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Original Article

Stevia rebaudiana loaded titanium oxide nanomaterials as an antidiabetic agent in rats

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ABSTRACT

Stevia rebaudiana (Bertoni) Bertoni, Asteraceae, is a plant with hypoglycemic and antihyperlipidemic properties. *S. rebaudiana* (SrB) has become a lead candidate for the treatment of the diabetes mellitus. However, chronic administrations of *S. rebaudiana* are required to cause the normoglycemic effect. Importantly, nanomaterials in general and titanium dioxide (TiO_2) in particular have become effective tools for drug delivery. In this work, we obtained TiO_2 nanomaterials with SrB at different concentrations (10, 20 and 30 μM) by sol–gel method. After this nanomaterials were characterized by Fourier transform infrared spectroscopy and transmission electron microscopy. Where it was demonstrated, the presence of the *S. rebaudiana* in TiO_2 nanomaterials, which were observed as hemispherical agglomerated particles of different sizes. The nanomaterials were evaluated in male rats whose diabetes mellitus-phenotype was induced by alloxan (200 mg/kg, *i.p.*). The co-administration of TiO_2-SrB (20 and 30 μM) induced a significant and permanent decrease in the glucose concentration since 4 h, until 30 days post-administration. Likewise, the concentrations of insulin, glycosylated hemoglobin, cholesterol, and triacylglycerides showed a significant recovery to basal levels. The major finding of the study was that the TiO_2-SrB (20 and 30 μM) has a potent and prolonged activity antidiabetic. TiO_2 can be considered like an appropriated vehicle in the continuous freeing of active substances to treat of diabetes mellitus.

Introduction

Traditional medicine principally derived from plants and currently represents a therapeutic potential for Diabetes mellitus (DM) (Laitiff et al., 2010; Malviya et al., 2010).

Stevia rebaudiana (Bertoni) Bertoni (SrB), also known as "sweet herb," is a shrubby perennial plant belonging to the Asteraceae family (Soejarto et al., 1982). SrB sheets containing approximately 4–15% stevioside, which have been isolated and characterized by chemical and spectral studies (Starratt et al., 2002).

SrB has various pharmacological properties including antioxidant activity, antihypertensive, antihyperlipidemic and cardiovascular protector (Chan et al., 1998). Recent studies have shown that the SrB stimulates insulin secretion from pancreatic β-cells and cause normolipidemia and normoglycemia in diabetic rats (Raskovic et al., 2004; Chen et al., 2005). In this sense, SrB provides evidence of his traditional use in the control of DM. However, it has been demonstrated that are required continuous administrations of SrB to achieve normoglycemic effect (Curry and Roberts, 2008). One way to address this problem is, to develop a controlled release system of SrB, in order to reduce the dose and make available SrB in the target sites and keep its prolonged activity. A variety of systems have been developed and used, including liposomes, micelles, dendrimers, and copolymers.

Currently, nanomaterials of titanium dioxide (TiO_2) have attracted attention as delivery potential systems (Barb et al., 2004). The TiO_2 nanomaterials are chemically inert, possess hydrophilic features and its synthesis isn't complicated, in addition, these present high mechanical strength and low toxicity (Son et al., 2007). Recently the nanomaterials have been implanted in the amygdala of rats with epilepsy and obtained optimal results, and found that the porous TiO_2 matrix release their contents in a controlled manner

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for a period approximately of 500 h (h) (López et al., 2010a,b). Here, we performed the synthesis of TiO_2 nanomaterials from solutions of titanium *n*-butoxide and the aqueous extract of SrB concentrate obtained from the dried leaves of the plant. This with the purpose of using it as a controlled release system SrB extract. For the above, this study aimed to evaluate the antidiabetic activity of intraperitoneal injection of TiO_2-SrB nanomaterials in a diabetic rat model.

Materials and methods

Preparation of SrB-extract

Stevia rebaudiana (Bertoni) Bertoni, Asteraceae, was grown on campus grounds of the Benemérita Universidad Autónoma de Puebla (BUAP), México. Dr. Albino Moreno-Rodríguez confirmed the identification, additionally; a sample was deposited in the botanical garden of the BUAP (file 21-09/14). Leaves SrB (10 g) washed and dried in an Elisa-550 oven for 24 h. Then it pulverized and macerated with a solution of ethanol and water (with a ratio of 80:20). The solution obtained from the mashing process is filtered and the SrB aqueous extract is placed on a rotary evaporator of the ESEVE-402-2 brand to eliminate to 80% of solvent (water and alcohol) under reduced pressure. Thus we obtain the SrB concentrated extract to 80 wt.

