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Curcumin nanoparticles have potential antioxidant effect and restore tetrahydrobiopterin levels in experimental diabetes

Gasem Mohammad Abu-Taweel [a], Mohamed F. Attia [b,*], Jihan Hussein [c], Enas Mahmoud Mekawi [d], Heba M. Galal [e,f], Eman Ibrahim Ahmed [g,h], Ahmed A. Allam [i], Mehrez E. El-Naggar [j,*]

[a] Department of Biology, College of Science, Jazan University, P.O. Box 2079, Jazan, 45142, Saudi Arabia
[b] Department of Chemistry, Clemson University, Clemson, SC, United States
[c] Medical Biochemistry Department, National Research Centre, Doki, Giza, Egypt
[d] Agricultural Biochemistry Department, Faculty of Agriculture, Benha University, Egypt
[e] Medical Physiology Department, Faculty of Medicine, Assiut University, Assiut, Egypt
[f] Department of Medical Physiology, College of Medicine, Jouf University, Sakaka, Saudi Arabia
[g] Pharmacology and Therapeutics Department, College of Medicine, Jouf University, Saudi Arabia
[h] Pharmacology Department, Faculty of Medicine, Fayoum University, Egypt
[i] Department of Zoology, Faculty of Science, Beni-Suef University, Beni-Suef, 65211, Egypt
[j] Textile Research Division, National Research Centre, 33 El Bohouth st., Dokki, Giza, Cairo, Egypt

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ABSTRACT

Diabetes is associated with an increase in the production of free radicals, reduction of tetrahydrobiopterin (BH4, THB) levels and reduced bioavailability of nitric oxide (NO) in the vascular walls. In this contribution, we probed explored the effective efficient role of curcumin nanoparticles (CUR-NPs) that prepared *via* solvent evaporation nanoprecipitation technique as potential system to attenuate endothelial dysfunction. In this technique, Tween 60 (polysorbate) was used as stabilizing agent for the prepared CUR-NPs and protect such nanoparticles from further agglomeration. BH4 levels and other parameters were estimated in diabetic rats. To this end, we dedicated 48 male albino rats, categorized into six groups; control (healthy rats), diabetic rats, along with four treated groups *via* oral administration of 0.2 mL/kg body weight/day of solutions of Tween 60 (60 mg/mL), free CUR (60 mg/mL), CUR-NPs1 (30 mg/mL), and CUR-NPs2 (60 mg/mL) for 30 days. Results showed that the mean level of malondialdehyde (MDA) has been significantly increased in diabetic group associated with a reduction of total antioxidant capacity, NO, and BH4 compared to control. These parameters were restored by the delivery of CUR-NPs – both doses in rats, compared with the two control groups that treated with Tween 60 and free CUR.

1. Introduction

Diabetes mellitus (DM) leads to multiple primarily vascular complications that affect vessels. In addition, increasing the development of precise reactive oxygen species (ROS) in all tissues from glucose autooxidation and protein glycosylation [1]. Furthermore, diabetes is known to be associated with an increase in the production of superoxide [2], reduction of tetrahydrobiopterin (BH4) levels [3] and a reduced bioavailability of nitric oxide (NO) in the vascular wall. It has been reported that superoxide interacts with NO to produce peroxynitrite, and consequently oxidizes BH4 resulting in an oxidative damage to endothelial nitric oxide (eNO). In such condition with a relative shortage of BH4, the enzyme may generate superoxide instead of NO [4]. In fact, food derived antioxidants have the potential for long term use as chemo-protective agents in different diseases with respect to oxidative stress such as liver diseases [5,6], renal dysfunction [7], and diabetes mellitus [8].

Curcumin is a well-known as a potential therapeutic natural product exhibiting super antioxidant [9], antidiabetic [10,11], anticancer [12], antihypertensive [13,14], anti-inflammatory [15], antiscleroderma [16], antipsoriatic [17], and antimicrobial activities [18] that help to cure various chronic diseases. It can also effectively cross the blood

* Corresponding authors.
E-mail addresses: mattia@clemson.edu (M.F. Attia), mehrez_chem@yahoo.com, mehrezeelnaggar@gmail.com (M.E. El-Naggar).

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barrier due to its polar structure and small molecular weight in diabetic rats, curcumin were found to improve insulin sensitivity, increase the antioxidant properties of pancreatic β-cells, and enhance hyperlipidemia [11,19] that are correlated with the reduction of free fatty acids (FFA) in plasma. However, the treatment of many diseases using plant extracts alone is still challenging or very slow approach, and require much efforts in order to enhance their antidiabetic efficacy. Although curcumin has multiple pharmacologic effects, its poor peroral bioavailability, low absorption by the digestive system and undergoes glucuronidation, and excretion rather than being released into the serum and systematically distributed limit its therapeutic efficiency. By encapsulating CUR in polymeric nanoparticles, dendrimers, nanogels, lipid nanoparticles, or even conjugating to metal or metal oxide nanoparticles, its hydrophilicity and bioavailability in tissues can be improved and thus, augment its impact in pharmacological domains.

