



The Effect of *Lepidium Sativum* L. Extract Loaded on Chitosan Nanoparticles in Experimental Rat Model of Osteoporosis.

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ABSTRACT:

Osteoporosis is a metabolic bone disorder influencing approximately 40% of postmenopausal females, caused by a suppression in bone mass leading to bone weakness and fractures. Herbal medicine commonly used rather than conventional drugs due to its minor adverse effects. *Lepidium sativum* L. (LS) or “hab arachad”, is known as an alternative medication for fracture treatment. The aim of this study is to evaluate the effect LS extract loaded chitosan nanoparticles on gene expression of miR-142-3p and miR-23a in experimental rat model of osteoporosis. The osteoporosis was induced in rats by subcutaneous injection with methylprednisolone (3.5 mg/kg b.wt / day) for 4 weeks. Then osteoporotic rats were orally treated with 400 mg/kg b.wt /day LS extract, chitosan nanoparticles (CN) and 400 mg/kg b.wt /day LS extract loaded on chitosan nanoparticles for 12 weeks. Our results showed that treatment with LS, CN and LS loaded on CN ameliorated the biochemical bone indices with variable degrees, with a final results of improved serum calcium and phosphorus. Treated rats showed a significant decrease in miR-23a expression and a significant increase in miR-142-3p expression which emphasized the improvement of osteoporosis. The effect of treatment with LS and LS loaded on CN was confirmed by histopathological observations. All of these effects together with the safety and no side effects of LS and LS loaded on CN make it a hopeful therapeutic agent for Osteoporosis management.

Keywords: Osteoporosis, *Lepidium sativum* L., Chitosan.

1. INTRODUCTION

Osteoporosis is aggressive bone disease caused by progressive suppression in bone mass and increasing risk of fracture. In osteoporosis, the bone mineral density (BMD) is decreased and the bone micro architecture worsens (Herlund et al., 2013). The cell types responsible for bone remodeling are osteoblasts, that make new bone, and osteoclasts, that cause bone resorption. When the balance between bone

synthesis and resorption is disturbed and resorption predominates formation, inflammation will cause bone-resorption disorder (osteoporosis) (Zheng et al., 2014).

The incidence of osteoporosis occurs via spontaneous elevation in proinflammatory, proosteoclastic, cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), and Interleukin -1 (IL-1) which causes

bone resorption by osteoclasts (Kanis et al., 2017). IL-1 plays a major role in bone resorption under abnormal circumstances. Bone tissue is very sensitive to IL-1, that controls both bone synthesis and bone resorption (Lee et al., 2010).

MicroRNAs (miRNAs) are endogenous, single short stranded non-coding RNAs nearly 22 nucleotides length, which are extensively found in higher organisms and control gene expression post-transcriptionally. miRNAs have important functions in bone homeostasis, such as the control of osteoblast and osteoclast differentiation (Taipaleenmäki et al., 2012). MiR-142-3p is significantly, particularly expressed during osteoclastogenesis (Fordham et al., 2016). MiR-23a enhances osteocyte differentiation (Zeng et al., 2017).

Herbal medicine is commonly used in place of chemical drugs due to its minor adverse effects. LS which is called locally “habarachad”, is belonging to Brassicaceae family, grown in the Middle East where LS is recommended traditionally for hypertension, diabetes, renal disease and phytotherapy (Juma, 2007).

Nanotechnology is developed as the science which has numerous applications such as delivery of certain minerals, nutrients or drugs. There has been a need for the development of nanotechnology-based delivery structures for the delivery of various drugs or to improve the bioavailability

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of particular micronutrients. Chitosan (CS) is the most used polymers in drug delivery because of its properties such as biocompatibility, biodegradability and non-toxicity with environmental safety (Saini *et al.*, 2015). The aim of this study is to evaluate the effect LS extract loaded chitosan nanoparticles on gene expression of miR-142-3p and miR-23a in experimental rat model of osteoporosis.

2. MATERIALS AND METHODS

Experimental animals

Our study was conducted on 50 albino male rats (body weight ranging from 200 to 250 g) which were purchased from animal house of Medical Research Institute, Alexandria University). Animals were housed in cages in a well-ventilated room ($25\pm 2^{\circ}\text{C}$), with a humidity of ($50\pm 10\%$) and kept on 12 hrs dark/ light cycle at the animal house.