Preparation of TiO_2-SrB nanomaterials by sol–gel method

The homogeneous solutions were prepared separately with different concentrations of the SrB concentrated extract (10, 20 and 30 μM). Each homogeneous solution containing 150 ml of anhydrous 1-butanol (99.8%, Sigma–Aldrich), 10 ml of deionized water and 0.5 g of polyvinylpyrrolidone (with an average molecular weight of 40,000, Sigma–Aldrich) and the required concentration of the SrB concentrated extract (10, 20 and 30 μM).

For each homogeneous solution was added 21.5 ml of titanium *n*-butoxide (97%, Sigma–Aldrich) in a reflux system at 70 °C with constant agitation. The final solution with properties of gel was immersed in a container with ice for 15 min at 3 °C. The solvent was removed on a rotary evaporator at 50 °C under vacuum conditions to finally obtain the TiO_2-SrB nanomaterials (López et al., 2010a,b).

Characterized of TiO_2-SrB nanomaterials

Infrared spectroscopy (FTIR)

The TiO_2-SrB nanomaterials were mixed with KBr (5 wt %) and pressed in transparent wafers. Fourier transform infrared spectroscopy was recorded using a Perkin-Elmer 1600 spectrophotometer (Perkin-Elmer, Shelton, CT) in the 4000–400 cm^{-1} range, and 32 scans were run for each measurement.

Scanning electron microscopy (SEM)

The particle size was measured using conventional Scanning electron microscopy (SEM, Zeiss; Carl Zeiss, Oberkochen, Germany), operated at 100 kV, with entry goniometer at the side and 0.4 nm point-to-point resolution, and attached to a CCD camera (MegaVision, Santa Barbara, CA).

Animals

Adult male Long Evans rats (230–250 g) were obtained from Bioterio "Claude Bernard" BUAP. Animals were individually housed in an environment with controlled temperature, humidity, and light conditions (12 h light: 12 h dark cycle), with free access to food and water. All procedures described in this study were performed in accordance to the Mexican Law of Animal Treatment and Protection Guidelines (NOM-062-ZOO-1999) and the Research Committee uses of laboratory animals of the BUAP (VIEP-3450-2013).

Induction of diabetes in rats

The animals were injected with alloxan dissolved in 0.1 M citrate buffer, pH 4.5 at a dose 200 mg/kg body weight (*i.p.*). The alloxan dose was selected based on various reports (Raskovic et al., 2004; Szkudelski, 2001). Three day after of the administration of alloxan, blood samples were taken and blood glucose levels were determined. Animals that had higher concentrations of glucose (150 mg/dl) were considered diabetic.

SrB-TiO_2 administration protocols

In the experiment a total of forty rats were used, which were divided in five groups ($n = 8$ per group).

Group 1: normal rats treated with TiO_2 nanomaterials (vehicle group)
Group 2: diabetic group treated with TiO_2 nanomaterials
Group 3: diabetic rats with TiO_2-SrB nanomaterials to 10 μM
Group 4: diabetic rats with TiO_2-SrB nanomaterials to 20 μM
Group 5: diabetic rats with TiO_2-SrB nanomaterials to 30 μM

The nanomaterials were injected by *i.p.* The route in all animals depending of treatment.

Subsequently, both the empty TiO_2 nanomaterials (1 g/kg in sterile water at 37 °C) and SrB (10, 20 and 30 μM) (1 g/kg in sterile water at 37 °C) were administered, respectively.

Method for determination of plasma blood glucose level

To evaluate the plasmatic concentration of glucose with respect at course of time in the animals injected with SrB nanomaterials, the animals were deprived of the food for 5 h before the determination. The blood glucose levels were determined at 0, 4, 8 and 24 as well as at 5, 10, 15 and 30 days after administration of each treatments. In the experiment the blood glucose level of the animals were estimated by Glucose Oxidase–Peroxidase Enzymatic Method using a digital glucometer (ACCU-CHEK brand active). Blood from the tail vein was collected.

Evaluation of the effect of TiO_2-SrB nanomaterials on hyperglycemia and hyperlipidemia induced by alloxan

At 31 days post-injection of TiO_2 and TiO_2-SrB, the animals of each experimental group were sacrificed by dislocation and by cardiac puncture. The blood samples were collected to measure glucose, insulin, glycated hemoglobin, cholesterol and triacylglycerides. Collected samples were centrifuged at $2 \times g$ for 5 min. Serum determinations of glucose, cholesterol and triacylglycerides were developed by enzymatic colorimetric assay, following the protocols respectively labeled according the BioSystem kits and analyzed in a semi-automated spectrophotometer (Bayer RA-50). Plasma insulin concentration was determined by an ELISA immunoassay (Diagnóstica Internacional), and antibody-antigen complex was determined at 415 nm in a Stat fax 2600 plate reader (WinerLab group). The glycated hemoglobin measuring was carried out by immunofluorescence method, following the protocol according the i-Chroma kit and it was analyzed in a detector of the same brand.