In the past few years, large number of studies have been reported on engineering nanoparticulate systems loading curcumin (CUR) exhibiting superior biological activities over conventional therapeutics. As example, the research study carried out by Reddy et al. stated the preparation of CUR loaded poly (lactide-co-glycolic acid) nanoparticles resulted in enhanced cellular uptake, and increased the antidiabetic action [20]. Likewise, in our recently research work, we aimed to synthesize CUR loaded poly(lactic acid)-poly(ethylene glycol) nanoparticles as an efficient antidiabetic agent [11]. Another study reported by Reeves et al. who have encapsulated curcumin in nanogel formulation by using amphiphilic poloxamer-cationic network as an effective anticancer agent [21].

Given the aforementioned facts, our study herein designates the formulation of CUR-NPs in large scale synthesis differ than the literature *via* using tween 60 (stabilizing agent) as excellent drug delivery system aiming to investigate the effective role of CUR-NPs in attenuating endothelial dysfunction through the estimation of tetrahydrobiopterin levels in experimental diabetes and modulating other parameters influenced by diabetes. Our designed system successfully validated as an efficient therapy in STZ-induced diabetic rats.

2. Materials and methods

2.1. Chemicals and animals

Tetrahydrobiopterin (BH4) (HPLC grade), streptozotocin (STZ), iodine, potassium iodide (KI), trichloroacetic acid (TCA), sodium hydroxide (NaOH), ascorbic acid, and curcumin (≥94 % (curcuminoid content), ≥80 % (Curcumin), CAS Number 458-37-7) were purchased from Sigma-Aldrich Co. USA. Tween 60 was obtained from R&M (UK). For experimental tools and characteristics of the resultant nanoparticles, deionized water (DI) was utilized. All chemicals were used without further purification. For diabetic experiment, 40 Male albino rats with mean weight were 180 ± 20 g were obtained from National Research Centre's (NRC) animal house, Egypt. These rats were kept in individual stainless cabinets at a temperature of 25 ± 2 °C and under 12 h light/12 h dark cycle, and were permitted to quickly adapt for one week prior to launch the animal experiment. The rats were freely exposed to water and a regular diet of rodent chow. The recommendations for animal ethical care and treatment followed the rules of the National Research Centre's (NRC) Ethical Committee.

2.2. Methods

2.2.1. Preparation of Curcumin nanoparticles (CUR-NPs)

CUR-NPs were prepared by dissolving 240 mg of curcumin powder in 40 mL of dichloromethane (DCM) under magnetic stirring for 15 min. The as-prepared solution was then added dropwise to 100 mL of deionized warm water containing 250 mg of Tween 60 as stabilizing agent with continuous stirring. After complete addition, the solution was conducted to ultrasonication waves of 100 kHz for 2 h. Subsequently, the sonicated solution was magnetically stirred at 1200 rpm for another 1 h. The orange colored precipitate was obtained by centrifugation at 5000 rpm for 30 min. Afterwards, the sample was kept at -80 °C for few hours followed by freeze drying at 0.1 mbar for 2–3 days for complete drying. The orange dried solid of CUR-NPs was obtained in appropriate theoretical yield and kept at room temperature for further study; characterization and application. The developed CUR-NPs powder was redispersed in deionized water in two different doses; 30 mg/mL (CUR-NPs1) and 60 mg/mL (CUR-NPs2) for oral administration in experimental rats.

2.2.2. Induction of diabetes and experimental design

Streptozotocin (STZ) (6.0 mg/100 g body weight) was dissolved in sodium citrate (50 mM, pH 4.5). The solution was injected intravenously in rats; fasting blood sugar was measured to prove the induction of diabetes after three days [22]. Forty-eight male albino rats (8 rats per group) were divided into 6 groups and classified as follows:

Group I (control) is healthy rats that orally administered with a vehicle. Group II is diabetic rats that orally administered with a vehicle. Group III is the diabetic rats orally administered with a solution of Tween 60 (60 mg/mL). Group IV is the diabetic rats orally administered with free CUR (60 mg/mL). Group V is the diabetic rats orally administered with CUR-NPs1 (30 mg/mL). Finally, group VI is the diabetic rats orally administered with CUR-NPs2 (60 mg/mL). All doses were 0.2 mL/kg.bw and were continued for 30 days [23].

2.3. Samples collection

Throughout the end of the experiment, all rats were maintained fasting for 12 h, the blood was drained and gathered in two tubes; the first tube includes sodium fluoride in order to assess the level of the fasting blood sugar while the second tube contains anti-coagulant material in order to separate the plasma for further evaluation. Then, the two tubes were centrifuged for 10 min using cooling centrifuge at 2400 rpm. For further estimations of the required biochemical parameters, plasma was separated and placed in fridge at -80 °C.

2.4. Characterizations

2.4.1. Physicochemical characterization and entrapment efficiency of CUR-NPs

UV-visible spectrophotometer (Shimadzu UV-1700, Japan) monitors the absorbance spectrum in the range between 200 nm and 800 nm wavelengths for the detection of the prepared CUR-NPs. *Transmission electron microscopy* (TEM; JEOL, JEM, 120 KV, Japan) was applied to examine the surface topology and morphology of the developed CUR-NPs. The sample was prepared by placing a drop of CUR-NPs solution on copper grid, stained and allowed to dry in air overnight then conducted to the TEM technique. The surface structure of the dispersed CUR-NPs sample on a carbon tape followed by drying the sample under nitrogen stream was imaged by *scanning electron microscopy* (SEM; JEOL JSM 840 microscope). *Dynamic light scattering microscope* (DLS; Malvern Zetasizer S90 series, UK) was used to determine the mean particle size and size distribution of CUR-NPs. The sample has been measured by dispersing 1 mg of the lyophilized CUR-NPs powder in 10 ml of deionized water. In order to evaluate the surface charge and stability on the surface of the nanoparticles at pH 7, *ζ-potential* of synthesized CUR-NPs were analyzed. In this analysis, 1000 μL of the sample in transparent, disposable zeta cells were utilized to determine the value of zeta potential.