Ethical statement

The approval of our study was according to "Institutional Animal Care and Use Committee" (IACUC), Alexandria University, Egypt (Approval No: AU01219091521). Experiments were made in exact accordance to guidelines and regulations of Egypt's guide for the care and use of experimental animals. All efforts were done to decline the distress of rats throughout the experiment.

Experimental design

Rats were classified into 2 groups: **Group I (control):** 10 normal males .**Group II:** 40 Osteoporotic males. The induction of Osteoporosis was done by subcutaneous injection with methylprednisolone (synthetic glucocorticoid) (3.5 mg/kg b.wt / day) for 4 weeks (Hulley *et al.*, 2002). Then this group was categorized into 4 subgroups (each group 10 rats): **Group IIA:** osteoporotic untreated males. **Group IIB:** Osteoporotic rats were orally treated with LS extract 400mg/kg b.wt /day for 12 weeks (Gabr *et al.*, 2017). **Group IIC:** Osteoporotic rats were orally treated with CN 10mg/kg b.wt / day for 12 weeks (Rashid *et al.*, 2018). **Group IID:** Osteoporotic rats were treated with LS extract 400 mg/kg b.wt /day loaded on CN 10mg/kg b.wt / day for 12 weeks (Rashid *et al.*, 2018).

After the end of treatment duration , all male rats were sacrificed using isoflurane inhalation then blood was obtained and centrifuged for 10 min at 4,000 rpm to get serum for biochemical analysis of calcium, phosphorus, alkaline phosphatase, tartrate-resistant, TNF- α and IL-1 and to obtain plasma for molecular analysis of miR-23a and miR-142-3p. Also femur tissues of the rats were collected for histopathological examination.

Extraction of *Lepidium sativum*

3000 g of LS seeds were completely grinded and mixed in absolute ethanol overnight. Filtration of The mixture and ethanol addition were done respectively. This procedure was repeated three times, and all the filtrates were evaporated in rotary evaporator. The obtained dried extract was weighted (Walvekar *et al.*, 2017).

Preparation of chitosan nanoparticles

By ionotropic gelation chitosan with sodium tripolyphosphate (TPP) anions, chitosan nanoparticles were synthesized Using magnetic stirring, CS (0.5%) was dissolved in acetic acid(1%) at room temperature TPP was dissolved at a 2 mg/ml ratio in deionized water. Using a 1 ml syringe, TPP solution was added to CS dropwise. A few

drops of glutaraldehyde were added as well (for particle cross-linking) while the mixture was continuously magnetically stirred at 1000 rpm for 30 minutes. TPP solutions produced a final CS to TPP mass ratio of 3:1 (Vishwakarma *et al.*, 2019)

Synthesis of Chitosan encapsulated *Lepidium sativum*

LS extract (10% w/w) was dissolved in ethanol, added dropwise to the CS solution, and stirred for one hour. In deionized water TPP was dissolved. Drops of glutaraldehyde were added and TPP(1%) solution was added dropwise to the chitosan at a ratio of 1:1 while the mixture was continuously magnetically stirred at 1000 rpm for thirty minutes. Ionic gelation between chitosan and TPP spontaneously generated nanocapsules. The solution was then centrifuged at 12,000 rpm for 15 minutes at 4°C to extract the non-entrapped particles.

Serum parameters

Calcium (Ca), phosphorus (P) were measured using colorimetric method [Lab-Care Diagnostics (India) PVT. Ltd], alkaline phosphatase (ALP) using kinetic method [Chema Diagnostica], tartrate-resistant acid phosphatase (TRAP), TNF- α and IL-1 using enzyme-linked immunosorbent assay (ELISA) [Chongqing Biospes C., Ltd].

Plasma miR-23a and miR-142-3p expression: using real time polymerase chain reaction (RT-PCR)

Total RNA purification was performed using the miRNeasy Serum/Plasma Advanced Kit. Reverse transcription reactions are done using miScript HiSpec Buffer. miScript Primer Assays, used in combination with the miScript SYBR Green PCR Kit, enable quantification of miRNA by RT-PCR.

