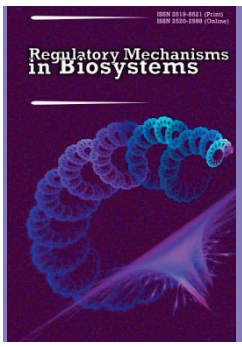

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Newly synthesized chitosan-stevioside-TPGS nanoparticles (CSdNPs) attenuate the effects of high doses of free stevioside in male rats via inhibition of PRAP-α gene expression

A. H. Abdul-Ameer, H. H. Kzar, H. K. Al-Awadi

Al-Qasim Green University, Babylon, Iraq

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Veterinary Medicine College, Al-Qasim Green University, Babylon, 51013, Iraq.
E-mail: hamza14shukri72@gmail.com

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This study investigated newly synthesized of chitosan-St-TPGS-NPs and chitosan-Sd-TPGS-NPs (CStNPs and CSdNPs) produced by a combination of sonication and emulsification/solvent evaporation method and in combination with the ionic gelation method with slight modifications. The newly synthesized CStNPs and CSdNPs were characterized by several technical methods such as SEM, TEM and FT-IR. In this study, 60 male Wistar rats were divided randomly into six groups. Each group included 10 animals with control group, stevia group (St), stevioside group (Sd), CNPs group, chitosan-stevia-TPGS nanoparticles (CStNPs) group, chitosan-stevioside-TPGS nanoparticles (CSdNPs) group. All the groups received their daily dosages orally for two months. After the end of the experiment, a blood sample was collected for estimation of the liver enzyme concentration (ALT, AST, ALP, and TSB), lipids profile (TC, TG, LDL-C, VLDL-C, and HDL-C), hematological parameters (RBCs, WBCs, Hb, and PCV, also FAS, FBG, and TyG index). Analysis was performed to assess the average change (AFC) in PPAR-α gene expression in all study groups. The results suggested that there is a significant difference in FAS (pg/mL) levels between the control group (494.2 ± 15.8) and the St or free Sd groups at the end of 2nd month (511.6 ± 16.2, and 561.7 ± 17.2), respectively. In addition the highly significant differences were registered between the Sd group in comparison with CNPs, CStNPs, and CSdNPs groups at the end of the experiment. On the other hand, the results of this study suggested that there is a significant difference in AFC between the control group (5.86 ± 0.58) and St or free Sd groups at the end of the 2nd month (3.00 ± 0.22, and 1.86 ± 0.12), respectively. In addition, highly significant differences were found between the Sd group (1.86 ± 0.12) and the CNPs, CStNPs, and CSdNPs groups at the end of the experiment (4.98 ± 0.25, 3.91 ± 0.24, and 4.02 ± 0.45). This study concluded that St and in large form Sd have harmful effects on the male liver of male rats. The newly synthesized (CStNPs and CSdNPs) should attenuate the risk of St and Sd via the activation of PPAR-α gene expression and inhibition of FAS.

Keywords: *Stevia*; stevioside; chitosan; TPGS; hepatic function; rats.

Introduction

The generic term used for food ingredients derived from the herb *Stevia rebaudiana* Bertoni, 1899 (Asteraceae family) has been called stevia and the more precise term for a group of intensely sweet compounds extracted and purified from *S. rebaudiana* is also called steviol glycoside (Dyduch-Siemińska et al., 2020). The predominant steviol glycosides found in *S. rebaudiana* has been called stevioside (Sd) and rebaudioside A (Pradhan & Dwivedi, 2016). *Stevia rebaudiana* is a natural sweetener herb (Gupta et al., 2013). Stevia leaves produce secondary metabolites (diterpene glycosides), which are about 300 times sweeter than sucrose (Goyal et al., 2009). Stevioside could not be decomposed by either digestive enzymes nor gastric juice in preclinical and clinical studies (Koyama et al., 2003). Oral Sd is not absorbed at the upper-small-intestine level, which probably results from its high molecular weight (Gardana et al., 2003).

In the lower gastrointestinal tracts of rats, mice and pigs Sd can be decomposed by bacterial intestinal flora (*Bacteroides* genus) to free steviol (Geuns et al., 2003). After Sd (750 mg per day) consumption, a human volunteer study showed that no significant levels of free steviol or any other steviol metabolite were present in the blood as well (Geuns et al., 2007). In rats, steviol was detected in a portal venous blood sample after oral administration of Sd (Wheeler et al., 2008). The receptors activated by peroxisome proliferators (PPARs) can be activated by ligands to regulate the gene expression of elements that are part of the nuclear receptor (NR) superfamily (Christofides et al., 2021). Three types of PPAR have been discovered: PPAR alpha (NR1C1), PPAR beta/delta (NR1C2), and PPAR gamma (NR1C3) (Michalik et al., 2006). The initial member of the PPAR family, PPAR-α as organelles play a role in lipid metabolism and detoxification within cells. Organelles present in the majority of plant and animal cells contain a variety of enzymes with complex functions and different metabolic processes, such as the breakdown of beta-oxidation and are involved in metabolism of fatty acids (FAs), bile acid, and cholesterol (Heidari et al., 2019).

