60.

Nano-Stevia exerts protective effects in the liver tissue of STZ-induced diabetic rats by changing the mRNA levels of PEPCK and GCK genes

As shown in Fig. 5A, the mRNA levels of PEPCK were significantly higher in diabetic rats than in non-diabetic control group. Compared with the diabetic group, STZ-induced diabetic rats treated by both stevia and nano-stevia exhibited significantly reduced levels of PEPCK. Nano-stevia displayed more powerful effects on reducing the mRNA levels of PEPCK compared to only stevia. Significantly decreased expression levels of GCK were seen in the liver of STZ-induced diabetic rats compared to non-diabetic control group. Interestingly, treatment with both stevia and nano-stevia markedly increased the expression levels of

GCK compared to diabetic control group (Fig. 5B). On the contrary, treatment with only nanoniosomes failed to create noticeable changes in the mRNA levels of GCK in the liver of STZ-induced diabetic rats compared to diabetic control group.

Nano-Stevia exerts protective effects in the liver tissue of STZ-induced diabetic rats by increasing the protein level of INSR.

Significantly reduced protein levels of INSR were observed in the liver of STZ-induced diabetic rats compared to non-diabetic control group (Fig. 6). Either stevia or nano-stevia treatment markedly increased protein levels of INSR, with nano-stevia exerting a more powerful effect than stevia. There were no significant differences between only nanoniosomes and diabetic control groups.