Real-time PCR

an miRNA-specific miScript Primer Assay (forward primer) and the miScript SYBR Green PCR Kit, which contains the miScript Universal Primer (reverse primer) (Table 1) and QuantiTect SYBR Green PCR Master Mix were used.

Table 1: Primers (Catalog No

Gene Name	Primers (Catalog No.)
hsa-miR-23a-3p	MS00031633
hsa-miR-142-3p	MS00031451
Hs_miR-25_3p (Reference gene)	MS00003227

The relative expression of the target gene was quantified relative to the expression of the reference gene in the same sample by calculating and normalizing the threshold cycles (Ct) values of target genes to that of miRNA 25-3p using $\Delta\Delta\text{Ct}$ method.

The relative expression of the genes in each sample is expressed as:

$$\Delta\text{Ct} = \text{Ct}_{\text{test gene}} - \text{Ct}_{\text{reference gene}}$$

Then the ΔCt of groups was normalized to ΔCt of control group:

$$\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{groups}) - \Delta\text{Ct}(\text{control group})$$

Finally the expression ratio or fold difference in expression was calculated from the formula:

$$\text{Normalized expression ratio} = 2^{-\Delta\Delta\text{Ct}}$$

Histopathological Examination Hematoxylin & Eosin (H & E) Stain

For cross-sectioning, the right tibia of each rat was removed, weighed, preserved in 10% (v/v) formaline, and embedded in paraffin wax. Haematoxylin and Eosin (H&E) was used to stain five micron slices, which were then seen under a light microscope..

Alizarin Red S Staining

For cross-sectioning, the right tibia of each rat was removed, weighed, preserved in 10% (v/v) formaline, and embedded in paraffin wax. Five micron sections were subjected to light microscopy after being stained with 1% Alizarin red S.

Statistical analysis

Data were analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp).. Results significance was judged at the 5% level. The used tests were: F-test (ANOVA): Pearson coefficient: To correlate between two normally distributed quantitative variables.

Results

characterization of prepared nanosuspension

In this study

Transmission Electron Microscopy(TEM) of prepared nanosuspension

In the present study, TEM images of CN and CN incorporated LS nanoparticles shown in figure (1). The nanoparticles have spherical shape, and mean size around 60 nm , 250 nm for CN and CN loaded LS nanoparticles respectively.

Fourier-transform infrared (FTIR) measurement

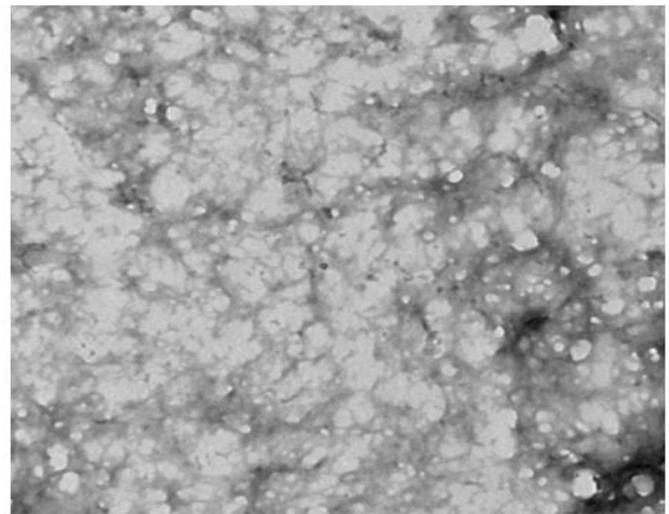
The FTIR spectra of CS matrix, CN and LS loaded CN are shown in figure (2). In CS nanoparticles spectra, the strong and wide peak in the 3500-3300 area is attributed to hydrogen-bonded O-H stretching vibration and the peak of 3438 cm^{-1} has a shift to 3323.27 cm^{-1} indicating an enhancement of hydrogen bonding. In nanoparticles the peak for N-H bending vibration of the amide II carbonyl stretch at 1650 cm^{-1} shifted to 1635.79 cm^{-1} . These results were attributed to the linkage between phosphoric and ammonium ion. So, we concluded that the tripolyphosphoric groups of TPP are linked with ammonium groups of CS In the LS loaded CS the peak for N-H bending at 1635.97 cm^{-1} was disappeared and new peaks at 1379.95 cm^{-1} and 1079.17 cm^{-1} has appeared and this is due to interaction between CS and LS and this confirm the loading of LS into CN.