The aim of this research is to describe the impact of newly synthesized chitosan-stevioside-TPGS nanoparticles (CSdNPs) on attenuating the effects of high doses of free stevioside in male rats via inhibition of PRAP-α gene expression.

Materials and methods

The Department of Physiology at Al-Qasim Green University provided an ethical permission letter (No: 334, 9/30/2023) to authorize the conducting of this scientific experiment in the College of Veterinary Medicine.

Stevia was harvested from the market in Babylon province, Iraq. The *Stevia* leaves were dried completely and the dried leaves crushed into small pieces using a mortar and pestle or grinder. 500 g (an appropriate

amount) of crushed *Stevia* leaves were weighed and put in (1000 mL) of distilled water and the distilled water brought to a boil. The mixture was stirred well with a glass stirrer and allowed to stand for about 30 minutes. After the standing time is complete, the beaker was removed from the heat source and the mixture allowed to cool to room temperature.The mixture was filtered using a filtration apparatus to remove the solid leaf particles. After complete extraction, the extract was stored in a refrigerator to preserve its stability if it was not used immediately.

Chitosan-Sd-TPGS Nanoparticles (CSdNPs) (stevioside loaded chitosan NPs) were prepared by combination of the sonication and emulsification/solvent evaporation methods and in combination with the ionic gelation method with slight modifications. Sixty mg of chitosan was dissolved in 4 mL of 0.2% (v/v) acetic acid and pH was brought to 6.0 with sodium hydroxide (NaOH). Chitosan at a concentration of 1% (w/v) was mixed with 1% acetic acid and stirred with a magnetic stirrer for 24 hours to ensure full dissolution of the chitosan in the solution. Following dissolution, the pH was set to 5 by adding 1 N NaOH. The prepared solution was mixed with 2 mL of chloroform containing of 40 mg of TPGS and 6 mg of Sd to form nanoparticle emulsion by using an ultrasonic probe sonication. The emulsion was stirred overnight to evaporate chloroform and nanoparticle formation. Four mL of 2 mg/mL of sodium tripolyphosphate (TPP) solution was added to the formulation that prepare by step 3 for cross-linking formation. The formulated nanoparticles were subjected to centrifugation for 10 min at 4,000 rpm to remove larger particles. The centrifugation was again done for 15 min at 12,000 rpm, and the clear supernatant was removed, and settled nanoparticles were washed with distilled water to remove the excess Sd that adhered to the surface of nanoparticles. A dispersion of resulting nanoparticles in distilled water was made by using vortex. The procedure for preparation of CStNPs was the same as described above but differed by the use of St extract instead of Sd. CNPs were prepared without St or Sd by the same method.

CNPs, CStNPs and CSdNPs were characterized by several techniques such as UV spectroscopy, particle size (PS), zeta potential (ZP), and polydispersity Index (PDI), FT-IR Analysis, TEM, SEM, and in vitro Sd release analysis.

In this study, 60 male Wistar rats were divided randomly into six groups. Each group included 10 animals: the control group, stevia group (St), stevioside group (Sd), CNPs group, chitosan-stevia-TPGS nanoparticles (CStNPs) group and the chitosan-stevioside-TPGS nanoparticles (CSdNPs) group. Group 1 (CONT): administration of normal drinking water for 8 weeks. Group 2 (St): administration of stevia extract (250 mg/kg/day) dissolved in drinking water for 8 weeks. Group 3 (Sd): administration of stevia (500 mg/kg/day) dissolved in drinking water for 8 weeks. Group 4 (CNPs): administration of CNPs (100 mg/kg/day) dissolved in drinking water for 8 weeks. Group 5 (CStNPs): administration of CSdNPs (250 mg/kg/day) dissolved in drinking water for 8 weeks. Group 6 (CSdNPs): administration of CSdNPs (500 mg/kg/day) dissolved in drinking water for 8 weeks.

Lipids profile, liver enzymes and blood parameters were assessed depending on kits instructions by spectrophotometric analysis.

Fatty acid synthase (FAS) (ng/mL) were investigated according to the protocol provided with the ELISA kit.

Genomic RNA was extracted from tissue using TransZol Up RNA Kit. The PPAR-α and GAPDH gene primers used in this study were provided by (Macrogen Company, Korea) as shown in Table 1.

Table 1
The PCR primers with their sequence

Primers		Sequence
PPAR-α	F	5'- ACGATGCTGTCCTCCTTGATG -3'
	R	5'- GCGTCTGACTCGGTCTTCTTG -3'
GAPDH	F	5'-ATGACTCTACCCACGGCAAG-3'
	R	5'-CTGGAAGATGGTGATGGGTT-3'

Data are presented as means ± standard deviation (x ± SD). The impact of treatments was statistically assessed using the one-way analysis of variance (one-way ANOVA) followed by Turkey's post-hoc test to adjust for multiple comparison treatments. Statistical significance was established at the $P < 0.05$ level.