Statistical analysis

The data were expressed as mean ± standard error (SE) for all experiments. Statistical analysis was developed through of ANOVA

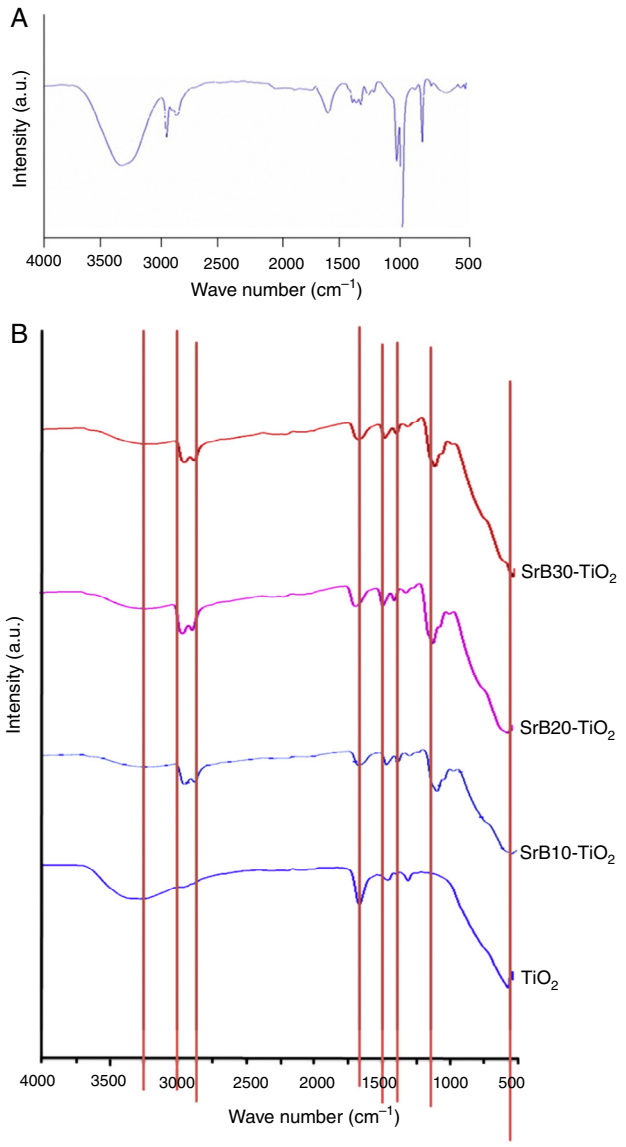

Fig. 1. (A) FTIR spectrum of SrB extract 80 wt%. (B) FTIR spectra of TiO_2, SrB/TiO_2-10, SrB/TiO_2-20 and SrB/TiO_2-30 nanomaterials.

and a confidence level of 95% and, when necessary, the means were compared using the Bonferroni test.

Results

Characterization of TiO_2-SrB nanomaterials

Infrared Spectroscopy (FTIR)

The FTIR spectrum of SrB extract is presented in Fig. 1A. The absorption band located at 3383 cm^{-1}, it corresponding to the stretching vibration mode ν_{OH}, which identifies the hydroxyl groups (OH^-), of water (H—OH), and the alcohol (ethanol, R—OH). The stretching vibration modes $\nu_{CH\ CH}$, of chemical species C—H are located to 2988 cm^{-1}. Mode ν_{CH} of asymmetric stretching vibration it locates at 2900 cm^{-1}. The vibration band corresponding to the bending type interactions mode ν_{OH} of the OH-groups, it is assigned of the surface hydroxyl groups of the material, to solvent and the coordinated deformation δ_{HOH} of Water be located at 1630 cm^{-1}. The wavenumber to 1425 cm^{-1} and 1410 cm^{-1}, they corresponding to the symmetrical stretching vibration ν_{COO} of the COO groups and deformation δ_{CH3} vibration of modes. The vibration bands corresponding to the asymmetric stretching vibration type ν_{COO} of carboxylate ions are located at 1593 cm^{-1}.

The IR spectrum of TiO_2 nanomaterials shows in the Fig. 1B. The absorption band at 3,339.1 cm^{-1}, correspond to the stretching vibration mode ν_{OH}, which identifies the hydroxyl groups (OH^-) of water (H—OH), the solvent (butyl alcohol, R—OH) and hydroxylation of gel (Ti—OH). The asymmetric stretching vibration modes of CH is located at 2939.2 cm^{-1}, correspond to the chemical species of the methyl ($-CH_3$) (Ti—O—CH_3) and ethoxy groups ($\equiv$Ti—OCH_2CH_3).