Zeta potential measurement

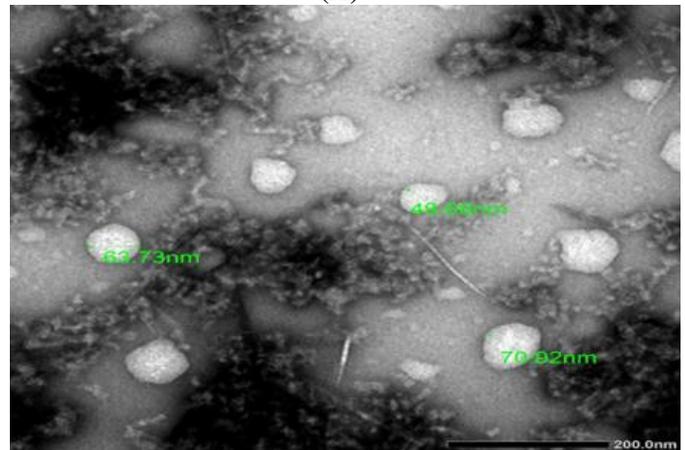
Our results demonstrated respective zeta potentials of CN, LS and CS loaded LS of 39.5, -21.1 and 6.41 mV respectively. These results showed that LS loading leads to a reduction of the particle's zeta potential and leads to stability of nanoparticles(figures 3-5).

Lepidium Sativum loaded CS nanoparticles Entrapment efficiency(EE) by UV-visible spectroscopic analysis:

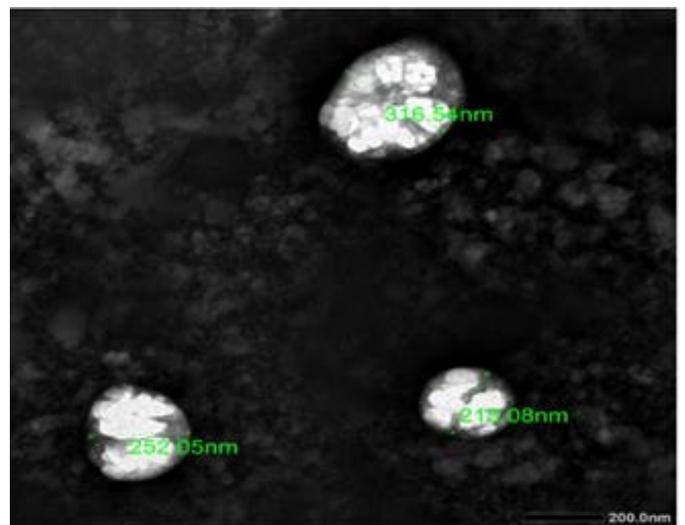
LS loaded CS nanoparticles EE value was calculated. The EE value was in range of 81% and this indicates that the enlargement of the LS in CS solution.



(A)

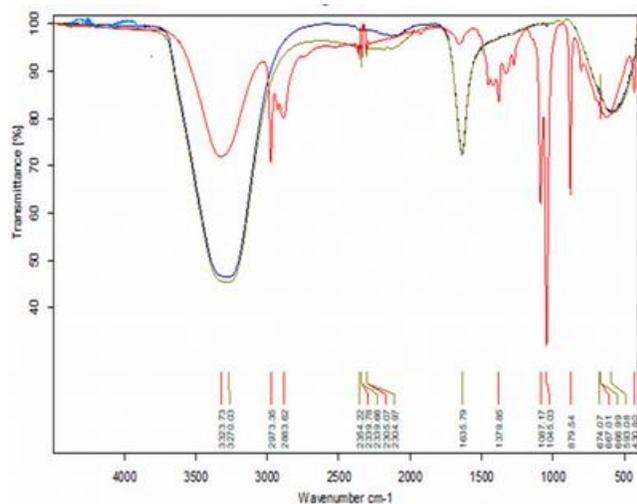


(A)



(B)

Figure (1): Transmission electron microscopy of nanoparticles (a) CS nanoparticles at different magnification (b) CS nanoparticles loaded with LS.