Results

UV-visible spectrum scanning of the synthesized CNPs, CStNPs and CSdNPs was performed after dissolving in 2% (v/v) glacial acetic acid and scanning at a wavelength range of (200–600 nm). The results of the present study recorded on UV-Visible spectrum showed an absorption peak at 293 nm, which corresponds to the p-p* transition of the CSdNPs (Fig. 1a). The results of the XRD of Sd loaded on chitosan-TPGS-NPs are displayed in Figure 1b. Partial sizes (PS), polydispersity index (PDI), and zeta potential (ZP) values of the CStNPs and CSdNPs were assessed and shown in Table 2 and Figure 1c.

Table 2
PS (nm), ZP (Mv), and PDI of nano formulations (x ± SD, n = 3)

Sample	PS, nm	ZP, mV	PDI
CNPs	103.1 ± 2.4^{a}	-38.92 ± 0.34^{a}	0.567
CStNPs	299.3 ± 12.6^{b}	-39.31 ± 0.39^{a}	0.775
CSdNPs	92.4 ± 4.9^{c}	-42.04 ± 0.08^{b}	0.832

Note: symbols [a], [b], and [c] show significant difference demonstrated by Turkey's post-hoc test.

SEM and TEM analysis is another useful tool for the surface morphology of synthesized chitosan nanoparticles (CNPs, CStNPs, and CSdNPs) (Fig. 1d, 1e, 1f). FT-IR analysis showed that the absorption of the stevia extract molecules was characterized by a stretching or bending of the C-H bond of alkane at the peaks of 877.61 and 2924.09 cm^{-1}, while the bending of the C=C bond of the alkane was found at the wavelength numbers 721.38 and 831.32 cm^{-1}. The peak at 1708.93 cm^{-1} indicated stretching vibration of the bond C=O, which was attributed to the indication of aliphatic compounds. The peaks at 1026.13, 1076.28, and 1143.79 cm^{-1} may be due to C-O. The presence of C-N has been attributed to the absorbance at 1143.79 cm^{-1}. Furthermore, the wave numbers 1409.96, 2924.09, 1371.39, and 3348.42 cm^{-1} are indicative of vibration bending or stretching in the bonds of the hydroxyl group (OH), associated with the presence of carboxylic acid. The NH vibrations at 1371.39 and 3348.42 cm^{-1} may be linked to the expansion vibration of an amine or aliphatic group (Fig. 2).

Chitosan exhibits excellent physico-chemical properties and specific interactions with proteins, cells, and living organisms and because of these properties the newly synthesized CStNPs and CSdNPs successfully loaded stevia and stevioside to target tissues such as the liver to complete metabolism with less risk factors. For this aim, the results of this study were supported with in vitro release stevioside analysis. Figure 3 shows the retention time of Sd in standard and samples at 293 nm measured by HPLC and the St release profile from the Sd loaded CSdNPs (3 formulations) differences in pH of experiment. The profile is compared with the dissolution profile of the free Sd in the same pH media and an equal concentration. The free Sd dissolution was 89% within two hours in comparison to the release profile of CNPs, CStNPs and CStNPs formulations within the same time, which were 45%, 35%, and 22%, respectively.