Drug entrapment efficiency: specific weight of the resultant CUR-NPs stabilized by Tween 60 surfactant was centrifuged at 15000 rpm for 60 min, and free CUR in the supernatant was measured by UV–vis spectroscopy (T80, Germany) at 423 nm. The entrapment efficiency (E.E%) of CUR loaded in Tween 60 was calculated following this equation (Eq. (1)).

Table 1
Gradient method for determination of biopterin and BH4 by HPLC [8].

Time (min)	Methanol (%)	Phosphate buffer (%)
0.0 – 2.0	90	10
2.1– 5.0	00	100
5.1– 10.0	100	00

$$\text{Entrapment efficiency (E. E\%)} = \frac{\text{amount of loaded curcumin}}{\text{total amount of curumin}} \times 100 \quad (1)$$

2.4.2. Biochemical assays

The fasting blood glucose was calorimetrically calculated using a previous method [24]. Plasma insulin level was estimated by ELISA using BioSource INS-EASIA Kit [25]. Plasma Lipid peroxidation was evaluated by the method of Ruiz-Larrea et al. [26] through measuring malondialdehyde (MDA) level. Total antioxidant capacity (TAC) was kinetically measured as described by Kankofer et al., [27] using commercial kits purchased from Glory Science Co. (Cairo, Egypt). Griess reagent was used to assess nitric oxide (NO) using the method reported by Moshage, et al. [28] in which nitrite, stable end product of radical nitric oxide, is often utilized as an indicator for the development of NO.

Biopterin and tetrahydrobiopterin (BH4) levels were estimated by High performance liquid chromatography (HPLC) with minor modification on the protocol described by Fekkes and Voskuilen-Kooijman [29]. Throughout, total biopterin was evaluated by using acid iodine for the oxidation of 7,8-dihydrobiopterin and BH4 to biopterin. Additionally, the indirect quantitation of BH4 was assessed *via* using alkaline iodine, resulting in side-chain cleavage of BH4 to pterin and only oxidizes 7,8-dihydrobiopterin to biopterin. The difference in the levels of biopterin between acid and alkaline oxidation results in BH4 concentration.

The chemicals were prepared as follow: *Iodine solution I,* was prepared by adding iodine (0.5 % w/v) to potassium iodide (1 % w/v) and trichloroacetic acid (TCA; 0.2 M). *Iodine solution II* was prepared by adding 0.5 % (w/v) iodine to potassium iodide (1 % w/v) and sodium hydroxide (0.2 M). Phosphate buffered solution was prepared by adding potassium phosphate buffer (15 mM), pH was adjusted to 6.5 by using ascorbic acid (1 % w/v).

In this method, 400 μL plasma were added to 100 μL of trichloroacetic acid (TCA, 1 M) at 4 °C; the mixture was then left for 15 min and centrifuged at 2500 *g* for another 15 min. Then, iodine solution I (50 μL) was added to the supernatant (350 μL) and transferred to an amber tube. After 1 h, 20 μL of ascorbic acid solution (1 % w/v) was added to reduce the excess of iodine. Sodium hydroxide (6 M, 20 μL) and iodine solution II (50 μL) was applied to the deproteinized plasma for BH4 evaluation. Afterward, samples were blended and incubated for 1 h and to be shielded from the light. The reaction was terminated by adding hydrochloric acid (HCL, 6 M, 20 μL) and ascorbic (1 % w/v, 20 μL). Both samples of the two protocols (acidic and alkaline) were centrifuged for 10 min at 2500 rpm. The supernatant was moved to a vial of an amber glass and directly injected into HPLC system.

Total biopterin and BH4 were assayed using HPLC technique, Agilent technologies 1100 series, equipped with a quaternary pump (G131A model). Separation was achieved on reversed phase column (250 mm x5 μm); temperature was adjusted at 50 °C using florescent detector with excitation and emission wavelengths of 360 and 440 nm, respectively. The mobile phase consists of potassium methanol/phosphate buffer, eluted by a gradient method as described in Table 1, at a flow rate of 0.4 mL/min.

