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Research Article

Nano-encapsulated Form of *Citrus medica* for Osteoporosis Treatment in Animal Model

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ABSTRACT

Although Rosiglitazone (R) is recommended for diabetes therapy, however, it increases the incidence of osteoporosis. Therefore, this study was conducted to investigate the efficiency of *Citrus medica* leaves extract nanoparticles (CM-NPs) against osteoporosis prompted by Rosiglitazone (R). In addition, pharmaceutical impacts of R with CM-NPs in treating diabetic-induced male rats were premeditated. Swiss albino rats (n= 60) were equally allocated in six groups including control (C), diabetes mellitus (DM), DM+R, DM+R+CM_{50}-NPs, DM+R+CM_{75}-NPs and DM+R+CM_{100}-NPs. Serum glucose, receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG) and β2-microglobulin concentrations were appraised. Bone mineral density (BMD) of each right femur was restrained. The results revealed that administration of R to DM-rats induced significant diminution in serum OPG level and increase in serum RANKL and β2-microglobulin levels which were improved in DM-R-CM-NPs rats. A significant decline in total BMD was found in DM+R treated rats which was repressed in DM+R-CM-NP rats. DM-rats treated with R showed significantly low gene expression of *OC, COL* genes and higher level of *ACP5* mRNA compared to control rats. However, the expression values due to R treatment were amended by CM-NPs administration. In addition, DM-rats treated with R showed significantly high incidence of MnPCEs and 8-OHdG/2-dG ratio which were inhibited by the administration of CM-NPs. Conclusion: The combination therapy of R and *Citrus medica* nanoparticles (CM-NPs) reduced the risk of osteoporosis in animal model.

Keywords: Rosiglitazone, *Citrus medica*, Nanoparticles, Osteoporosis, Gene expression, MnPCEs, DNA damage

INTRODUCTION

High levels of blood glucose and enhancement of hyperglycemia are the most symptoms of Diabetes mellitus (DM). Additionally, vascular complications, indisposition and death are synchronized in diabetic cases[1]. The diabetes Type1 is resulting from the autoimmune destruction of the insulin-producing beta cells in the pancreas. However, diabetes Type2 is considered as non-insulin dependent. It is resulting by glucose overproduction or by dysfunction of β-cell or insulin opposition at target cells[2-4].

Rosiglitazone, a thiazolidinedione is a high affinity ligand. It is considered as activator of the peroxisome proliferators-activated receptor-gamma (PPAR-g). It acts by increasing sensitivity to endogenous insulin and accordingly reducing blood glucose. Most of the Rosiglitazone effects are mediated via this transcription factor[5,6]. The PPAR-g1 isoform is communicated in several cell types such as osteoblasts, muscle cells, adiposities and macrophages[7]. Bone forming cells namely Osteoblasts are sharing the precursor of the common mesenchymal in bone marrow cells with adipocytes[8,9].

Current study revealed that a significant decrease in total bone mineral density was perceived in group diabetic rats preserved with rosiglitazone[10,11]. It has been found that rosiglitazone-activates PPAR-g2 functions as a dominant negative regulator of osteoblast diversity[12]. Thus, there is wide diversity of newer therapeutic agents/strategies being examined for the treatment of T2DM, most of all currently under preclinical and early clinical stages of drug improvement[4].

Citrus medica have been used in the traditional herbal medicine as anti-diabetic herb[13-15]. The *Citrus medica* have many chemical constituents which put it as favorite herb in several ethno medicines. Citrus fruits contain limonoids and flavonoids which are recognized to possess antitumor and anti-inflammatory effects. The pectin are existing widely in Citrus peels which are known to retain blood sugar depressing and decrease cholesterol level. Sah et al.[15] reported antihyperglycemic impact and anti-oxidant effect of *Citrus medica* leaf in streptozotocin prompted diabetic rats. Fruit juice of *Citrus medica* possess hypoglycemic and hypocholesterolemic activities.

Nanotechnology has been used in medicine as a basic science tool[16,17] in therapeutic applications. Nanoparticles offer a non-toxic and efficient carrier system for battered-delivery and enhanced drug bioavailability within the cells, tissues, or both. An effort has been made in the present study to explore one of the modern ways of pharmaceutical mediations to formulate nanoencapsulation of the leaves extract of *Citrus medica*.

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In view of the pharmacological properties of *Citrus medica*, this study was planned to assess the effect of *Citrus medica* nanoparticles against Rosiglitazone induced osteoporosis and also investigate the pharmacodynamic effects of Rosiglitazone with *Citrus medica* nanoparticles in treating hyperglycemia of diabetic rats.

MATERIALS AND METHODS

Drugs and chemicals

Rosiglitazone was provided as a gift from APEX Company (Egypt). It was suspended in 1% Tween 80. Rosiglitazone was orally controlled in a dose of 10 mg/kg[18]. Streptozotocin (STZ) was obtained from Sigma–Aldrich (USA).

Induction of experimental diabetes

Diabetes was induced in 12 h fasted rats with single Intraperitoneal injection (i.p.) injection of STZ (50 mg/kg,[19] inserted in citrate buffer (0.01 M, pH 4.5). Normal control group was injected with only citrate buffer. Animals were considered diabetic when their blood glucose level exceeded 250 mg/dl[20] and were included in the study after 72 h of STZ injection.

Plant material

The leaves of *C. medica* L. were collected from private farm in Giza Governorate and dried instantly for extraction assay. The leaves of the plant were authenticated by Department of Botany, Agriculture and Biology Research Division, National Research Center, Giza,, Egypt.

Preparation of the extract

Citrus medica L. fresh leaves were air – dried in shade, pulverized and weighed to give 1kg of powder. It was macerated with petroleum ether, concentrated at low temperature and reduced pressure to obtain 9.0 g residue (18%w/v).