The bending mode (ν_{OH}) localized to 1635.9 cm^{-1}, correspond to OH groups of water, which are present on the surface of TiO_2 nanomaterial. They are mainly associated with the humidity of the nanomaterial, the solvent and the coordinated deformation mode of water. In the range (from ~1400 cm^{-1} to ~1300 cm^{-1}) of electromagnetic radiation, are localized the bending vibration of symmetrical type of the COO^- groups, the vibration asymmetric deformation of the CH groups and the vibration mode of the deformation scissor of the δ_{CH3} of the CH_3 groups. In the near infrared region, the vibration modes ν_{Ti-O} type bending of the metal-oxygen interaction, they localized at 745.3 cm^{-1}, to 640.2 cm^{-1} and to 498.8 cm^{-1}. The TiO_2 nanomaterials doped with 20 μM and 30 μM of the extract SrB, present similar stretching vibration modes: (ν_{OH}) and (ν_{CH}) and bending vibration modes: (ν_{OH}), (δ_{HOH}), (ν_{COO}), (ν_{CH}), (ν_{CH3}), (δ_{CC}), (ν_{CO}) and (ν_{Ti-O}), as than those observed in the TiO_2 nanomaterial undoped.

The decrease in intensity of the absorption bands, mainly of OH^- vibration modes corresponding to the humidity (at 1635.9 cm^{-1}) of SRB-TiO_2 with 10, 20 and 30 μM nanomaterials, may be due to the interaction to SrB aqueous extract with the surface of TiO_2 nanoreservoir, as shown in scheme of Fig. 2. It is also important to note that under these experimental conditions is impossible to talk about the formation of polymorphs of TiO_2 relative to the concentration of the aqueous extract of SrB.

Scanning electron microscopy (SEM)

The Fig. 3A, show the SEM studies of TiO_2 nanomaterial with a traced area to 100,000 magnifications. The texture of the TiO_2 has shown the form of agglomerated particles with an average particle diameter of 100 nm. The micrograph of TiO_2 nanomaterial with SrB-10 μM; present in a swept area to 100,000×. The micrograph of the TiO_2-SrB-10 μM nanomaterial present in a swept area of 100,000 magnifications, formless particles with an average area of 70 nm

Fig. 3B, show the micrograph of the nanomaterial of TiO_2 with 20 μl of SrB (SrB-TiO_2-20 μl), which presented a swept area to 100,000 magnifications, it showed hemispherical type particle with an average area of 60 nm. Similarly, the Fig. 3C show the micrograph of SrB/TiO_2-30 nanomaterial with an average spherical particles size of 4 nm that are located within the matrix of TiO_2. In Fig. 3D, the increasing in the concentration of the aqueous extract of SrB in TiO_2 nanomaterials, we note that decreases the average particle size of nanomaterials of 100 nm (for TiO_2) to 4 nm (for SrB-Ti_2-30 μl and we concluded that the molecules of the aqueous extract of SrB were present in the mesh of TiO_2.

Effect of TiO_2-SrB nanomaterials treatment about hyperglycemia induced by alloxan

The results of body weight and blood glucose measurements are shown in Fig. 4A. As expected in the course of 30 days, the body weights were decreased in the diabetic group treated with TiO_2 but not in the normal rats treated with TiO_2. In hyperglycemic animals treated with nanomaterials with TiO_2-SrB the body weight was recovered of concentration-dependent manner. The animals treated with the nanomaterials TiO_2-SrB at 20 and 30 μM showed a similar weight to the vehicle group during the 4 weeks that developed in the experiment.