D:\FTIR Measurements\ATR Results\Extract.1	Extract	Instrument type and/ or accessory
D:\FTIR Measurements\ATR Results\CS NPs.0	CS NPs.0	Instrument type and/ or accessory
D:\FTIR Measurements\ATR Results\Ex@CS NPs.0	Ex@CS NPs.0	Instrument type and/ or accessory

Figure (2): FTIR of LS, CS nanoparticles and LS loaded CS nanoparticles.

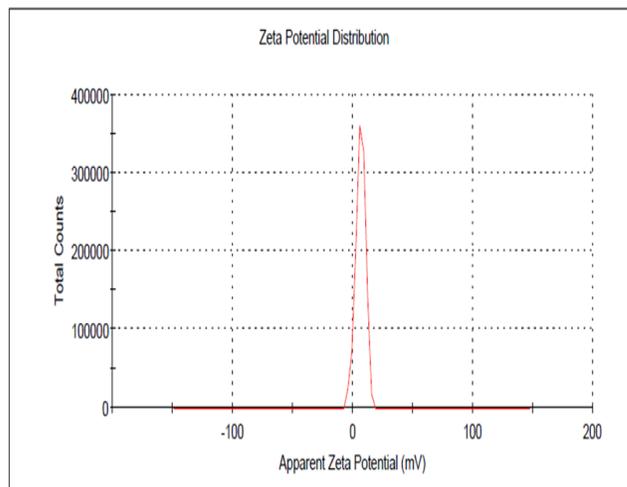


Figure (5): Zeta potential of LS loaded on CS nanoparticles.

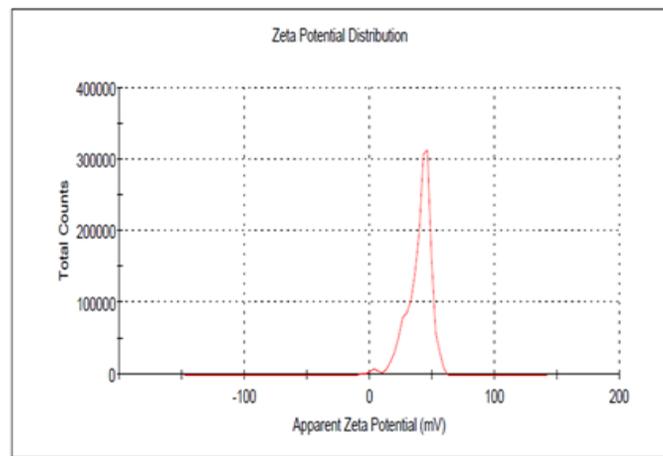


Figure (3): Zeta potential of chitosan nanoparticles

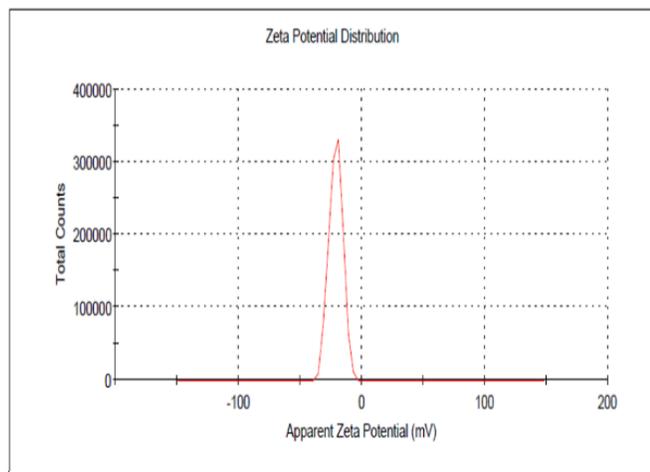


Figure (4): Zeta potential of LS.