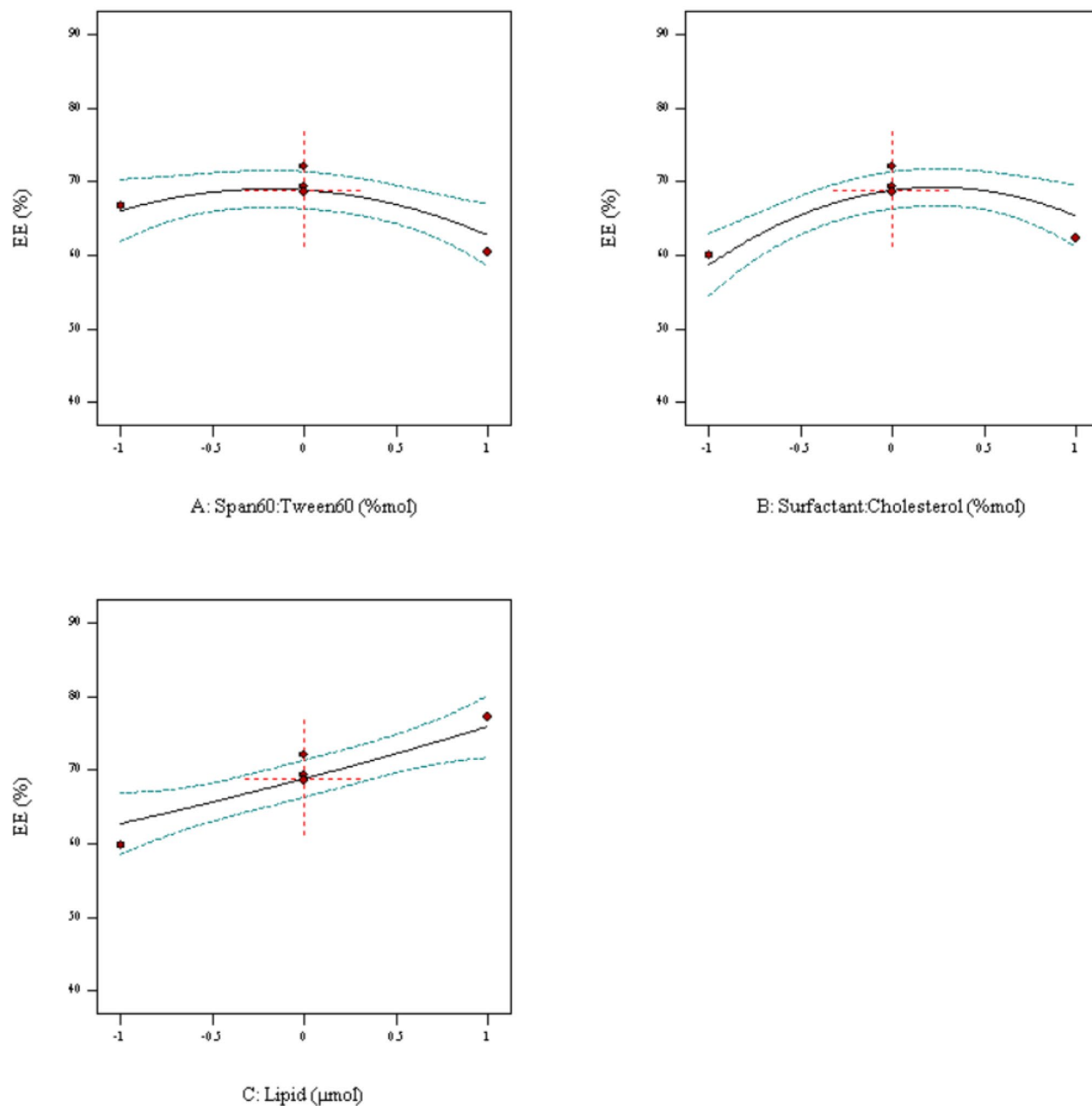


Fig. 2 Box-Behnken method for encapsulation efficiency (EE) as a function of the parameters

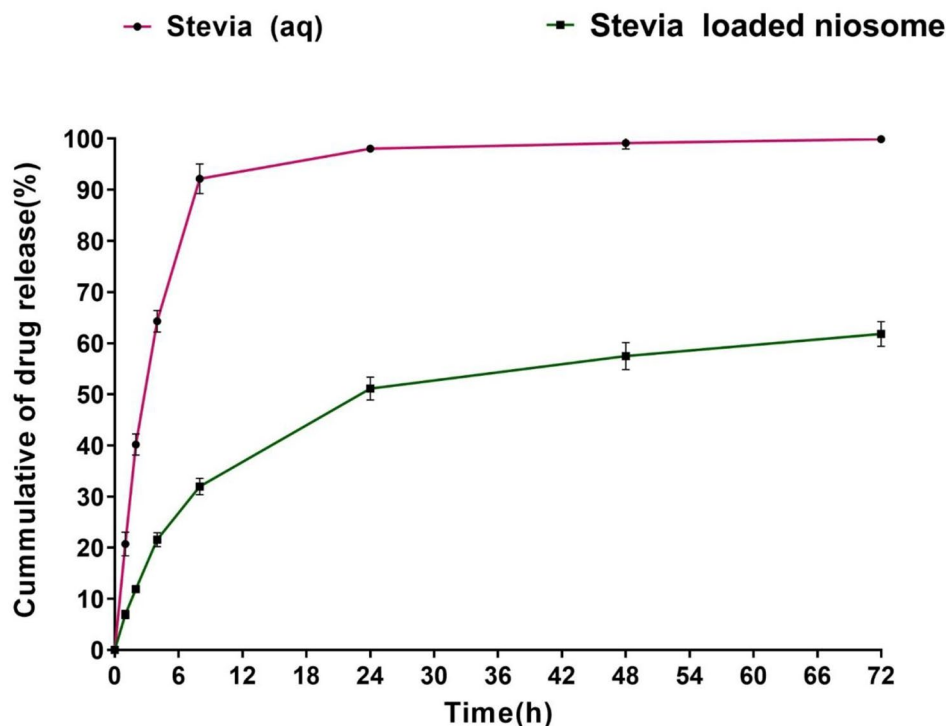
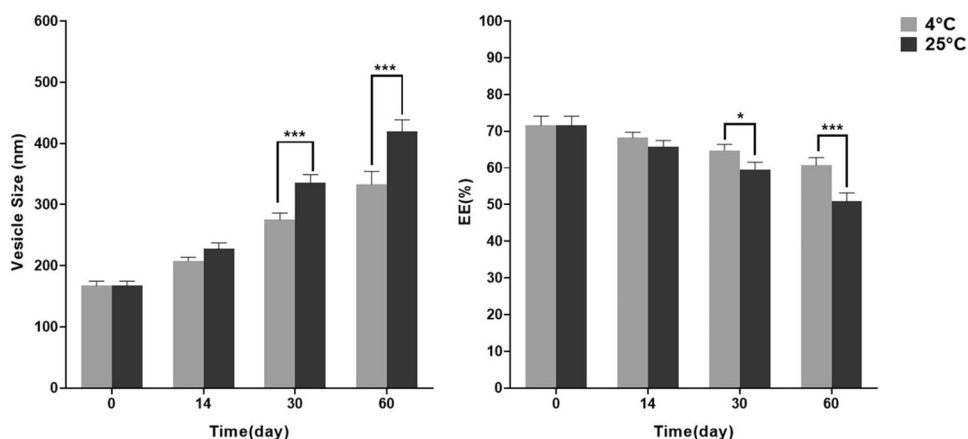
Nano-Stevia exerts protective effects in the liver tissue of STZ-induced diabetic rats by reducing apoptotic cell death