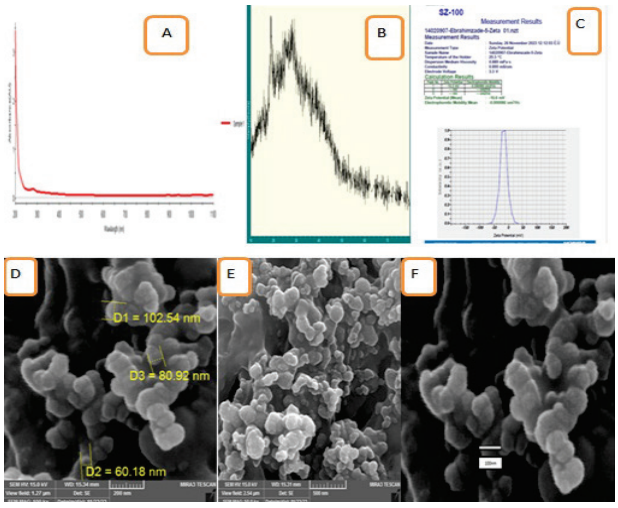

Fig. 1. Characterization of CNPs, CStNPs and CSdNPs

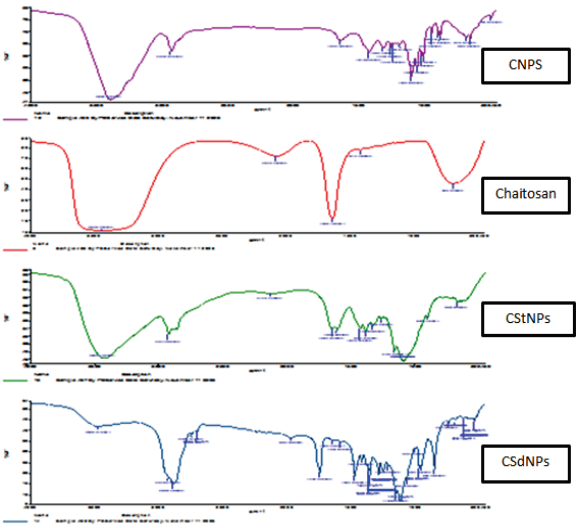

Fig. 2. FT-IR spectrum of CNPs, chitosan, CSdNPs, and CStNPs

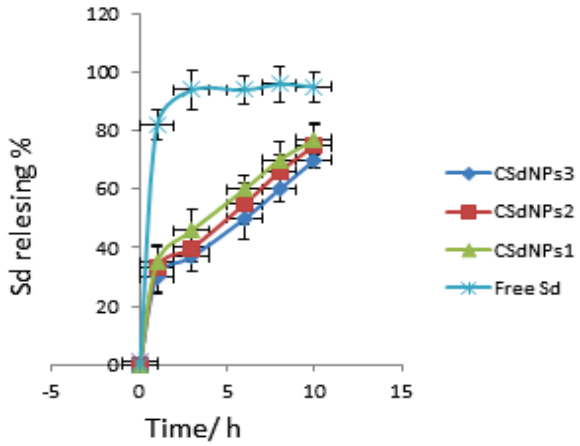

Fig. 3. In vitro release profile of Sd from CSdNPs at 37 ºC (x ± SD, n = 3)

The results of this study showed that there is a significant difference ($P < 0.05$) in ALT levels between the control group and St, free Sd, groups at the end of 1st month and the end of 2nd month of experiment: 46.7 ± 1.5, 70.4 ± 2.3, and 97.4 ± 4.2 IU/L, respectively (Table 3).

Table 3
Liver parameters levels at the end of second month of the study groups (x ± SD, n = 10)

Parameter	Control group	Stevia group (St)	Stevioside group (Sd)
Alanine transaminase (ALT), IU/L	46.7 ± 1.5[a]	70.4 ± 2.3[b]	97.4 ± 4.2[c]
Aspartate transaminase (AST), IU/L	88.3 ± 2.4[a]	100.2 ± 3.2[b]	136.9 ± 4.9[c]
Alkaline phosphatase (ALP), IU/L	113.7 ± 6.7[a]	127.8 ± 6.3[b]	242.3 ± 9.6[c]
Total serum bilirubin (TSB), mg/dL	0.311 ± 0.041[a]	0.378 ± 0.024[a]	0.544 ± 0.092[b]

Note: see Table 2.

The results of this study showed that there is a significant difference ($P < 0.05$) in AST levels between control group and St, free Sd, groups at compared in the end of 1st month (87.1 ± 2.2, 92.4 ± 3.9, and 102.4 ± 5.2 IU/L) and the end of 2nd month of experiment (90.3 ± 2.4, 100.2 ± 4.1, and 136.9 ± 4.4 IU/L, respectively).

A significant difference ($P < 0.05$) in ALP (IU/L) levels was found between the control group and St, free Sd, groups at the end of 1st month (110.1 ± 4.8, 121.1 ± 5.9, and 198.2 ± 8.8 IU/L) and the end of 2nd month of the experiment (113.7 ± 6.7, 127.8 ± 6.3, and 242.3 ± 9.6 IU/L, respectively).

A significant difference ($P < 0.05$) in TSB (mg/dL) levels was found between the control group and St or free Sd groups at the end of 1st month (0.30 ± 0.02, 0.33 ± 0.09, and 0.43 ± 0.02 mg/dL) and the end of 2nd month of experiment (0.311 ± 0.041, 0.378 ± 0.024, and 0.544 ± 0.092 mg/dL, respectively, Table 3).

The results obtained in the current study suggested that there is a significant difference ($P<0.05$) in TC (mg/dl) levels between control group and St or free Sd groups at the end of 1st month (81.1 ± 3.1, 95.0 ± 4.3, and 146.0 ± 5.9), respectively. In addition, we observed highly significant differences ($P < 0.001$) between the Sd group (in comparison with CNPs, CStNPs, and CSdNPs groups at the end of experiment (85.0 ± 3.8, 99.0 ± 4.1, and 104.4 ± 6.1, Table 4).

Table 4
Lipids profile levels at the end of 2nd month of the study groups (x ± SD, n = 10)

Parameter	Control group	Stevia group (St)	Stevioside group (Sd)
TC, mg/dL	81.1 ± 3.1[a]	108.0 ± 4.3[b]	146.0 ± 5.9[c]
TG) mg/dL	56.4 ± 2.3[a]	78.4 ± 3.5[b]	130.1 ± 7.1[c]
HDL-C, mg/dL	50.1 ± 3.1[a]	56.8 ± 4.2[b]	31.8 ± 7.1[c]
LDL-C, mg/dL	19.7 ± 1.1[a]	35.5 ± 1.9[b]	88.6 ± 4.4[c]
VLDL-C, mg/dL	11.3 ± 0.9[a]	15.7 ± 1.3[b]	26.0 ± 2.2[c]

Note: see Table 2.

The data obtained by our research suggested that there is a significant difference ($P < 0.05$) in TG (mg/dL) levels between the control group (53.4 ± 2.8) and St or free Sd groups at the end of 1st month (63.6 ± 3.1, and 112.0 ± 6.2, respectively). In addition, highly significant differences ($P < 0.001$) were observed between the Sd group (in comparison with CNPs, CStNPs, and CSdNPs groups at the end of experiment (61.2 ± 2.3, 64.4 ± 2.9, and 98.7 ± 4.3).