Saponification of Petroleum ether

Petroleum ether extract of *Citrus medica* L. (1g) was saponified by reflux overnight with 50 ml of 20% alcoholic potassium hydroxide (KOH) at room temperature. Fatty solution was acidified using 5N hydrochloric acid (HCl), followed by extraction with ether. The ether extract was collected, washed three times with water, dried over anhydrous sodium sulfate (Na_2SO_4) then, lastly dried via evaporation.

Formation of Citrus medica Loaded Nanoparticles (SON)

To prepare the poly-lactic-co-glycolic acid (PLGA) encapsulation of *Citrus medica* extract, solvent displacement technique of Samadder et al.[21] deployed under optimal conditions. To 20 mL of an aqueous solution of F68; w/v stabilizer (1% polyoxyethylene-polyoxypropylene), an organic phase mixture containing 10 mg of dried *Citrus medica* extract dissolved in 3 mL acetone along with the addition of 50 mg PLGA in a drop wise routine (0.5 mL/min). Stirring the mixture continuously was performed at room temperature until complete evaporation of the organic solvent; the redundant stabilizer was detached by centrifugation at 2500 g at 4ºC for 30 minutes. The pellet was re-suspended in Milli-Q water, washed three times and the nanoparticles obtained were kept in a suspension at 4ºC until supplementary use.

Transmission electron microscopy

The particle size and shape were characterized using high resolution transmission electron microscopy (HR-TEM) JEM 2100 LB_6 under operating voltage of 200 kV to investigate the micrograph of prepared PLGA encapsulation of *Citrus medica* extract under operating voltage of 200 kV for different samples (Fig. 1).

Experimental Animals

Sixty adult albino male rats (100-120 g, acquired from the Animal House Colony, Giza, Egypt) were sustained on standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy, 0.012 MJ) and water *ad libitum* at the Animal House Laboratory, National Research Center, Dokki, Giza, Egypt. After an acclimation period of 1 week, animals were allocated into 6 groups (10 rats/ group) and housed individually in filter-top polycarbonate cages, housed in a temperature-controlled (23 ± 1°C) and preciously illuminated (12 h dark/light cycle) room free from any source of chemical contamination. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of National Research Center, Egypt.

Experimental design

Animals were divided into following 6 groups. Each group consists of 10 rats:

Group 1 – control: oral saline (C); Group 2- rats were injected by single i.p. dose of STZ (50 mg/kg dissolved in citrate buffer to induce diabetes; Group 3 – diabetus mellitus induced-rats: Rosiglitazone (10 mg/kg bw/day in one dose per oral for 21 days,[11]) (DM + R); Group 4 – diabetus mellitus induced-rats: Rosiglitazone (10 mg/kg bw/day in one dose per oral for 21 days)+50 mg/kg bw/day of *Citrus medica* L. nanoparticles (1/20 of the minimum lethal dose,[22]) in one dose per oral for 90 days starting from the last dose of Rosiglitazone (DM+R+ CM-NPs); Group 5 – diabetus mellitus induced-rats: Rosiglitazone (10 mg/kg bw/day in one dose per oral for 21 days)+75 mg/kg bw/day of *Citrus medica* L. nanoparticles (1/15 of the minimum lethal dose,[22]) in one dose per oral for 90 days starting from the last dose of Rosiglitazone (DM+R+CM-NPs); Group 6 – diabetus mellitus induced-rats: Rosiglitazone (10 mg/kg bw/day in one dose per oral for 21 days)+100 mg/kg bw/day of *Citrus medica* L. nanoparticles (1/10 of the minimum lethal dose,[22]) in one dose per oral for 90 days starting from the last dose of Rosiglitazone (DM+R+ CM-NPs).

Sample Collections

At the end of the tentative period, blood samples from fasting rats were withdrawn from retro-orbital venous plexus under diethyl ether anesthesia in dry clean centrifuge tubes and left to clot. Blood samples were drained at several intervals: on days 21, month 1, month 2 and month 3. Animals were anesthetized with ether, and blood was collected from retro-orbital puncture. Serum was then alienated for the estimation of glucose (Table 1,[23]). Blood samples were centrifuged at 3000 rpm for 15 min at 4°C where the clear sera were separated and immediately stored at –20ºC in a clean plastic Eppendorf till analyses. The animals were then promptly sacrificed and the right femurs were garnered. Each right femur bone was carefully cleaned; length and weight were recorded

and then stored in formalin buffer 10% for dual energy X-ray absorptiometry (DEXA) [a means of measuring bone mineral density (BMD)]. Bone mineral density of each right femur were dignified via DEXA using Norland XR46, version 3.9.6/2.3.1 instrument (Norland X-R-46 version 3.9.6, Peachtree City, GA, USA) equipped with dedicated software for small animal measurements. This technique provided an integrated measure of right femur proximal, distal and total areas.

Analytical Determinations

Serum osteoprotegerin (OPG) and receptor activator of auclear factor-κB Ligand (RANKL) levels were determined by enzyme linked immunosorbent assay (ELISA) technique using R&D Elisa (Sorin Biomedica, Eti-System, Denlay Instruments Ltd, England) kit as described by O'Brien et al-[24] and Teng et al[25] respectively. While, serum β2-microglobulin level was assayed by ELISA procedure using International Immuno-Diagnostics kit (Orgentec Diagnostika GmbH, Mainz, Germany) as designated by Crisp et al[26].