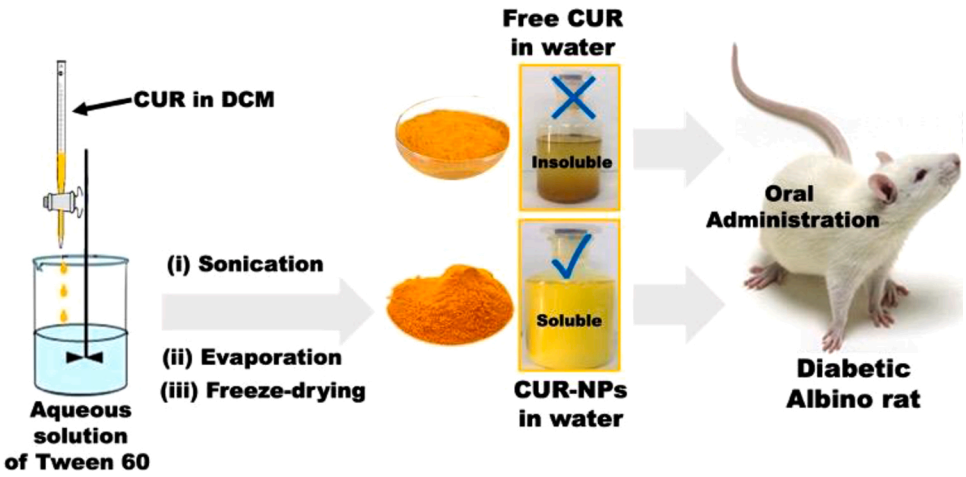
2.5. Statistical analysis

Results were expressed as mean ± standard error. Data were analyzed by one-way ANOVA (SPSS) version 16 followed by LSD test to compare significance between groups. Difference was considered significant when P value is < 0.05.

3. Results and discussion

The story began with the different nanoformulation approach of curcumin nanoparticles (CUR-NPs) compared with common methodologies in literature through using only stabilizing agent (*i.e.* tween 60 without a carrier by means of solvent evaporation nanoprecipitation method) with the help of ultrasonication yielding stable particles with mean size of 40−50 nm as described in Scheme 1. The resultant CUR-NPs also did not change their physical properties after freeze drying process. Before testing them with experimental rats, we intensively characterized them by such advanced tools, *i.e.* UV–vis, TEM, SEM, DLS in order to investigate their physicochemical properties comprising the size, morphology, and surface charge.

First off, the solubility test was used to confirm the improved absorbability properties of CUR-NPs. it was remarked that the prepared CUR-NPs were very fine powder and readily dispersed in water, unlike free parent curcumin (CUR) which has poor water solubility as clearly represented in Fig. 1A (top-right inset). The improved solubility of CUR-NPs in water could be attributed to its high surface area and the presence of amphiphilic coating agent tween 60 which promote dissolution. By using spectrophotometric measurements, both free CUR and CUR-NPs were measured in the range of wavelengths; 250–750 nm which



Scheme 1. Two consecutive steps: (i) Schematic representation depicts the preparation of CUR-NPs by solvent evaporation nanoprecipitation method. (ii) Solubility of free CUR and CUR-NPs and their oral administration to diabetic albino rats.

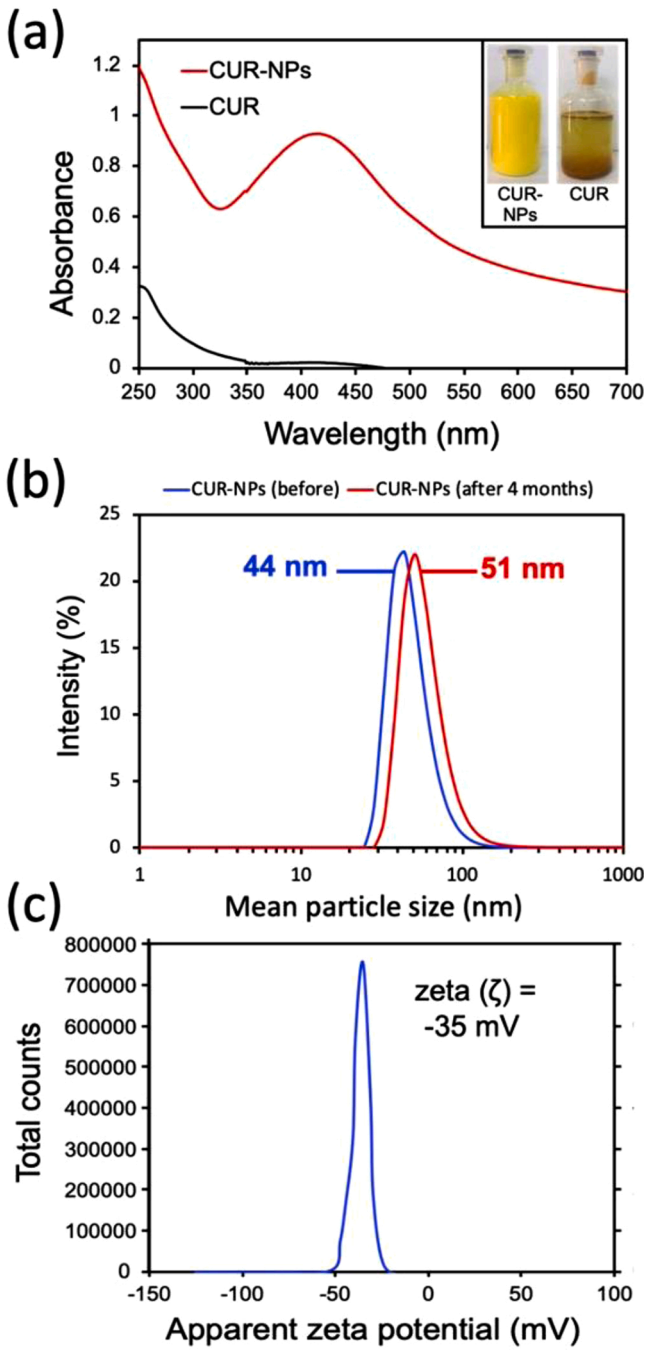


Fig. 1. Characterization of the resultant CUR-NPs by UV–vis spectroscopy (a), DLS for particle size determination (b), and Zetasizer for zeta potential (surface charge) (c).

displayed the assigned absorption band at 423 nm corresponding to CUR material where no peaks appeared for free CUR in water solvent. The absorbance data is in agreement with the results obtained by Alam et al. [30] and Ghosh et al. [31].