A significant difference ($P < 0.05$) was found in HDL-C (mg/dL) levels between control group (52.4 ± 3.1) and St or free Sd groups at the end of 1st month (55.8 ± 4.1, and 39.9 ± 2.0, respectively). On the other hand, highly significant differences ($P < 0.001$) were found between the Sd group and CNPs, CStNPs, and CSdNPs groups at the end of experiment (53.6 ± 3.02, 67.8 ± 4.43, and 68.1 ± 4.59).

The results obtained by the current study suggested that there is a significant difference ($P < 0.001$) in LDL-C (mg/dL) levels between the control group (14.8 ± 1.1) and the St or free Sd groups at the end of 1st month (29.5 ± 2.3, and 66.7 ± 4.2), respectively. In addition, highly significant differences ($P < 0.001$) were found between the Sd group (in comparison with CNPs, CStNPs and CSdNPs groups at the end of experiment (19.2 ± 2.2, 18.3 ± 1.2, and 16.6 ± 1.3, respectively).

The results of our research indicated that there is a significant difference ($P < 0.001$) in VLDL-C (mg/dL) levels between the control group (10.7 ± 0.1) and the St or free Sd groups at the end of 1st month (12.7 ± 2.4, and 22.4 ± 3.1), respectively. In addition, we recorded highly significant differences ($P < 0.001$) between the Sd group (in comparison with CNPs, CStNPs and CSdNPs groups at the end of experiment (12.2 ± 1.1, 12.9 ± 1.2, and 19.7 ± 2.1) (Table 4, Fig. 4 and 5).

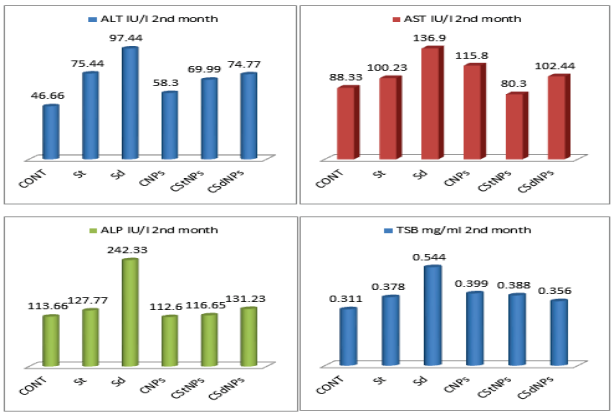

Fig. 4. Liver parameters levels in study groups

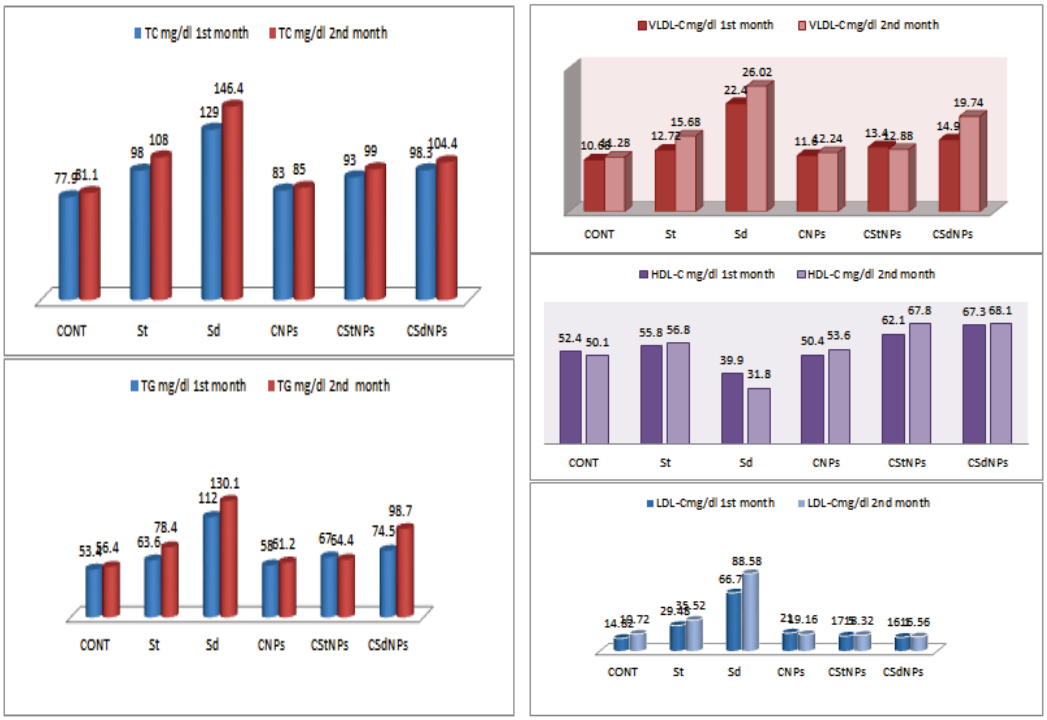

Fig. 5. Lipids profile parameters levels in study groups

The data obtained in the presented study suggested that there is a significant difference ($P < 0.05$) in RBCs ($x10^6/mm^3$) between control group (5.49 ± 0.19) and the free Sd groups at the end of 2nd month (7.72 ± 0.21) but no statistical differences ($P > 0.05$) between control and the St group (5.05 ± 0.09). In addition, we noted highly significant differences ($P < 0.001$) between the Sd group (7.72 ± 0.21) in comparison with CNPs, CStNPs and CSdNPs groups at the end of experiment (4.72 ± 0.05, 5.42 ± 0.05, and 6.28 ± 0.08, Table 5).