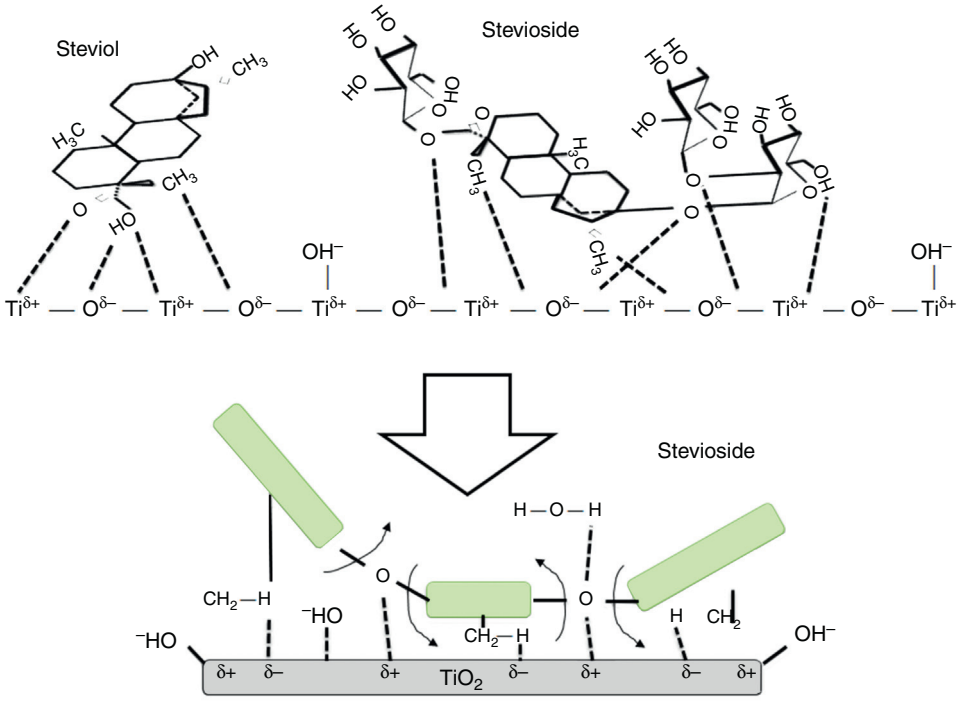

Fig. 2. Scheme of interaction to SrB with the surface of TiO_2 nanomaterials.

In treated rats with alloxan the blood glucose levels at 4, 8 or 24 h, increased (160–180 mg/dl), respect to the vehicle group (80.43 mg/dl) (Fig. 4B). Treatment with TiO_2-SrB nanomaterials in alloxan-treated animals showed a progressive decrease in glucose levels at 4, 8 and 24 h after administration, which, when it was compared with the alloxan + TiO_2 treated group, was observed a significant difference in the animals treated with TiO_2-SrB 20 and 30 μM (Fig. 4B). (One way ANOVA, Bonferroni post-test, $p < 0.05$).

Moreover, comparing the glucose concentration at 5, 10, 15 and 30 days in the hyperglycemic rats after administration of TiO_2-SrB, was observed, a significant decrease in the treated animals with TiO_2-SrB nanomaterials at 20 and 30 μM with respect to the alloxan + TiO_2 group (Fig. 4 C). In this regard, the decrease of glucose levels was observed during 30 days after administration of TiO_2-SrB at a concentration of 20–30 μM (one-way ANOVA, Bonferroni post-test, $p < 0.05$).

Laboratory studies, developed 31 days after administration of treatments, show a significant increase in the concentration of glucose (86%) of the hyperglycemic group + TiO_2, compared to the normal rats treated with TiO_2 (Fig. 5A and B). Consequently, the concentration of glycosylated hemoglobin in the alloxan + TiO_2 group, was higher (38%) compared to the normal rats + TiO_2 (Fig. 5C) (one-way ANOVA, post-Bonferroni test, $p < 0.05$).

Moreover, the animals of groups of alloxan + TiO_2-SrB 20 and 30 μM, had a lower concentration of glucose (20% and 56%) and glycated hemoglobin (25% and 41%) compared to the alloxan + TiO_2 group (Fig. 5A and C). Also, groups with alloxan + TiO_2-SrB 20 and 30 μM showed a higher insulin concentration compared to

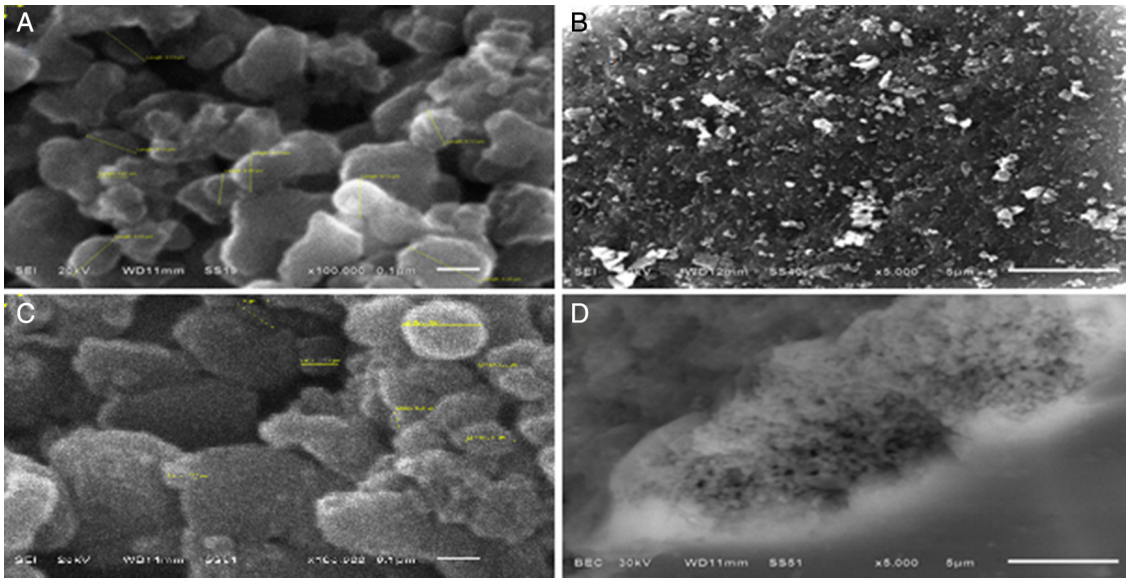

Fig. 3. (A) Micrograph of TiO_2. (B) SrB/TiO_2-10. (C) SrB/TiO_2-20, and (D) SrB/TiO_2-30 nanomaterials.