Next, we determined the mean particle size and size distribution of the developed CUR-NPs by DLS technique (Fig. 1B) demonstrated particle diameter of nearly 44 nm with narrow size distribution and the polydispersity index was very low (PDI < 0.1). The size of the same particle patch was then evaluated after 4 months of preparation and it was found to be centered at approximately 51 nm affirming the high stability of the particles. Moreover, the zeta potential was also determined by zetasizer revealing highly negative surface charge of about −35 mV – another good indication for the high physical and chemical stability of CUR-NPs. The purpose for the examination of CUR-NPs zeta potential is to predict the stability of the formed nanoparticles along with time. The signal negative charge could be ascribed to the chemical structure of CUR-NPs that stabilized *via* Tween 60.

To further demonstrate the particle size and morphological shape, both TEM and SEM microscopes were utilized to image the CUR-NPs. Fig. 2–a1, a2 shows TEM pictures in different magnifications a spherical, regular, and monodispersed particles' shape with dense black color supporting the DLS data (Fig. 1B). The particles exhibited good homogeneity and uniformity in the sample solution and inherent contrast properties.

Fig. 2–b1, b2 signifies SEM images of the developed CUR-NPs demonstrates granular particles with high monodispersity and smooth surface having approximately the same size as in TEM images. Overall, both TEM and SEM characterizations were found to be coherent with DLS measurements.

After CUR-NPs characterization, we evaluated CUR entrapment efficiency (E. E%) in Tween 60-stabilized CUR-NPs by means of UV-vis where found to be 93.1 ± 3.6 %.

Next, the six groups were divided in order into control, STZ-induced diabetic rats, group treated with free CUR, group treated with Tween 60, group treated with CUR-NPs1, and group treated with CUR-NPs2.

To induce diabetes, STZ was injected into healthy rats as previously described by Uchiyama et al., [32]. Fasting blood sugar was significantly increased in the diabetic group with simultaneous reduction in insulin levels with respect to control group.

The elevation in serum glucose level of diabetic group may be correlated with the destruction of ß-cells in the pancreas by STZ [33]. Thus, cells were not capable of producing insulin. This in turn, accumulated blood glucose. The reduction in the mean values of insulin levels recorded in the present study was also reported earlier [34].

Next, the diabetic rats were treated orally with Tween 60, free CUR, CUR-NPs1, and CUR-NPs2 as represented in Fig. 3. A remarkable increase in glucose level for diabetic group was detected (*i.e.* $>$ 240 mg/mL) compared to control (healthy rats) (∼ 80 mg/mL) after STZ induction. Obviously, Tween 60 treated diabetic group did not show change in glucose level. The free CUR resulted in slight decrease in glucose level (∼ 200 mg/mL) of the diabetic rats. In contrast, CUR-NPs1 and CUR-NPs2 exhibited significant similar reduction in glucose level (∼ 140 mg/mL). Together, this supports the diabetic treatment by use of CUR-NPs (Fig. 3A). On balance, after STZ induction, the insulin level reduced from 12 to 8 μIU/mL as shown in Fig. 3B. A regular increase obtained with the treatments of Tween 60, CUR, CUR-NPs1, and CUR-NPs2 demonstrating a strong effect of both CUR-NPs over the other two groups.

More evidently, the MDA (oxidant indicator) in diabetic group was significantly increased along with high reduction in both TAC and NO (antioxidant factors) compared to the control group (Fig. 4). Results recorded regular decline in MDA levels as well as regular upgrade in both TAC and NO levels when treated with Tween 60, CUR, CUR-NPs1, and CUR-NPs2, respectively. Interestingly, both CUR-NPs showed higher efficiency than free materials and the performance of CUR-NPs2 outperformed CUR-NPs1, providing values closer to healthy rats.

In general, the effective role of CUR could be attributed to the fact that it is a polyphenolic non-flavanone compound which is pharmacologically active and has antioxidant, anti-inflammatory and also antiproliferative activities as mentioned earlier. It has been reported that, the antioxidant activity of CUR is equivalent to vitamins E and C [35]. Several mechanisms have been postulated for the antioxidant and anti-inflammatory properties of CUR to play this role. This may be related to the capability of CUR to reduce the free fatty acids (FFA) by elevation of beta-oxidation activity [36]. This decreased endogenous polyunsaturated fatty acid synthesis [37] and inhibited TNF- α [38].