Table 5
Blood parameters in study groups (x ± SD)

Groups	RBCs, 10^6/mL	WBCs, 10^3 IU/L	Hb, g/dL	PCV, g/dL
Control	5.49 ± 0.19^a	7.88 ± 0.78^a	11.66 ± 1.99^a	36.44 ± 2.79^a
St	5.05 ± 0.09^a	8.23 ± 1.09^b	9.77 ± 2.21^b	38.44 ± 2.71^b
Sd	7.72 ± 0.21^b	9.89 ± 1.23^c	8.54 ± 2.23^c	46.32 ± 2.98^c
CNPs	4.72 ± 0.05^c	7.74 ± 0.89^a	11.04 ± 1.77^a	36.87 ± 1.23^a
CStNPs	5.42 ± 0.05^a	7.99 ± 0.78^a	10.92 ± 1.98^d	37.53 ± 2.77^b
CSdNPs	6.28 ± 0.08^d	8.01 ± 1.01^d	11.99 ± 1.82^a	40.22 ± 2.54^d

Note: see Table 2.

The findings of the presented research suggested that there is a significant difference ($P < 0.001$) in FAS (pg/mL) levels between the control group (494.2 ± 15.8) and the St or free Sd groups at the end of 2nd month (511.6 ± 16.2 and 561.7 ± 17.2), respectively. In addition, we found highly significant differences ($P < 0.001$) between the Sd group (561.7 ± 17.2) in comparison with the CNPs, CStNPs and CSdNPs groups at the end of the experiment (513.5 ± 12.3, 493.9 ± 11.4, and 527.3 ± 12.5, Fig. 6).

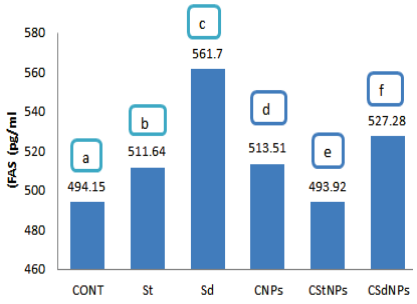

Fig. 6. FAS (pg/mL) levels of all study groups at 2nd months period (different small letters (*a–f*) indicated highly significant differences at $P < 0.001$)

We investigated the gene expression of PRPA-α mRNA by quantitative RT-PCR analysis and estimation the average fold change (AFC) of expression of this gene in all study groups. The findings of molecular examination revealed a significant variation in values of the amplification plot of PRPA-α mRNA as target gene as well as GAPDH as housekeeping gene as shown in Figure 7.

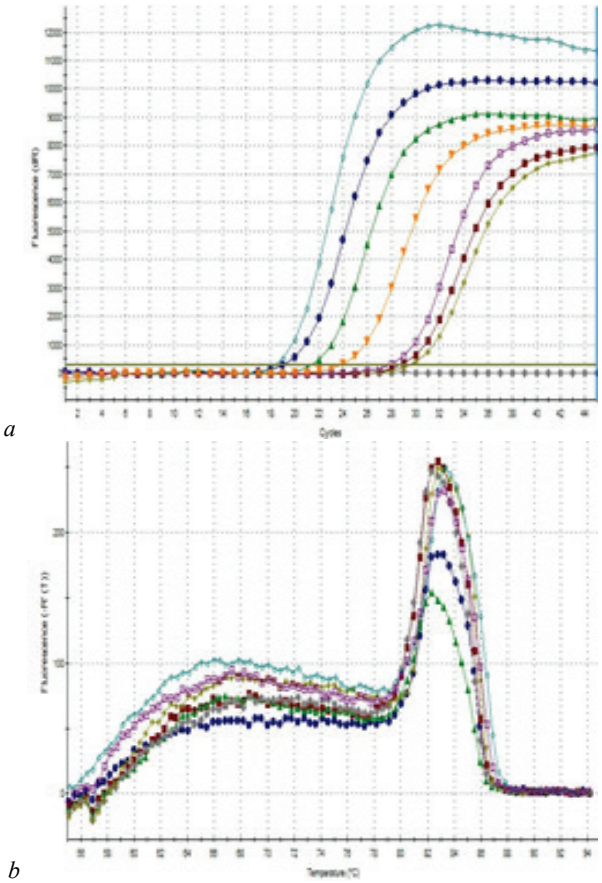

Fig. 7. Amplification plots (*a*) and melting curves (*b*) of PRPA-α mRNA and GAPDH genes generated byMx3005P Stratagene system

The average fold change (AFC) was assessed in all study groups by using the following formula: AFC = 2 – ΔΔCt = 2 – (Ct of GOI–Ct of HKG gene) treated-(Ct of GOI–Ct of HKG gene) untreated.

For example, AFC (St group) = 2 - (Ct of PRPA-α –Ct of GAPDH) St - (Ct of PRPA-α – Ct of GAPDH) CONT.