Expression of osteogenic genes

I. Isolation of total RNA

Total RNA was extracted from the bone samples obtained from the intertrochanteric region of the proximal femur of male rats by the standard TRIzol® Reagent extraction method (Invitrogen, Germany). Briefly, bone samples were pulverized under liquid nitrogen and homogenized in 1 ml of TRIzol® Reagent. Afterwards, the homogenized sample was incubated for 15 minutes at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol® Reagent was added. Then the samples were vigorously vortexed for 15 seconds and incubated at room temperature for 3 minutes. The samples were centrifuged for no more than 12,000 x g for 15 minutes at 4 ºC. After centrifugation, the mixture was parted into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA persisted exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per 1 ml of TRIzol® Reagent used for the initial homogenization. Afterwards, the samples were incubated at 15 to 30ºC for 10 minutes and centrifuged at not more than 12,000 x g for 10 minutes at 4 ºC. The RNA was precipitated forming a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 ml of 75% ethanol. The samples mixed and centrifuged at no more than 7,500 x g for 5 minutes at 4 ºC. The supernatant was removed and RNA pellet was air-dried for 10 minutes. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip.

Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used instantly for reverse transcription (RT).

II. Reverse transcription (RT) reaction

The complete Poly(A)$^+$ RNA isolated from male rat bone samples was transliterated into cDNA in a total volume of 20 µl using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM consisted of 50 mM MgCl2, 5x reverse transcription (RT) buffer (50 mMKCl; 10 mMTris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M- MuLV reverse transcriptase. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was clogged by heating for 5 min at 99 °C. Subsequently the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through quantitative real time-polymerase chain reaction (qRT-PCR).

III. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd., Germany), 0.5 µL 0.2 µM sense primers, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each qRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers**.** Each experiment comprised a distilled water control.

The quantitative values of RT-PCR (qRT-PCR) of osteogenic genes (OC = osteocalcin, COL = collagen and ACP5= 5 acid phosphatase) were normalized on the bases of tubulin β (TUB-B) and ß-actin expression (Table 2).

At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

Calculation of Gene Expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula found in the manufacturer's instruction pamphlet:

$Ef = 10^{-1/slope}$

Efficiency (%) = (Ef – 1) x 100

The relative quantification of the target to the reference was determined by using the

$2^{-\Delta\Delta CT}$ method if Ef for the target (OC, COL and ACP5) and the reference primers (β-Actin) as follows:

$\Delta C_{T(test)} = C_{T(target,\ test)} - C_{T(reference,\ test)}$,

$\Delta CT(calibrator) = C_{T(target,\ calibrator)} - C_{T(reference,\ calibrator)}$,

$\Delta\Delta CT = \Delta C_{T(Test)} - \Delta C_{T(calibrator)}$.

The relative expression was calculated by $2^{-\Delta\Delta CT}$.

Table 1: Comparative effect of Rosiglitazone alone and Rosiglitazone with CM-NPs on serum glucose level in diabetic rats.

Treatment	Glucose level (mg/dl)				
	Day 0*	Day 21	Mon1	Mon2	Mon3
Control	83.7 ± 12.4	84.2 ± 14.3[b]	82.8 ± 12.6 [b]	85.9 ± 13.3 [b]	82.5 ± 12.7 [b]
DM	336.7 ± 24.2	331.8 ± 23.4[a]	338.2 ± 25.7 [a]	339.4 ± 22.6 [a]	337.1 ± 21.3 [a]
DM+R	339.3 ± 21.6	108.2 ± 12.8[b]	111.6 ± 10.1 [b]	113.5 ± 11.2 [b]	111.3 ± 10.4
DM+R+CM_{50}-NPs	340.4 ± 23.1	110.7 ± 11.1 [b]	110.2 ± 9.6 [b]	104.8 ± 9.7 [b]	98.9 ± 8.7 [b]
DM+R+CM_{75}-NPs	338.1 ± 19.7	114.9 ± 10.3 [b]	102.1 ± 8.7 [b]	99.1 ± 9.3 [b]	91.3 ± 8.2 [b]
DM+R+CM_{100}-NPs	341.3 ± 25.2	112.6 ± 13.2 [b]	89.2 ± 8.4 [b]	87.3 ± 8.5 [b]	86.1 ± 8.1 [b]

Day 0*: The day when blood sugar reach >250 mg/dl (2-4 days after STZ treatment); DM: Diabetus mellitus; R: Rosiglitazone; Citrus medica; NPs: Nanoparticles; [a, b] Mean values within column with unlike superscript letters were significantly different ([a:] $P<0.001$, [b:] $P<0.01$, Scheffé-Test).

Micronucleus Test by Acridine Orange Fluorescent Staining

Acridine orange staining of erythrocytes was performed according to Ueda et al.[27]. To evaluate this assay, ten animals from each treatment were sacrificed after exposure period. The bone marrow cells were collected from both femora and re-suspended in a small volume of fetal calf serum (FBS; Sigma) on a 0.003% acridine orange-coated glass slide. The slide was then covered with a cover glass to prepare bone marrow specimens. Slides were dried overnight and fixed with methanol for 10 min. Bone marrow specimens were examined in a blinded manner using fluorescence microscopy at 600X or higher magnification with a blue excitation wavelength (e.g. 488 nm) and yellow to orange barrier filter (e.g., 515 nm long pass). Two slides per animal were labeled to get blinded micronuclei scoring. The number of micronucleated polychromatic erythrocytes (%MnPCEs) was measured at a rate of 3000 polychromatic erythrocytes (PCEs) per animal.