Eventually, we estimated total biopterin and tetrahydrobiopterin (BH4) parameters to prove both diabetes induction and their treatments as seen in Fig. 5. It is noted that both parameters were significantly

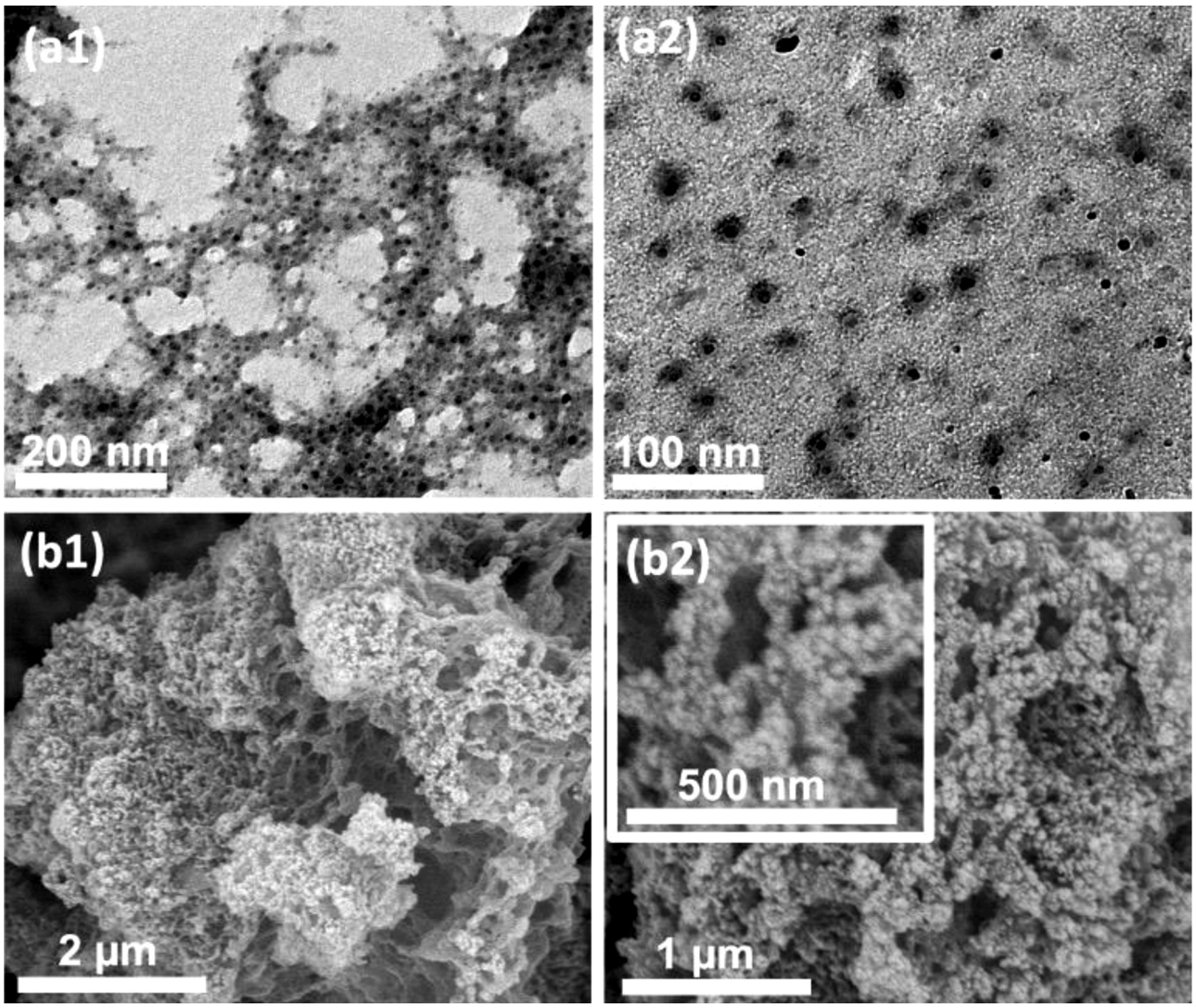


Fig. 2. Transmission electron microscopy (TEM) (a1, a2) and Scanning electron microscopy (SEM) (b1, b2) images of CUR-NPs in different magnifications demonstrating regular, monodispersed, and spherical particles.

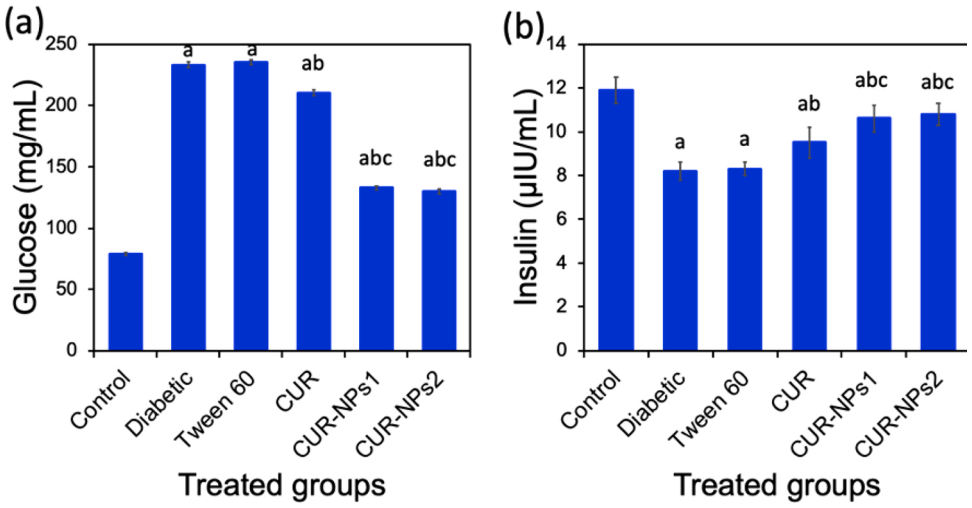


Fig. 3. Fasting blood sugar levels (a) and insulin levels (b) in all studied groups. a: significant difference compared to control group, b: significant difference compared to diabetic group, c: significant difference compared to Tween 60 treated group. Significant p value <0.05. Data presented as mean ± SE.