Serum Calcium (Ca) levels.

Serum calcium levels results; as shown in table (2), declares that osteoporotic rats (untreated) have significant decline in Ca level compared to healthy group. Meanwhile both groups treated with CS nanoparticles and L.S loaded on CN showed significant elevation in Ca level compared with the untreated osteoporotic rats. While the rats treated with LS showed relative elevation in Ca compared to osteoporotic untreated rats.

Serum Phosphorus (P) levels.

The results of serum level of P; as shown in table (2), declares that the osteoporotic untreated rats had non-significant decline in P level compared to control rats. On the other hand all other treated rats had non-significant elevation in P level compared to both the control and osteoporotic untreated rats.

Serum Alkaline phosphatase activity (ALP).

Serum ALP activity results ; as shown in table (2), that osteoporotic untreated rats had a non-significant elevation in ALP activity compared with normal rats. Meanwhile all treated osteoporotic rats had a non-significant differences in ALP activity compared to normal rats and osteoporotic untreated animals While the osteoporotic rats treated with LS loaded on CN had a significant decline in ALP activity compared to osteoporotic rats treated with LS.

Table (2): Showing serum Ca level (mg/dl), serum phosphorus level (mg/dl), serum ALP activity (U/l) in all studied groups.

	Healthy control (n = 10)	Untreated osteoporotic rats (n = 10)	Treated osteoporotic rats		
			LS (n = 10)	Chitosan nanoparticles (n = 10)	LS loaded on chitosan nanoparticle (n = 10)
Calcium level (mg/dl)	7.28 ± 0.14	6.35 ^a ± 0.35	6.99 ± 0.09	7.15 ^b ± 0.08	7.10 ^b ± 0.10
% of change 1		↓12.8	↓4.0	↓1.8	↓2.5
% of change 2			↑10.1	↑12.6	↑11.8
P level (mg/dl)	7.99 ± 0.24	7.87 ± 0.29	9.30 ± 0.60	8.71 ± 0.30	8.79 ± 0.26
% of change 1		↓1.5	↑16.4	↑9.0	↑10.0
% of change 2			↑18.2	↑10.7	↑11.7
ALP activity U/l	169.4 ± 6.98	201.4 ± 6.86	214.8 ± 12.40	175.1 ± 17.72	162.3 ^c ± 10.65
% of change 1		↑18.9	↑26.8	↑3.4	↓4.2
% of change 2			↑6.7	↓13.1	↓19.4

Data was expressed by using Mean ± SE.

Pairwise comparison between each 2 groups was done using Post Hoc Test, (Tukey)

a: significant with healthy control group at $p \leq 0.05$

b: significant with untreated osteoporotic group at $p \leq 0.05$

c: significant with osteoporotic group treated with LS at $p \leq 0.05$

d: significant osteoporotic group treated with chitosan nanoparticles at $p \leq 0.05$

% of change 1: from the control group

% of change 2: from the untreated osteoporotic group

Serum level of Tartrate-resistant acid phosphatase (TRAP).

Serum TRAP level results as shown in table (3) revealed that all osteoporotic rats (treated and untreated) had a significant elevation in TRAP level compared to normal rats. While osteoporotic rats treated with CN had a significant decline TRAP level compared with the untreated osteoporotic group and the osteoporotic group treated with LS. On the other hand the osteoporotic rats treated with LS loaded on CN had a significant elevation in serum TRAP level compared to osteoporotic rats treated with CS nanoparticles.

Serum level of Tumor necrosis factor alpha (TNF- α).

TNF- α results as shown in table (3) explaining that all osteoporotic rats (treated and untreated) had significant elevation in TNF- α level compared to normal rats. Meanwhile the osteoporotic rats treated with LS had a significant increase in of TNF- α level compared with osteoporotic (untreated) rats while osteoporotic rats treated with CN had a significant decrease compared to osteoporotic

rats treated with LS. On the other hand the osteoporotic rats treated with LS loaded on CN had significant elevation in TNF- α level compared to osteoporotic untreated rats and a significant decline compared to osteoporotic animals treated with LS.

Serum level of Interleukin-1 (IL-1).