HPLC Measurement of 8-Hydroxy-2-deoxyguanosine (8-OHdG) and 2-deoxyguanosine (2-dG)

DNA was extracted from rat bone marrow by homogenization in buffer containing 1% sodium dodecyl sulphate, 10mM Tris, 1mM EDTA (pH 7.4), and an overnight incubation in 0.5mg/ ml proteinase K at 55°C. Homogenates were incubated with RNase (0.1mg/ml) at 50°C for 10 min and extracted with chloroform/isoamyl alcohol. The extracts were mixed with 3M sodium acetate and two volumes of 100% ethanol to precipitate DNA at −20°C. The samples were washed twice with 70% ethanol, air-dried for 15min and dissolved in 100µl of 10mM Tris/1mM EDTA (pH 7.4). DNA digestion was performed as previously designated[28]. The adduct 8-OHdG was measured with high-performance liquid chromatography (HPLC) furnished with a CoulArray system (Model 5600). Analysts were detected on two coulometric array modules, each containing four electrochemical sensors attached in series, which allows identification targets based on reduction potential. UV detection was set to 260nm. The HPLC was controlled and the data acquired and analyzed using CoulArray software. The mobile phase was composed of 50mM sodium acetate/5% methanol at pH 5.2. Electrochemical detector potentials for 8- OHdG and 2-dG were 120/230/280/420/600/750/840/900mV and the flow rate was 1ml/min.

Statistical analysis

All data were analyzed using the General Liner Models (GLM) technique of Statistical Analysis System[29] followed by Scheffé-test to assess significant differences between groups. The values are uttered as mean ± SEM. All statements of significance were based on probability of $P < 0.05$.

RESULTS

Serum glucose levels

The antihyperglycemic effect of the Rosiglitazone with CM-NPs on the fasting serum glucose levels in diabetic rats was shown in Table 1. Diabetic-induced rats revealed extremely high levels ($P<0.001$) of glucose compared with healthy rats. Administration of Rosiglitazone reduced significantly the glucose levels compared with DM-rats. Moreover, regular treatment of CM-NPs after Rosiglitazone administration led to a dose dependent fall in serum glucose levels and continued constant till 3 months with CM-NPs treatment. Administration of DM-rosiglitazone-rats with high dose of CM-NPs revealed highly significant decrease in serum glucose levels compared with the DM-rats.

Serum OPG, RANKL and β2-microglobulin levels

The data in Table 3 indicated the effect of CM-NPs administration on serum osteoprotegerin (OPG), Receptor Activator of Nuclear factor-κB Ligand (RANKL) and beta 2-microglobulin (β2- microglobulin) levels in rosiglitazone-induced secondary osteoporosis in male rats. Administration of rosiglitazone to DM-rats caused significant decrease in serum OPG level concomitant with significant increase in serum RANKL and β2-microglobulin levels in comparison to healthy control rats. However, DM-rosiglitazone-rats treated with several doses of CM-NPs showed significant increase in serum OPG level, *RANKL and β2-microglobulin* as compared to DM-rats or DM-rosiglitazone-rats. Moreover, the administration of high dose of CM-NPs to DM-rosiglitazone-rats for the protection against osteoporosis revealed highly significant increase in serum OPG level with a concomitant significant decrease in serum RANKL and β2-microglobulin levels as compared with the DM-rats or DM-rosiglitazone-rats (Table 3).

Bone mineral density (BMD) levels

Table 2: Primer sequences of osteogenic genes

Genes	Sequence 5´ to 3		Accession no.
	Forward primer	Reverse primer	
OC	CCAGCGGTGCAGAGTCCAGC	GACACCCTAGACCGGGCCGT	NM_199173.3
COL1A1	AGCGGACGCTAACCCCCTCC	CAGACGGGACAGCACTCGCC	NM_000088.3
ACP5	GATCTCCAAGCGCTGGAACT	TGGTCTGTGGGATCTTGAAGTG	Logar et al. (2007)
TUB-B	GAGGGCGAGGACGAGGCTTA	TCTAACAGAGGCAAAACTGAGCAC C	NM_001069.2
β-actin	GGAGATTACTGCCCTGGCTCCT A	GACTCATCGTACTCCTGCTGCTG	Deng et al. (2012)

OC = osteocalcin, COL = collagen, ACP5= acid phosphatase, TUB-B = tubulin β

Table 3: Levels of serum OPG, RANKL and β2-microglobulin in diabetic rats treated with rosiglitazone alone or combined with CM-NPs.

Treatment	*OPG (ng mL-1)*	*RANKL (pg mL-1)*	*β2-microglobulin (µg mL-1)*
Control	3.21 ± 0.43^{a}	52.16 ± 10.02^{c}	0.19 ± 0.03^{b}
DM	1.43 ± 0.32^{d}	171.64 ± 13.54^{a}	0.31 ± 0.04^{a}
DM+R	1.27 ± 0.33^{d}	194.82 ± 14.11^{a}	0.36 ± 0.03^{a}
DM+R+CM_{50}-NPs	1.94 ± 0.27^{c}	104.52 ± 09.76^{b}	0.24 ± 0.01^{b}
DM+R+CM_{75}-NPs	2.71 ± 0.31^{b}	97.21 ± 8.22^{b}	0.21 ± 0.02^{b}
DM+R+CM_{100}-NPs	2.97 ± 0.66^{ab}	88.11 ± 7.43^{b}	0.19 ± 0.03^{b}

DM: Diabetus mellitus; R: Rosiglitazone; Citrus medica; NPs: Nanoparticles; ^{a,b,c,d} Mean values within column with unlike superscript letters were significantly different ($^{a:}$ $P<0.01$, $^{b,c,d:}$ $P<0.05$, Scheffé-Test).

Table 4. Bone mineral density (BMD) in proximal, distal and total areas of femur bones of diabetic rats treated with rosiglitazone alone or combined with CM-NPs.