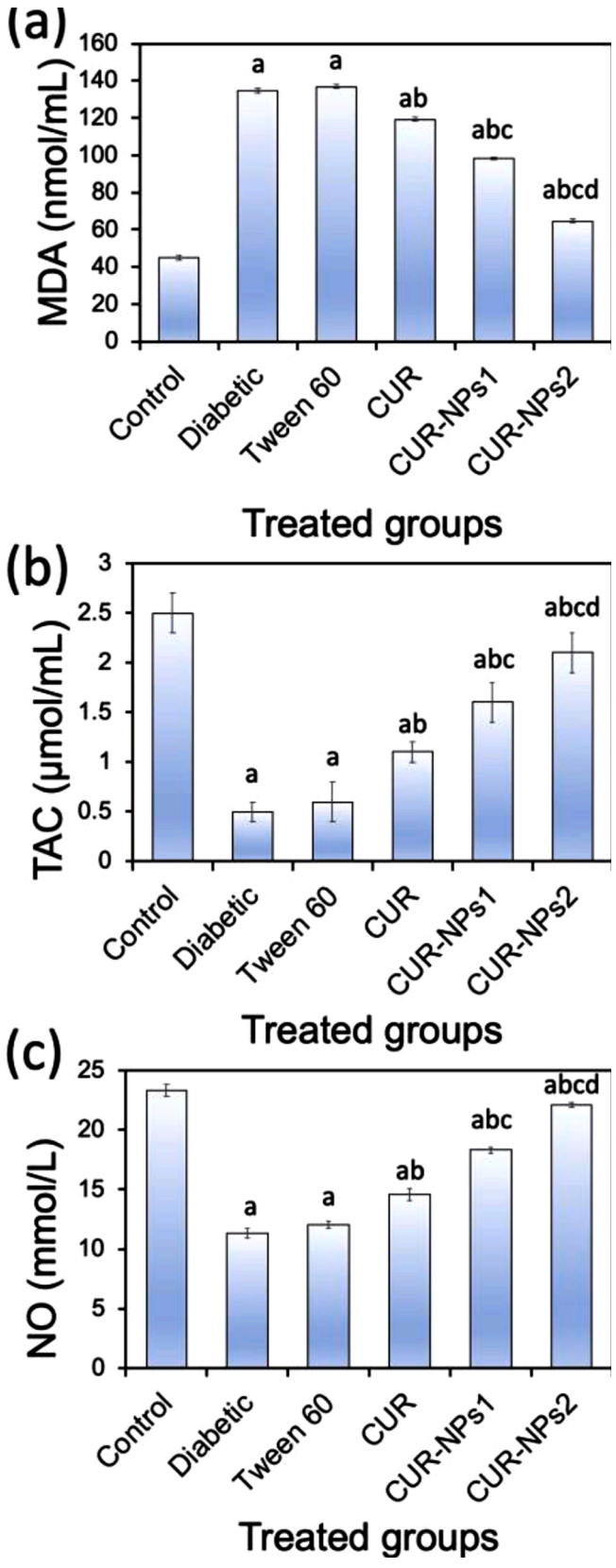


Fig. 4. malondialdehyde (MDA), Total antioxidant capacity (TAC) and nitric oxide (NO) in different studied groups. a: Significant difference relative to control group, b: significant difference relative to diabetic group, c: significant difference relative to Tween 60 treated group, d: significant difference relative to CUR-NPs1 treated group. Significant p value <0.05. Data presented as mean ± SE.