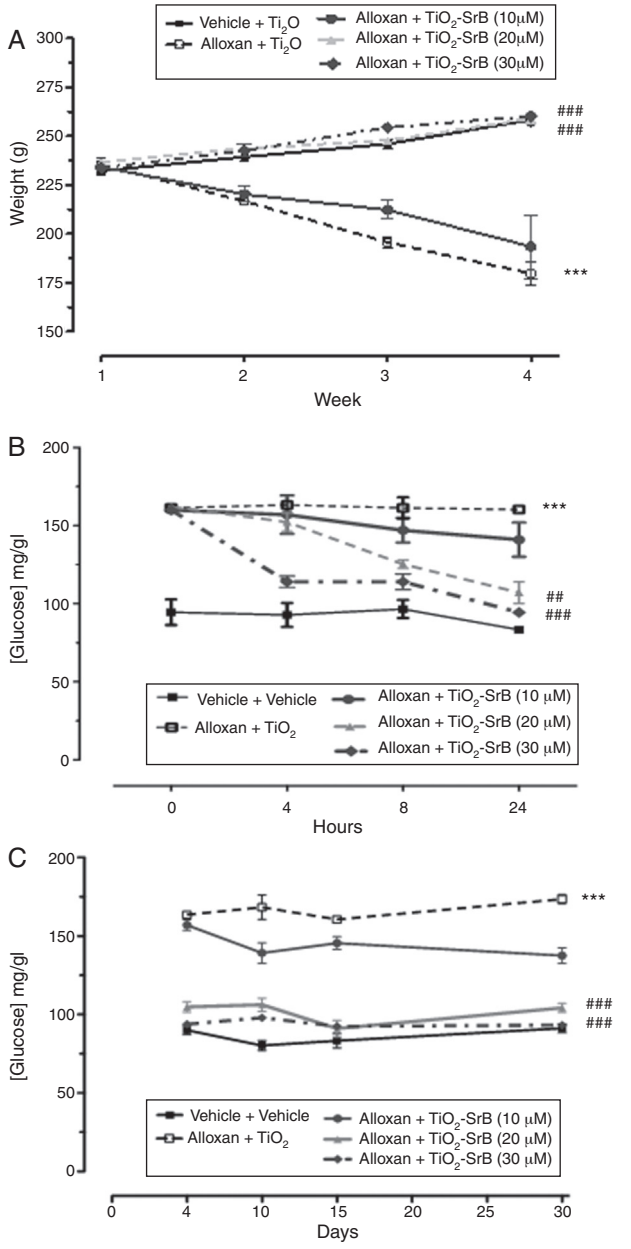

Fig. 4. Effect of administration of TiO_2-SrB nanomaterials on the weight and the time course of the glycemic levels in treated rats with alloxan. The animals were weighed every week for 4 weeks (A). The blood glucose levels were determined at 0, 4, 8 and 24 h (B), as well as 5, 10, 15 and 30 days (C) after administration of the nano-TiO_2 SrB (10, 20 and 30 μM) in diabetic rats (alloxan, 200 mg/kg). The vehicle group were administered with the vehicle, and with empty nanomaterials (Vehicle and alloxan + TiO_2) under the same experimental conditions ($n = 8$ per group). The data show the average weight (g) and glucose concentration (mg/dl) ± SE of each group respectively. (One-way ANOVA, Bonferroni post-test, $^{***}p < 0.001$: Vehicle vs alloxan + TiO_2; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$: alloxan + TiO_2-SrB vs alloxan + TiO_2).

the group with alloxan + TiO_2 (80% and 100%) (Fig. 5B). (One way ANOVA, Bonferroni post-test, $p < 0.05$). This showed for the first time, that nanomaterials TiO_2-SrB (20 and 30 μM) reversed hyperglycemic effect induced by alloxan, due to the biochemical parameters measured (glucose, glycosylated hemoglobin and insulin) are statistically similar to the vehicle group + TiO_2.