Treatment	*BMD-proximal (mg cm^{-2})*	*BMD-distal (mg cm^{-2})*	*BMD-total (mg cm-2)*
Control	114.8 ± 11.2^{a}	113.4 ± 9.7^{a}	113.1 ± 8.2^{a}
DM	78.1 ± 4.3^{b}	77.6 ± 6.8^{b}	76.4 ± 5.6^{b}
DM+R	73.4 ± 5.2^{b}	72.4 ± 7.3^{b}	72.3 ± 7.4^{b}
DM+R+CM_{50}-NPs	89.5 ± 11.6^{ab}	88.7 ± 11.2^{ab}	89.2 ± 10.6^{ab}
DM+R+CM_{75}-NPs	97.3 ± 14.8^{a}	95.5 ± 9.3^{a}	95.0 ± 15.3^{a}
DM+R+CM_{100}-NPs	112.6 ± 12.1^{a}	110.4 ± 11.2^{a}	111.2 ± 12.4^{a}

DM: Diabetus mellitus; R: Rosiglitazone; Citrus medica; NPs: Nanoparticles; ^{a,b,} Mean values within column with unlike superscript letters were significantly different ($^{a:}$ $P<0.01$, $^{b:}$ $P<0.05$, Scheffé-Test).

The results in Table 4 validated the effect of CM-NPs administration on bone mineral density of proximal, distal and total areas of femur bones in rosiglitazone. The DM-rats cured with rosiglitazone showed significant decrease in BMD of the proximal, distal and total measured areas in comparison to the healthy male rats. On the other hand, DM-rosiglitazone-rats treated with low dose of CM-NPs increased slightly the three measurements of BMD compared to DM-rosiglitazone-rats. However, DM-rosiglitazone-rats treated with medium or high doses of CM-NPs caused significant increase in BMD of the three areas as compared to the DM-rosiglitazone-rats (Table 4).

Expression changes in osteogenic genes

The quantitative values of RT-PCR (qRT-PCR) of osteogenic genes (*OC, COL and ACP5*) are summarized in Figures 2-4. The results revealed that DM-rats preserved with rosiglitazone showed significantly lower expression values of *OC, COL* genes in comparison to the healthy male rats (Figs 2&3). While, DM-rosiglitazone-rats treated with low, medium and high doses of CM-NPs caused noteworthy increase in *OC* and *COL* expression as compared with the DM-rosiglitazone-rats. Moreover, highest expression levels of *OC* and *COL* genes were showed in DM-rosiglitazone-rats treated with the high dose of CM-NPs designed for the protection against osteoporosis (Figs 2&3).

Regarding *ACP5* gene, the current results revealed that DM-rats treated with rosiglitazone showed significantly higher expression values of *ACP5* mRNA in comparison to the healthy male rats (Fig. 4). However, DM-rosiglitazone-rats treated with low, medium and high doses of CM-NPs caused significant decrease in *ACP5* expression as compared with the DM-rosiglitazone-rats. Moreover, lowest expression levels of *ACP5* gene were showed in DM-rosiglitazone-rats treated with the high dose of CM-NPs (Figs 2&3).

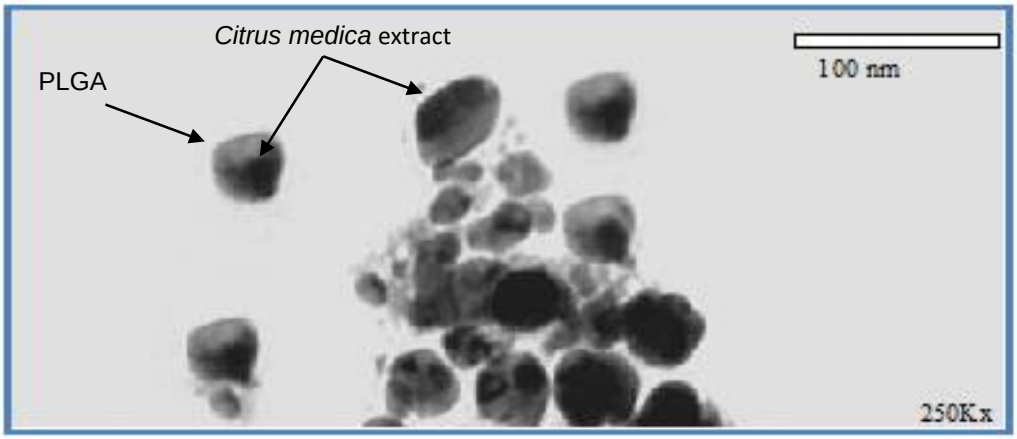

Figure 1: Cross-sectional transmission electron microscopy image of the of poly-lactic-co-glycolic acid (PLGA) encapsulation of *Citrus medica* nanoparticles.

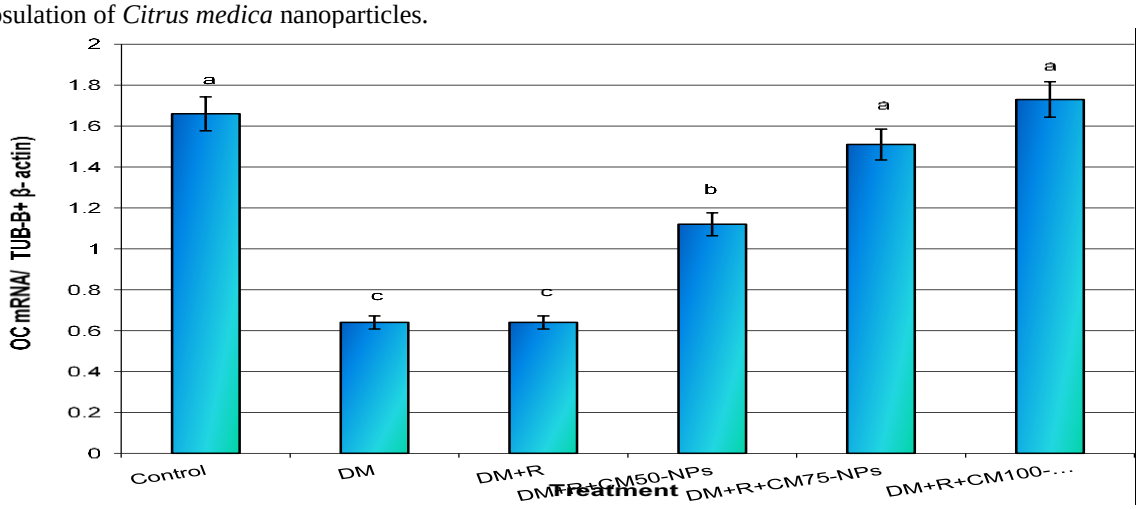

Figure 2: The alterations of OC mRNA in bone tissues isolated from intertrochanteric region of the proximal femur of male rats treated with rosiglitazone alone or combined with CM-NPs. Data are presented as mean ± SEM. [a,b,c]Mean values within tissue with unlike superscript letters were significantly different ([a:] $P<0.01$, [b, c] $P<0.05$, Scheffé-Test).