The number of apoptotic cells significantly increased in the liver of STZ-induced diabetic rats compared to non-diabetic control group. Reducing the number of apoptotic cells was more pronounced in the liver of STZ-induced diabetic rats treated by both stevia and nano-stevia (Fig. 7). No significant

differences were seen between only nanoniosomes and diabetic control groups.

Discussion

One of the most commonly used compounds to establish T2DM in animal models is STZ [28]. Upon STZ treatment, excessive reactive oxygen species (ROS) production leads to DNA alkylation and fragmentation, increasing lipid

Fig. 3 Release profile of stevia and nano-stevia during 72 h**Fig. 4** Stability of optimum stevia loaded niosomes during 2 months of storage at 4 ± 2 °C and 25 ± 2 °C, (* p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001)

peroxidation, reduced synthesis and release of insulin, and organ dysfunction [29, 30]. In this study, T2DM was induced by STZ to establish an accepted model for recognizing the pathophysiology of T2DM and evaluating protective effects of therapeutic agents. Intraperitoneal injection of STZ (50 mg/kg) could give rise to changing the expression of the mRNA levels of PEPCK and GCK genes, the protein level of INSR, and enhancing apoptotic cell death in the liver of STZ-induced diabetic rats. Both stevia and nano-stevia were capable of reducing apoptotic cell death in the liver of STZ-induced diabetic rats by targeting PEPCK, GCK, and INSR. It has been reported that diabetic condition is associated with increasing rate of hepatic gluconeogenesis. Hence, targeting the gluconeogenic pathway by therapeutic agents

can be considered an efficient mechanism to attenuate liver damage in STZ-induced diabetic rats and control blood glucose levels [31, 32]. PEPCK was known to play a key role in the first step of gluconeogenesis in which oxaloacetate is converted into phospho-pyruvate [33]. A large number of previous studies have shown an increased expression of PEPCK in the liver of STZ-induced diabetic rats [34–36]. In keeping with these previous studies, our findings showed that the mRNA levels of PEPCK markedly increased in the liver of STZ-induced diabetic rats that reversed by both stevia and nano-stevia. Likewise, a previous study by our group showed that STZ-induced diabetic rats treated by stevia and nano-stevia could exhibit noticeable reduction in blood glucose levels [37]. This can be contributed to regulation of the

Fig. 5 The effects of intra-gastrically administration with saline, nano-niosome, Stevia, and Nano-Stevia on mRNA levels of (A) PEPCK and (B) GCK. Significant differences: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to control group. ## $P < 0.01$ and ### $P < 0.001$ compared with diabetic control group. \$ $P < 0.05$ and \$\$\$ $P < 0.001$ compared with nano-niosome (n=8)