Our results suggested that there is a significant difference ($P < 0.001$) in AFC between the control group (5.86 ± 0.58) and St or free Sd groups at compared in the end of 2nd month (3.00 ± 0.22, and 1.86 ± 0.12), respectively. In addition, highly significant differences ($P < 0.001$) were found between the Sd group (1.86 ± 0.12) in comparison with CNPs, CStNPs and CSdNPs groups at the end of the experiment (4.98 ± 0.25, 3.91 ± 0.24, and 4.02 ± 0.45), as shown in Figure 8.

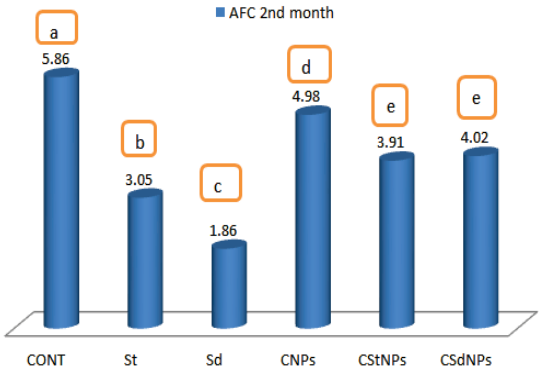

Fig. 8. AFC in study groups at the end of experiment 2nd month (different small letters (*a–e*) indicated highly significant differences at $P < 0.001$)

Discussion

Many studies have suggested the benefits of stevia extract and its product stevioside over sugar and artificial sweeteners, but it is still not a very popular sugar substitute and there is still a lack of knowledge of the risks of its daily consumption instead of table sugar especially by diabetics. This study was successful in the new synthesis of CStNPs and CSdNPs and with acceptable characterization of these NPs to reduce the expected risk of free St and Sd. The results of the (XRD) of Sd loaded on chitosan-TPGS-NPs. The XRD pattern shows broad peaks, indicating that the St-loaded CNPs have a mostly amorphous structure and this means that nanoparticles lack a long-range arrangement of atoms and the lower crystallinity of Sd in nanoparticles compared to pure Sd indicates that it is well dispersed within the chitosan matrix. This may be due to interactions between Sd and chitosan molecules, which prevent Sd molecules from forming a highly ordered crystalline structure. SEM analysis is another useful tool for the surface morphology of synthesized chitosan nanoparticles (CNPs, CStNPs, and CSdNPs). From the surface SEM micrographs it appears that the crosslinked chitosan was gradually produced after cross linking with sodium tripolyphosphate in alkaline solution.

The results confirm the crosslinking of chitosan with stevia extract and stevioside and agree with other studies in the same line such as (Hamza et al., 2022). The particles of self-assembled chitosan were found to have a size range from 277 to 731 nm. In the study of Nguyen et al. (2016) CNPs size showed a mean between 166 and 1230 nm when synthesized through ionotropic gelation with tripolyphosphate (TPP) coupled to spray dryer. The CNPs had an average size distribution of between 300–750 nm, according to the results obtained by Ha et al. (2018), using the LMS taken under normal conditions. The TEM image also revealed a comparatively rugged texture on the surface of the chitosan nanoparticles. The CNPs biosynthesized from Pelargonium graveolens as per El-Naggar et al. (2022) were described to be spherical and well dispersed. Larger CNPs are difficult to transport throughout biological membranes; however, smaller-sized particles can achieve this effectively and help deliver the drug payload in a controlled fashion (Duraisamy et al., 2022).

The smaller the particle, the more likely higher payloads and storage stability will be possible on a drug, increasing the absorption potential as well as improving parenteral delivery times (Slimani et al., 2022). The present results were agreement of many studies on chitosan as a polymer for extracts and drugs loading (Badwan et al., 2015; Garg et al., 2019; Yanat & Schroën, 2021). A new study has suggested that the chitosan-based drug delivery systems have been developed for various routes of administration, including oral, ophthalmic, transdermal, nasal, and vaginal, allowing targeted and sustained release of drugs (Desai et al., 2023). The present study shows significant effects of St and Sd on ALT (IU/L) in serum of male rats that were orally administered daily doses of both. It is important to note that many factors can influence liver health, including an individual's overall diet, lifestyle, and any pre-existing medical issues (Ramos-Tovar et al., 2019). A few studies have explored the potential hepato-protective effects of stevia in animal models, suggesting that it may exert antioxidant and anti-inflammatory effects (Kurek & Krejpcio, 2019). In a study by Abou Zaid et al. (2019), chitosan nanoparticles formulations showed decrease in ALT levels compared to free St and Sd groups and this is supported by other studies which reported that CNPs stimulates sustained release of drugs loading and decrease the risk factors related to overdose administration. Another of these antioxidant products is an important polysaccharide of marine origin, chitosan, which is prepared from the exoskeletons of crustaceans.