Micronucleus formation

Effect of CM-NPs against rosiglitazone-induced osteoporosis on MnPCEs formation in the bone marrow cells of male rats is summarized in Figure (5). The results revealed that DM-rats treated with rosiglitazone showed significantly higher incidence of MnPCEs in comparison to healthy male rats. On the contrary, DM-rosiglitazone-rats treated with low, medium and high doses of CM-NPs caused significant decrease in MnPCEs formation as compared to DM-rosiglitazone-rats. Furthermore, lowest incidence of MnPCEs was showed in DM-rosiglitazone-rats treated with the high dose of CM-NPs for the protection against osteoporosis.

MAA-QDs induce 8-hydroxy-2-deoxyguanosine (8-OHdG) generation

Assessment of "8-OHdG" generation in bone marrow cells of rat genome following CM-NPs treatment against rosiglitazone as an alternate oxidative stress was summarized in Figure 6.

The results indicated that "8-OHdG" levels in healthy control rats reached value about 6.2±0.7 "8-OHdG" per 10^5 dG. However, generation of 8-OHdG/2-dG ratio following rosiglitazone treatment of DM-rats showed significantly higher ratio (16.7±1.9) in comparison to the healthy male rats (6.2±0.7). DM-rosiglitazone-rats treated with low, medium and high doses of CM-NPs caused significantly low ratio of 8-OHdG/2-dG generation as compared to those in DM-rosiglitazone-rats. Moreover, low ratio of 8-OHdG/2-dG generation was showed in DM-rosiglitazone-rats treated with the high dose of CM-NPs which was relatively similar to that in healthy male rats.

DISCUSSION

The present study exposed that experimental induction of diabetes in rats found a significant increment in serum glucose. Diabetes was brought chemically using streptozotocin, which is a toxic compound for β-cell[30]. It produces oxygen radicals in the body, which cause pancreatic injury through demolition of β-cells of the islets of Langerhans; leading to massive reduction in insulin release and improved blood sugar in animals[30] .

However, there was a significant and a dose dependant decrease in serum level of glucose with CM-NPs treatment. The MeOH extract of the defatted leaves of *C. medica* L. proved to be rich in flavonoids[31]. Phenolic compounds are reported to be antioxidant[32] as well as Vit C, E and carotenoids are known also by their great antioxidant activity[33]. Flavanones and flavanols were

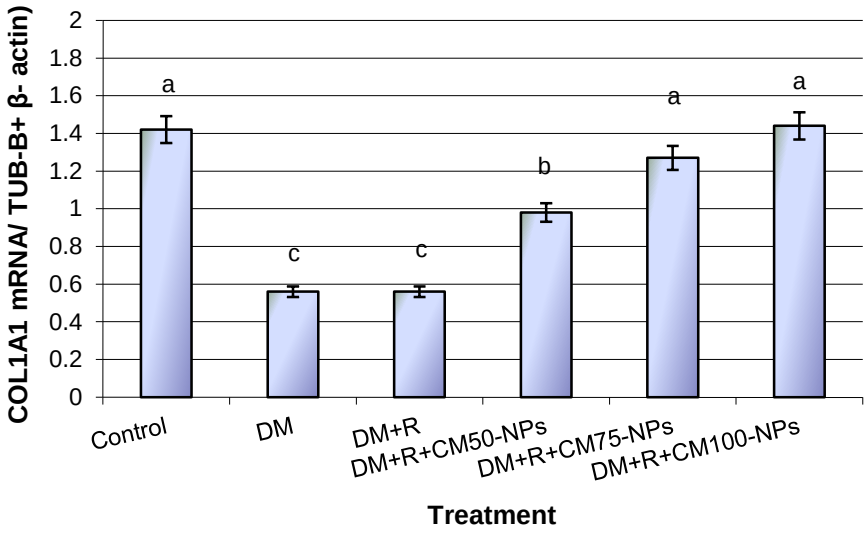

Figure 3: The alterations of COL1A1 mRNA in bone tissues isolated from intertrochanteric region of the proximal femur of male rats treated with rosiglitazone alone or combined with CM-NPs. Data are presented as mean ± SEM. [a,b,c] Mean values within tissue with unlike superscript letters were significantly different ([a:] $P<0.01$, [b, c] $P<0.05$, Scheffé-Test).