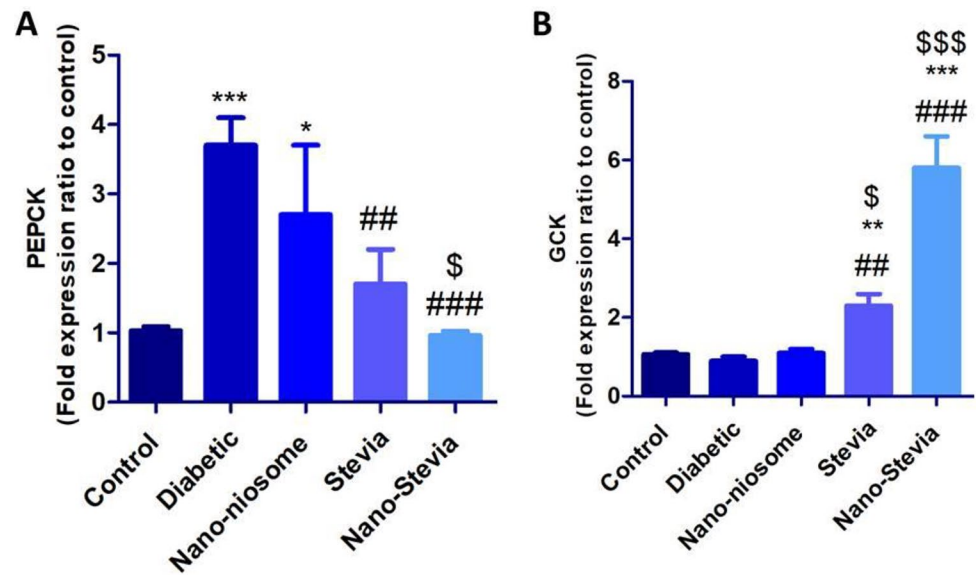
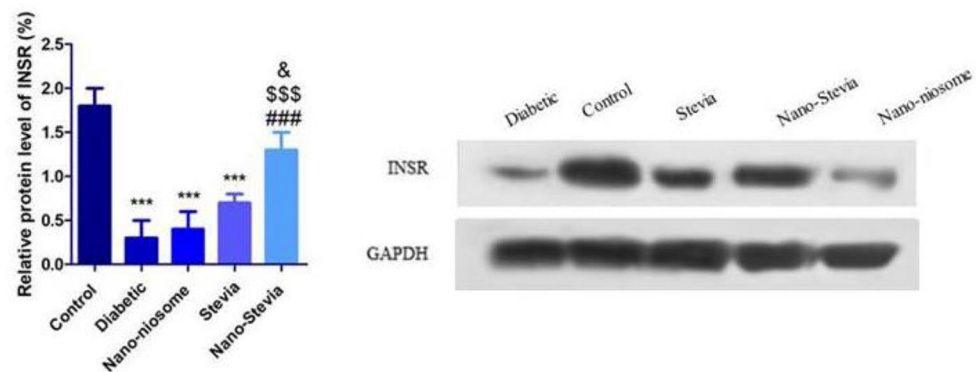


Fig. 6 The effects of intra-gastrically administration with saline, nano-niosome, Stevia, and Nano-Stevia on protein levels of INSR. Significant differences: *** $P < 0.001$ compared to control group. ### $P < 0.001$ compared with diabetic control group. \$\$\$ $P < 0.001$ compared with nano-niosome. & $P < 0.05$ compared to Stevia (n=4)



gluconeogenic pathway by stevia and nano-stevia. It seems that there is a close relationship between increasing PEPCK expression and apoptotic cell death in diabetic conditions [38]. In addition, it has been reported that decreasing cellular ATP production, reducing insulin secretion and glucokinase (GCK) expression occurs in high-glucose-treated cells during apoptosis. In fact, exposure to a chronically high dose of glucose give rise to reducing interactions between GCK and mitochondria which in turn results in enhancing Bax binding to mitochondria and cytochrome C release [39]. GCK overexpression has demonstrated abilities for reducing apoptotic cell death during diabetic conditions. A previous study has shown that hepatic GCK expression was suppressed more than 60% in diabetic subjects with HbA1c > 7.0 [40]. In agreement with these previous studies, our findings demonstrated that reducing GCK expression in the liver of STZ-induced diabetic rats enhanced apoptotic cell death that reversed by both stevia and nano-stevia. In the line with present study, several previous reports show

that protective effects of stevia against degenerative disorders are associated with reducing apoptotic cell death [41, 42].

Reducing total INSR abundance in liver tissue during different diseases is associated with increasing apoptotic cell death [43]. In the line with this previous study, our findings indicate that the protein levels of INSR markedly decreased in the liver of STZ-induced diabetic rats that reversed by both stevia and nano-stevia. The present study might have some limitations. One of the major limitations of this study is that some parameters are measured by RT-PCR only which cannot confirm the protein level.