TiO_2-SrB nanomaterials treatment effect on hyperlipidemic effects induced by alloxan

The results show that the diabetic group treated with TiO_2 increased the cholesterol and triacylglycerides levels respect to

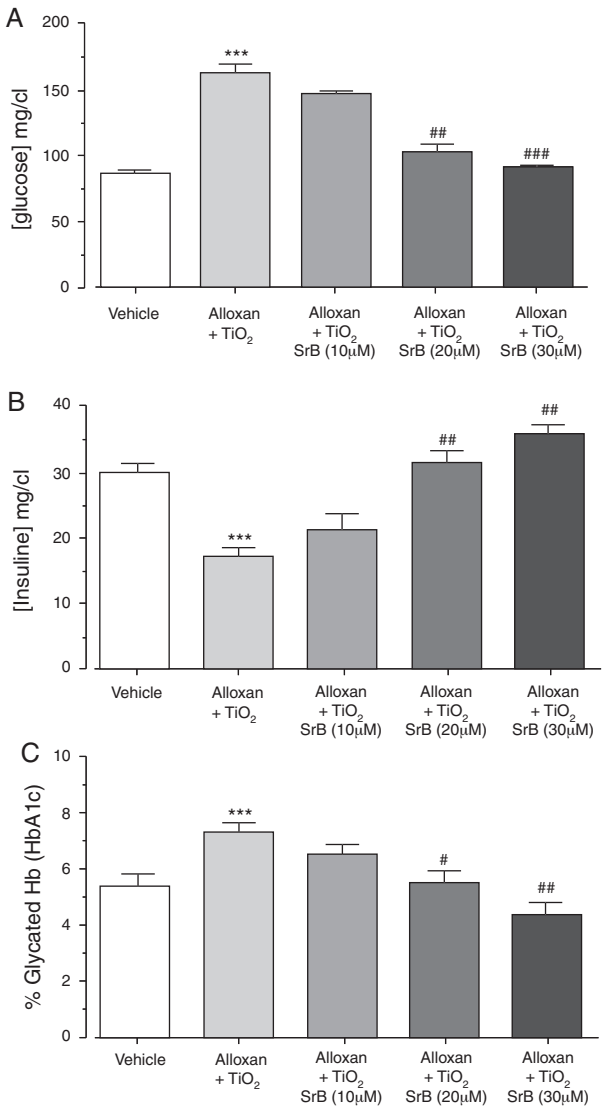

Fig. 5. Effect of administration of TiO_2-SrB nanomaterials on glucose, insulin and glycosylated hemoglobin in administered rats with alloxan. Quantification was performed in plasma at 31 days after administration of TiO_2-SRB (10, 20 and 30 μM) or TiO_2 in treated rats with alloxan (200 mg/kg) or vehicle. The graph (A) glucose concentration (mg/ml) in plasma and shown in graphs (B) and (C) insulin concentration (mU/ml), and the percentage of glycosylated hemoglobin, respectively are shown. The data show the mean ± SE of each group respectively. (One-way ANOVA, Bonferroni post-test, $^{***}p < 0.001$: Vehicle vs alloxan + TiO_2 and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$: alloxan + TiO_2-SrB vs alloxan + TiO_2).

normal rats treated with TiO_2 (227% and 185%, respectively) (Fig. 6). Moreover the treatment with TiO_2-SrB nanomaterials to 20 or 30 μM in diabetic rats decreased the cholesterol (62 and 64%, respectively) and triacylglycerides levels (59 and 63%, respectively) respect to diabetic rats with TiO_2 (Fig. 6A and B). (One way ANOVA, Bonferroni post-test, $p < 0.05$). Treated animals with TiO_2-SrB nanomaterials to 10 μM did not modify the biochemical parameters evaluated.

Discussion

In the present study, we synthesize the TiO_2-SrB nanomaterials by the sol–gel method, which were characterized by FTIR and SEM, showing the interaction of *Stevia rebaudiana* (Bertoni) Bertoni, Asteraceae, nanomaterials with TiO_2, as agglomerates of different sizes. Subsequently, we investigate the antidiabetic activity of