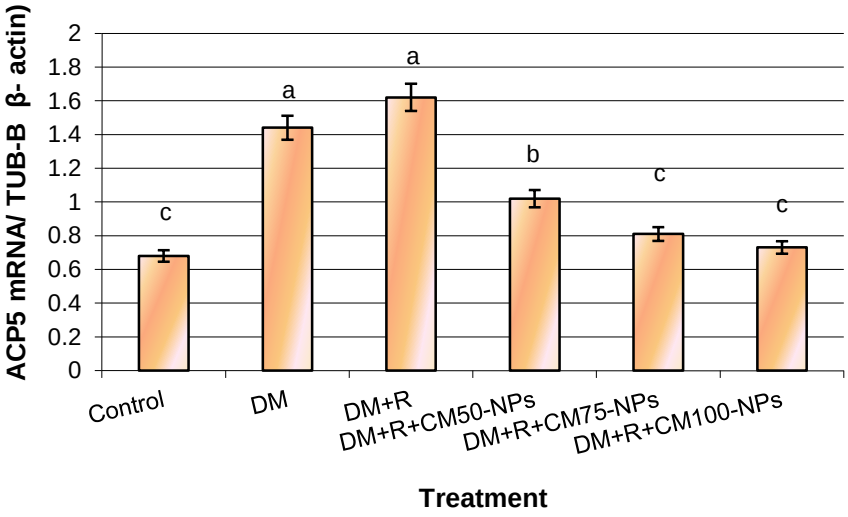

Figure 4: The alterations of ACP5 mRNA in bone tissues isolated from intertrochanteric region of the proximal femur of male rats treated with rosiglitazone alone or combined with CM-NPs. Data are presented as mean ± SEM. [a,b,c] Mean values within tissue with unlike superscript letters were significantly different ([a:] $P<0.01$, [b, c] $P<0.05$, Scheffé-Test).

reported to act as biological antioxidant in cell cultures and offer some confidence against the early stage of diabetes[34]. They also normalize the blood glucose altering the glucose regulatory enzymes[34]. Furthermore, they decrease glucose levels and improve glycolytic and gluconeogenetic enzymes in tissues[35]. The possible mechanism by which CM-NP brings about its antihyperglycemic action may be through potentiation of the pancreatic secretion of insulin from islet β-cell or due to enhanced transport of blood glucose to the peripheral tissue.

Treatment with natural herbs is likely to be fraught with minor side effects compared to the presently used synthetic oral medicated preparations like Rosiglitazone that show adverse effect on bones[36,11]. Osteoporosis is a frequent and important disorder associated with aging and results in bone fracture. It is well established that Rosiglitazone acts on PPAR-γ1 and PPAR-γ2, as a results of alternative promoter usage and alternative splicing[37]. The present study revealed that Rosiglitazone administered (10 mg/ kg) results in significant bone loss, possibly through its interaction with PPAR-γ2 isoforms, which is critical for the regulation of osteoblast and adiposities differentiation[38].

The present study showed that treatment with Rosiglitazone resulted in significant reduction in serum

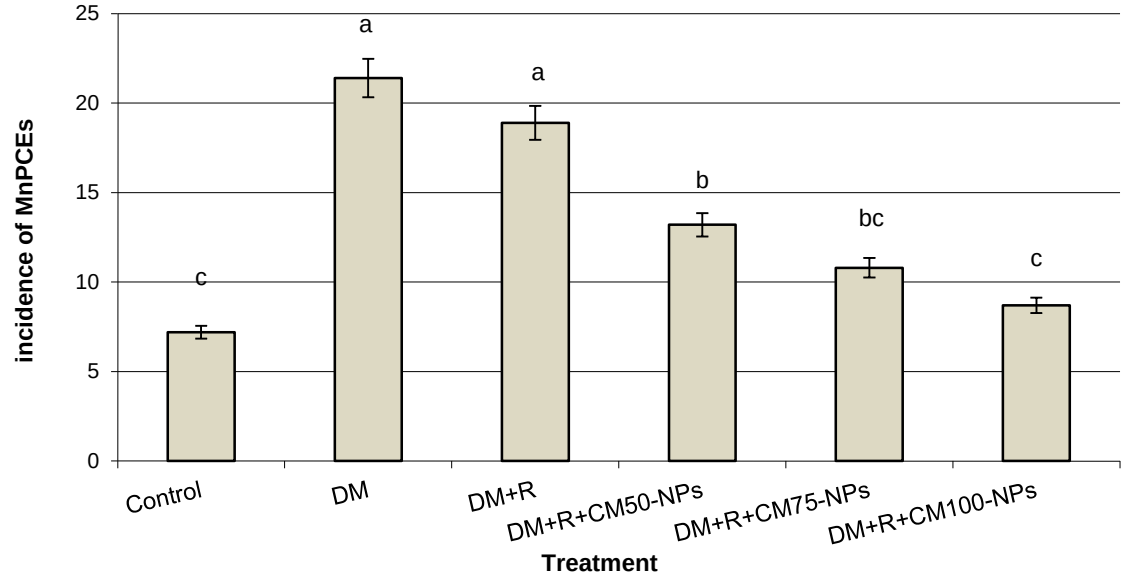

Figure 5: Micronucleated polychromatic erythrocytes (MnPCEs) of male rats treated with rosiglitazone alone or combined with CM-NPs. Data are presented as mean ± SEM. [a,b,c] Mean values within cell samples with unlike superscript letters were significantly different ([a:] $P<0.01$, [b, c] $P<0.05$, Scheffé-Test).