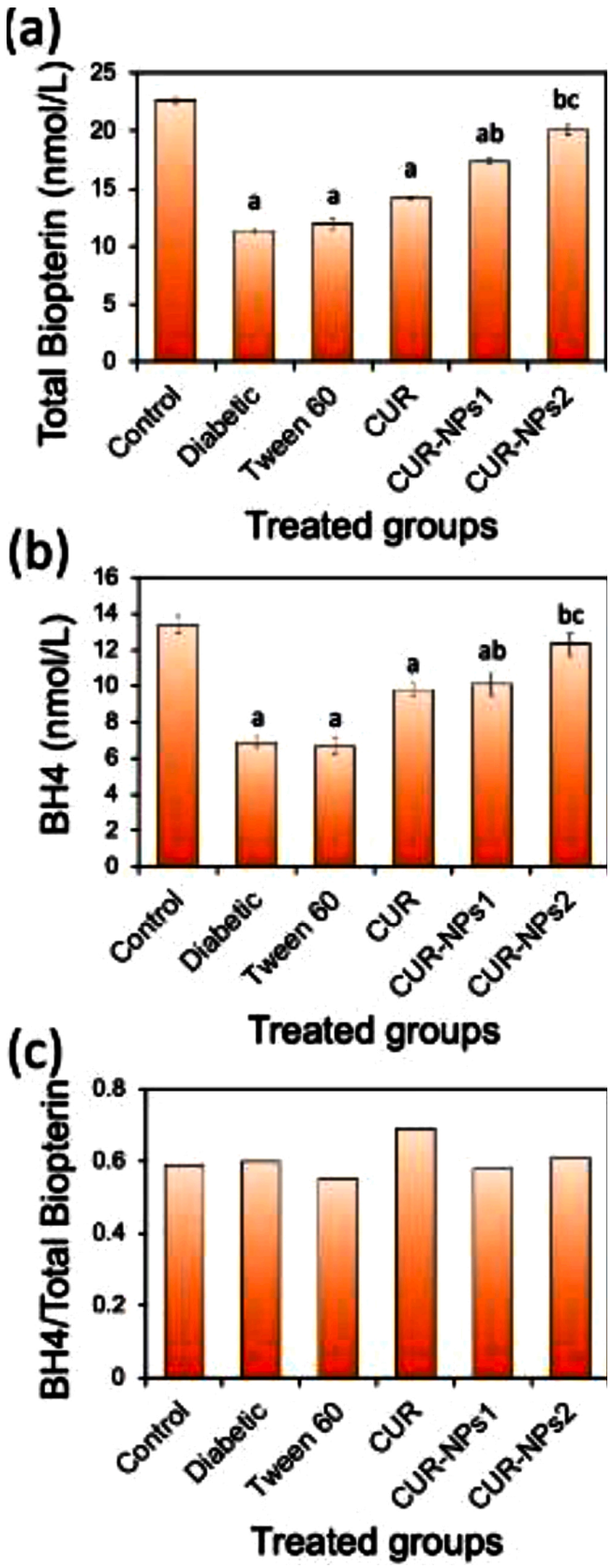


Fig. 5. Total biopterin (a), tetrahydrobiopterin (BH4) (b), and BH4/total biopterin (c) levels in all studied groups. a: Significant difference relative to control group, b: significant difference relative to diabetic group, c: significant difference relative to Tween 60 treated group, d: significant difference relative to CUR-NPs1 treated group. Significant p value <0.05. Data presented as mean ± SE.

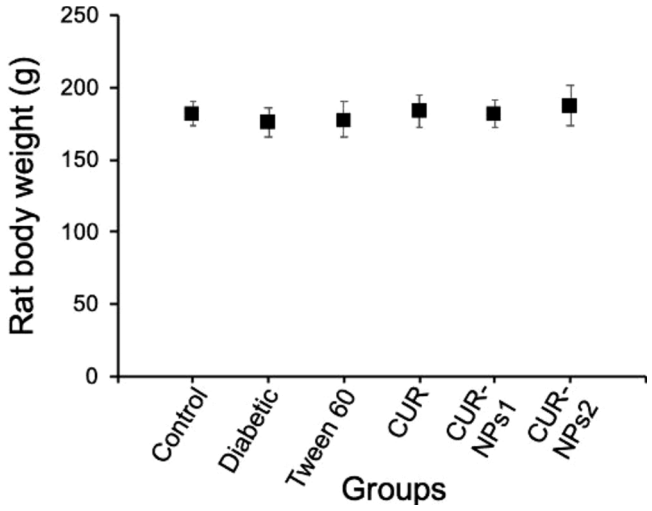


Fig. 6. Rats body weight of all treated groups compared with control showing negligible change in the weight after the treatment course.

dropped in diabetic group compared to healthy group. The reduction may be due to the decline of GTP-cyclohydrolase I protein expression; the first controlling enzyme for de novo synthesis of BH4 [39]. When treating the diabetic group with different materials, we observed regular increase in total biopterin and BH4 for Tween 60, CUR, CUR-NPs1, and reaching close values in CUR-NPs2 to control. Notably, CUR supplementation significantly increased these values (biopterin and tetrahydrobiopterin) in treated groups compared to diabetic group in our study, which likely due to the stimulation of BH4 synthesis in endothelial cells by boosting the expression of GTP-cyclohydrolase I protein. Amazingly, the restoration of BH4 levels with sepiapterin, which is a precursor for the BH_4 in the pathway of salvage, such as dietary arginine supplementation, normalized NO synthesis in STZ-diabetic rats endothelial cells [40].

Eventually, we examined the rat body weight of all groups to investigate the impact of CUR treatment on the animal's health before and after diabetes (Fig. 6). We indeed observed negligible change in their weights of all studied groups.

Overall, our data showed that CUR-NPs had remarkable antidiabetic efficiency compared to Tween 60 and free CUR (control) in rats. We also found that CUR-NPs in each dose of the two studied doses exhibited similar recovery to glucose and insulin levels. However, CUR-NPs2 revealed slightly higher antidiabetic performance over CUR-NPs1 in case of oxidant/antioxidant enzymes as well as in total biopterin and BH4 levels signifying the effective role of nanoparticles' application in medicine over conventional therapies.

4. Conclusion

This contribution describes the development of high stable CUR-NPs in the presence of Tween 60 as stabilizing agent. The synthesis was carried out by the means of solvent evaporation method aiming to be utilized as efficient antidiabetic agent in rats. Physicochemical characterization (UV-absorbance determination, TEM, SEM, and DL techniques) of CUR-NPs was performed. Thereafter, CUR-NPs in separate two doses were found to significantly regulate glucose and insulin levels, improved oxidative stability, restored BH4 levels, and improved the bioavailability of NO in the vascular wall. Results showed that CUR-NPs was more efficient than Tween 60 and free CUR. Based on the aforementioned data, it was observed that CUR-NPs2 exhibited higher superiority than CUR-NPs1 as antidiabetic agent in model diabetic rat.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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