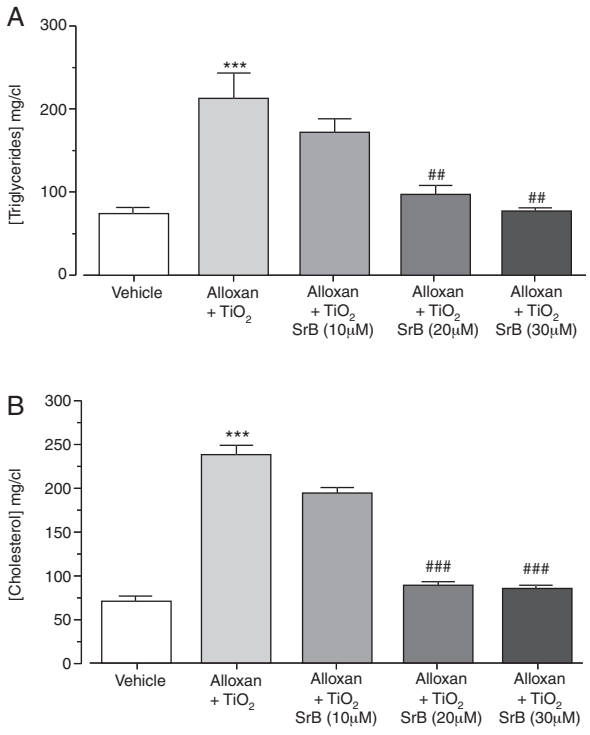

Fig. 6. Effect of administration of TiO_2-SrB nanomaterials on concentration of cholesterol and triacylglycerides in administered rats with alloxan. Quantification was performed in plasma at 31 days after administration of TiO_2-SrB (10, 20 and 30 μM) or TiO_2 in treated rats with alloxan (200 mg/kg) or vehicle. The graph (A) is shown cholesterol concentration (mg/dl) and in the graph (B) triacylglycerides (mg/dl) are shown respectively. Data show the mean concentration ± SE of each group respectively. (One-way ANOVA, Bonferroni post-test, $^{***}p < 0$. Vehicle vs alloxan + TiO_2 and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$: alloxan + TiO_2-SrB vs alloxan + TiO_2).

TiO_2-SrB nanomaterials in diabetic rat model induced with alloxan, which is a toxic hepato-pancreatic agent, to generate a similar metabolic changes to the DM. The alloxan is employed as an experimental diabetogenic to evaluate the effectiveness of new drugs with hypoglycemic properties (Szkudelski, 2001).

Glycemia and lipemia evaluations, determines the degree of metabolic damage that exists in experimental animals. This study shows that the treatment with TiO_2 alone have no effect on glucose and lipids values, while TiO_2-SrB at 20 or 30 μM caused a hypoglycemic and antihyperlipidemic effect in diabetic rats induced with alloxan, which is a toxic glucose analog, which selectively destroys insulin-producing beta cells in the pancreas when is administered to rodents (Jeppesen et al., 2000), and causes that secreted insulin are insufficient to regulate blood glucose, therefore in this study the rats exhibited continuous hyperglycemia. This model produces a decrease in the insulin levels in the animals, similar to the reported in several studies. When the rats are treated with TiO_2-SrB to 20 or 30 μM produce a hypoglycemic effect from 4 h to 31 days after the treatment. These results reveal the importance of TiO_2 nanomaterials as continuous and sustained release drugs delivery system into the bloodstream.

According to Jeppesen et al. (2000) the SrB exerts a hypoglycemic action which is analogous to sulfonylureas, because it blocks K^+ channels in pancreatic β-cells remaining, depolarized the membrane and promotes channel opening of voltage-dependent Ca^{+2}. Wherein the increase of intracellular Ca^{+2} is crucial to induce insulin secretion from pancreatic β-cells and thus cause the decrease of blood sugar, reaching normal values biologically (Raskovic et al., 2004).

According our results it appears that the treatment with SrB into TiO_2 nanomaterials on diabetic rats stimulate of remaining β-pancreatic cells and induce the insulin secretion constantly. This insulin promotes the decrease of glucose and glycated hemoglobin levels in the plasma of the diabetic rats (Park and Cha, 2010). It is noteworthy that interaction, SrB, with nanomaterials TiO_2 decreases progressively, once they are in the aqueous medium, which provides the blood stream, therefore, the SrB is released and distributed peripherally to reaching the sites of action and exercise the respective pharmacological effects. According as suggested by some work with nanomaterials of TiO_2 (López et al., 2010a,b, 2015). However, it is necessary to demonstrate this process in the future.

In DM, high lipid levels are present and play an important toxic role. The present study show high lipid levels in diabetic rats induced with alloxan but the treatment with nanomaterials of TiO_2-SrB normalize the profile lipids demonstrating the hypolipidemic effect of SrB. However, the molecular mechanism to regulating glucose or lipids of the SrB are unknown. At the moment, studies indicate that SrB has antioxidant and antiapoptotic effects (Shivanna et al., 2013), which probably helps to enhance the SrB therapeutic effect. According to Misra et al. (2011) suggest that Stevia extract produced good antidiabetic effects together with lesser loss in body weight and propose that can be used in the preparation of cough syrups and cold beverages for diabetes patients (Himanshu et al., 2011).

For above expose, this study describes for the first times that is necessary the delivery system like TiO_2 nanomaterials to release SrB and generate regulation on hyperglycemia and hyperlipidemia in diabetic rats induced with alloxan. Our results open a new opportunity for the treatment of the DM; however more studies are needed to better understand the potential use of TiO_2-nanomaterials and SrB in metabolic diseases.

Author contributions

AL, MGC, ST, AD, BV, GI, GC and AMR design the study and wrote the protocol. AL, AD and ST performed the experiments. AL, AD, AMR, ST, BV and GC managed the literature searches and analysis; MGC and AD undertook the statistical analysis. All contributing authors have approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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