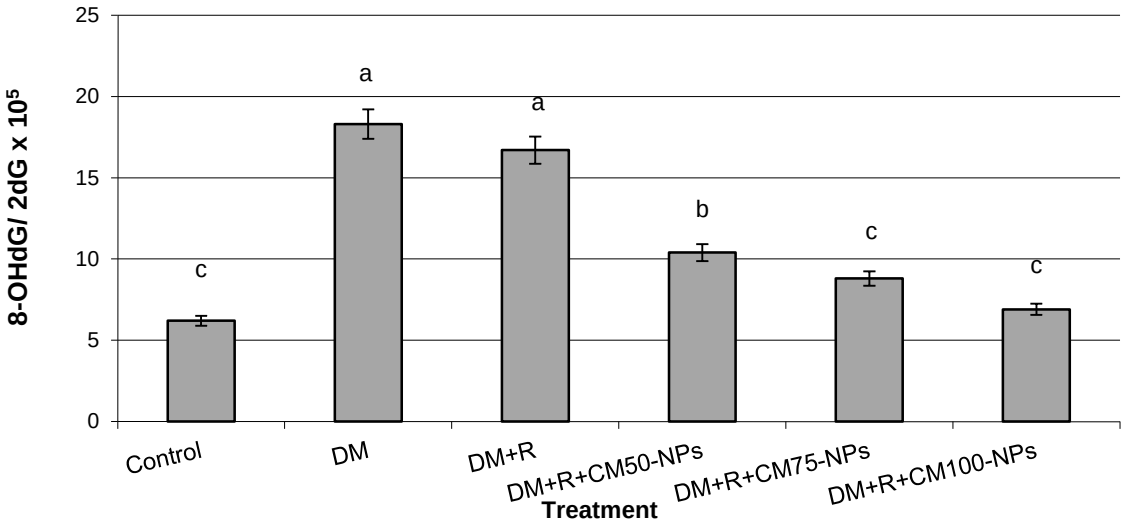

Figure (6): Generation of 8-OHdG in rat bone marrow genome following rosiglitazone alone or combined with CM-NPs. DNA damage was expressed as the ratio of oxidized DNA base (8-OHdG) to non-oxidized base (2-dG) in bone marrow DNA. Results are expressed as mean±SEM of data from at least ten samples. [a,b,c] Mean values within cell samples with unlike superscript letters were significantly different ([a:] $P<0.01$, [b, c] $P<0.05$, Scheffé-Test).

OPG level. This coincides with the findings of Lazarenko et al[39], who reported that Rosiglitazone affect the level of OPG mRNA and diminishes the production of OPG from osteoblasts and/or marrow cells of mice. While, the CM-NPs administration produced a significant increase in the serum OPG level in Rosiglitazone treated rats. CM-NPs has a direct effect on the osteoblast cells stimulating OPG expression[40].

Moreover, the current study revealed that the treatment with Rosiglitazone caused a significant elevation in the serum RANKL and β2-microglobulin levels. These findings agreed with that of Cho et al.[41], who reported that Rosiglitazone promote osteoclastogenesis via increasing RANKL, β2-microglobulin and decreasing the OPG expression. CM-NPs administration caused a significant decrease in serum RANKL and β2-microglobulin levels in the DM- Rosiglitazone- treated rats. It has been reported that significant reduction in serum RANKL level were resulted in Citrus extracts treated groups as compared to that in untreated OVX rats[42].

The action mechanism of CM-NPS in inhibiting bone loss may be resulting from the flavonoids hesperidin in CM extract indicating that intake of the citrus flavonoids might be useful in preventing symptoms arising from estrogen deficiency. In agreement with these findings Chiba et al.[43] demonstrated that hesperidin not only has cholesterol-lowering effects, but also prevents bone loss in ovariectomized (OVX) mice without substantial effects on the uterus, indicating that supplementation of the citrus flavonoids is useful in inhibiting symptoms arising from estrogen deficiency. Our results emphasized that citrus flavonoids prohibited bone loss at all three regions of the femur, indicating that citrus flavonoid was effective on both cortical and trabecular bones.

The present study revealed that DM-rats treated with Rosiglitazone showed significantly low levels of OC, COL expression and higher expression of ACP5 mRNA in bone samples compared with healthy rats. This finding was consistent with previous studies that Rosiglitazone treatment reduced collagen and osteocalcin expression levels in bone marrow cells[41]. However, DM-rosiglitazone-rats treated with CM-NPs caused adverse effect on OC, COL and ACP5 expression compared with DM-rosiglitazone-rats. Trzeciakiewicz et al.[44] and Kannampalli et al.[45] reported also that citrus flavonoids hesperetin increased the expression of osteogenic genes in rodent primary osteoblasts and decreased the level of acid phosphatase in several tissues of rats. They proposed that hesperetin may adjust osteoblast differentiation through bone morphogenetic protein signaling and may influence the mineralization process by modulating osteopontin expression. We could also show that the expression of osteoinducing genes such as osteocalcin and collagen1α1 by rats osteoblasts during CM-NPs supplementation were improved. It was shown, that osteocalcin mRNA and synthesis correlates with calcium deposition in rat osteoblast[46]. Moreover, osteocalcin and collagen 1 promotes osteoblasts differentiation[48,49].

The current study indicated that DM-rats treated with Rosiglitazone showed significantly higher incidence of MnPCEs and generation of 8-OHdG/2-dG ratio compared with healthy rats. Oz Gul et al.[49] found also that treatment of type-2 diabetes patients with Rosiglitazone increased sister-chromatid exchange, total chromosome aberrations, and micronucleus formation. In contrary, DM-rosiglitazone-rats treated with CM-NPs caused significant decrease in MnPCEs formation and generation of 8-OHdG/2-dG ratio. In agreement with our finding, Hosseinimehr et al.[50] described that citrus flavonoids hesperidin significantly protects against genotoxicity induced by the radiotracer ^{99m}Tc-MIBI in lymphocytes.

The molecular mechanisms underlying the protective effects of hesperidin against genotoxic agents are not clear. Hesperidin has been shown to have antioxidant activity against the cellular oxidative stress concomitant with neurodegenerative diseases[50]. This flavonoid also attenuated decreases of glutathione peroxidase and glutathione reductase activity and decreased DNA damage in H2O2-induced PC12 cells[51], and also inhibited low-density lipoprotein oxidation[52]. Oral administration of hesperidin has protective effects against gamma radiation-induced hepatocellular damage and oxidative stress in rats[53].

CONCLUSION

The combination therapy of R and *Citrus medica* nanoparticles (CM-NPs) reduced the risk of osteoporosis in insulin resistance rats.

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