Conclusion

In conclusion, the findings of this study suggested that both stevia and nano-stevia are capable of reducing apoptotic cell death in the liver of STZ-induced diabetic rats by regulation of the gluconeogenic pathway and the insulin receptor (INSR). Our findings showed that nano-stevia could

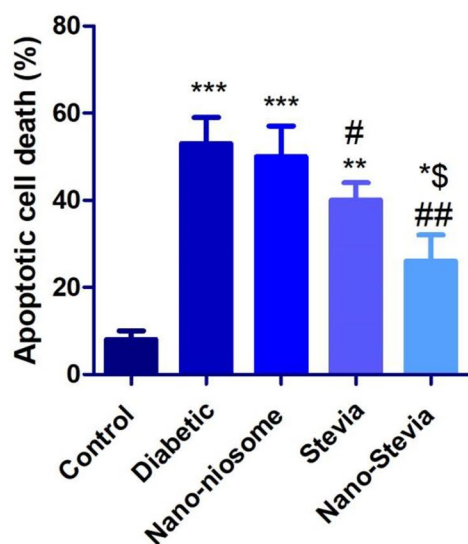
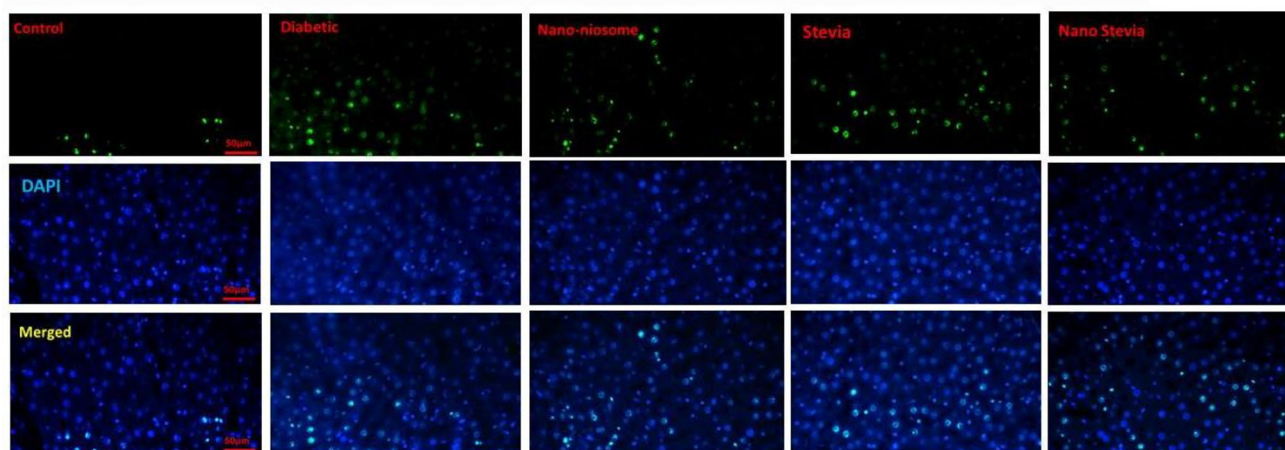


Fig. 7 The effects of intra-gastrically administration with saline, nano-niosome, stevia, and nano-stevia on apoptotic cell death in the liver of STZ-induced diabetic rats. Tunel assay 50 μ m (apoptotic cells = green dot, DAPI stained nucleus: blue dots). Significant differences: *

attenuate the liver injury caused by STZ-induced diabetes in rats by targeting PEPCK/GCK genes, INSR pathway and apoptosis. Our results showed that nano-stevia has more powerful effects in reducing diabetes related liver injury in rats. Increasing stability of stevia in a nanocarrier might explain these differences. This study has some limitations. One of the limitations is that more evaluations using Western blot are required to approve our results since there is the frequent mismatch between mRNA transcription and protein translation.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s40200-023-01278-2>.

$P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to control group. # $P < 0.05$ and ## $P < 0.01$ compared with diabetic control group. \$ $P < 0.05$ compared with nano-niosome ($n = 4$)

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Authors' contribution M.N: Conceptualization, Supervision, Visualization, Validation, Writing - review & editing, M.M-A, E.GH, F.A, and F.KH: Writing - original draft, Investigation, Formal analysis, Resources, and N.M: review & editing.

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Data Availability The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Declarations The Ethics Committee of the Islamic Azad University approved all the experiments and protocols. All the efforts were carried out to minimize animals suffering.

Competing interest The authors declare that there are no conflicts of interest.

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