Hyaluronate has gained significant attention as a biomedical material, due to its anti-tumor (Jeon et al., 2003), immune-stimulatory (Neimert-Andersson et al., 2011) and antimicrobial activity (Ong et al., 2017). This is observed to be in agreement with previous findings, which had reported that chitosan nanoparticle treatments exerted the same effect on diabetic rats through its capability of reducing oxidative stress and enhancing endogenous antioxidant defenses (Wang et al., 2016). On the other hand, the decrease in liver markers observed with chitosan may be partly due to antioxidant compounds involved in chitosan and stevia that help to reduce liver injury. Ozdek et al. (2023) reported that the administration of chitosan can strengthen the antioxidant defense system of liver tissue and may decrease oxidative stress.

The results for lipid profile such as TC (mg/dL) are supported previously by the results of liver enzymes as discussed above and show an increase in levels of ALP (IU/L). Consumption of St or Sd at higher doses may stimulate the activation of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA) and stimulate hepatic cholesterol synthesis. The results of the present study are not in same line with other studies (Curry & Roberts, 2008; Abo Elnaga et al., 2016; Brijesh & Kamath, 2016) that suggest hypolipidimia activity of St. In contrast, a study by Iman (2011) showed significant effects of St on lipid profile. Sharma et al. (2023) reported that stevia extract has a hypolipidaemic effect and can be used to reduce the risk of CVD in future. The results of our research concur with those of a study from Pakistan (Ahmad et al., 2020). A study conducted by Abo Elnaga et al. (2016) investigated the impact of stevia aqueous extract on the hematology of female rats.

The results indicated that the extract led to an increase in the hematology of female rats. These findings were consistent with previous research and suggested that there were no significant changes in various blood parameters among the different groups of rats, except for a decrease in MCHC and MPV in the stevia sweetener groups compared to the negative control group. While stevia and its derivatives have been associated with numerous health benefits (Rojas et al., 2018; Kurek & Krejpcio, 2019), their effects on hepatic metabolism and autophagy are still not fully understood. Therefore, the primary objective of this study was to explore the influence of stevia derivatives on hepatic fatty acid metabolism in rats. A prior study published by (Kurek & Krejpcio, 2019) demonstrated regulatory effects of the supplementation of rebaudioside A, steviol glycosides, and stevioside on the blood lipid profile (they normalised elevated serum triacylglycerols, total cholesterol and low-density lipoprotein cholesterol concentrations) in high-fat (HF) fed streptozotocin-induced diabetic rats. These changes in FAS (pg/mL) levels in different study groups may be due to St and Sd which are daily consumed and accumulation of its by-products in the blood and in finally in the liver and effects on gene expression of FAS gene or on some transcription factors involved on FAS protein synthesis. Some previous studies such as (Matsusue et al., 2004) show that the hepatic expression of this gene determines a number of processes in the course of diabetes as it is crucial for lipid and glucose metabolism. On the other hand, Park et al. (2022) reported the effects of *Stevia*, a low-calorie sweetener, and stevioside on hepatic steatosis and autophagy in

hepatocytes, as well as in db/db mice, which ultimately show reduced the body and liver weight and levels of serum triglyceride, total cholesterol, and hepatic lipogenic proteins.

Tao et al. (2019) suggested that the nonalcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease closely associated with metabolic syndrome, but there are no validated pharmacological therapies. The present study aimed to investigate the effect of chitosan nano formulations on NAFLD. From the results of the present study, increase in FAS (pg/ml) and other lipid profile parameters indicate induced hepatic fat accumulation by accelerating lipogenesis and depressing fatty acid oxidation and these results are in agreement with results of other studies (Nakamura & Terauchi, 2013; Lv et al., 2019). On the other hand, the present results show that the CStNPs and CSdNPs formulations modified the AFC of PPAR-α and statistically increased the gene expression to be near to the control group. A study on curcumin-loaded chitosan nanoparticles suggested that this was more effective at inhibiting oxidative damage and controlling lipogenesis and pyroptosis in the hepatic tissues of FEN-exposed rats (Alqahtani et al., 2023). Coll et al. (2009) found that PPAR-α is widely expressed and has a significant impact on the regulation of energy balance and the breakdown of lipoproteins. This research suggests that PPAR-α plays a crucial role in controlling lipid and glucose metabolism (Bensinger & Tontonoz, 2008).

The present study focused on the synthesis and characterization of bioactive molecule-loaded chitosan nanoparticles (CStNPs and CSdNPs) with respect to their hepatoprotective potential. Their actions were evaluated in experimental rats with free St and Sd-induced steatotic fatty liver disease. An important factor in the onset of early NAFLD and then nonalcoholic steatohepatitis NASH is the modulatory role played by the PPAR-α (Todisco et al., 2022). Multiple research studies have failed to examine how stevia and stevioside impact the liver function of male rats. Our discoveries indicate that these substances could impact liver enzymes, markers of inflammation, and the general health of the liver. This study suggested that stevia and stevioside may interact with metabolic pathways related to liver function in a mechanistic way in model male rats.

Conclusion

In conclusion, the St extract and especially free Sd have high risk factor for liver fatty (NALFD) and hyperlipidemia in blood of rats when administered for a period of 2 months. This study suggests that Sd consumption decreases the gene expression fold (AFC) of the transcription factor (PPAR-α) that controls β-oxidation of fatty acids and promotes fatty acid synthesis through activation of FAS.

Authors declare no conflict of interest recorded in this study.

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