IL-1 results as shown in table (3) revealed that all osteoporotic rats (treated and untreated) had significant increase in IL-1 level compared to normal rats. Meanwhile the osteoporotic group treated with LS showed significant increase in IL-1 level compared with osteoporotic (untreated) rats. While the osteoporotic rats treated with CN had a significant decline in IL-1 level compared with the osteoporotic rats treated with LS. On the other hand the osteoporotic rats treated with LS loaded on CN had significant elevation in IL-1 level compared to osteoporotic untreated rats and osteoporotic rats treated with CN.

Table (3) Showing serum TRAP level (pg/ml), serum TNF- α level (pg/ml) and serum IL-1 level (ng/L) in all studied groups.

	Healthy control (n = 10)	Untreated osteoporotic (n = 10)	Treated osteoporotic rats		
			rats LS (n = 10)	Chitosan nanoparticles (n = 10)	LS loaded on chitosan nanoparticle (n = 10)
TRAP (pg/ml)	31.51 ± 2.15	7534.7 ^a ± 1154.3	6856.0 ^a ± 1324.6	1931.4 ^{abc} ± 227.1	8340.3 ^{ad} ± 1263.5
% of change 1		↑23812.1	↑21658.2	↑6029.5	↑26368.7
% of change 2			↓9.0	↓74.4	↑10.7
TNF-α (pg/ml)	8.42 ± 0.18	674.0 ^a ± 14.31	1249.4 ^{ab} ± 37.61	808.1 ^{ac} ± 81.59	987.1 ^{abc} ± 71.11
% of change 1		↑7904.8	↑14738.5	↑9497.4	↑11623.3
% of change 2			↑85.4	↑19.9	↑46.5
IL-1 (ng/L)	7.27 ± 0.41	155.5 ^a ± 10.83	272.3 ^{ab} ± 26.56	130.9 ^{ac} ± 7.91	296.5 ^{abd} ± 29.51
% of change 1		↑2038.9	↑3645.5	↑1700.6	↑3978.4
% of change 2			↑75.1	↓15.8	↑90.7

Data was expressed by using Mean \pm SE.

Pairwise comparison between each 2 groups was done using Post Hoc Test, (Tukey)

a: significant with healthy control group at $p \leq 0.05$

b: significant with untreated osteoporotic group at $p \leq 0.05$

c: significant with osteoporotic group treated with LS at $p \leq 0.05$

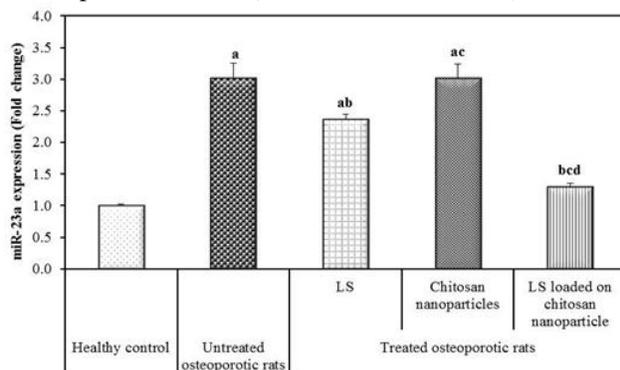
d: significant osteoporotic group treated with chitosan nanoparticles at $p \leq 0.05$

% of change 1: from the control group

% of change 2: from the untreated osteoporotic group

miR-23a expression (Fold change).

miR-23a expression results as shown in figure (6) declared that osteoporotic rats (untreated and treated with LS and treated with CS nanoparticles) had a significant upregulation miR-23a compared to control. Meanwhile osteoporotic rats treated with LS had a significant suppression in miR-23a compared to osteoporotic untreated animals. While osteoporotic group treated with CN had a significant upregulation in miR-23a level compared with osteoporotic animals treated with LS. On the other hand osteoporotic rats treated with LS loaded CN had significant suppression in of miR-23a level compared with osteoporotic untreated rats and both osteoporotic animals (treated with LS and CN).



miR-142-3p expression (Fold change)

miR-142-3p expression results as shown in figure (6) declared that osteoporotic (untreated) group had a significant suppression in miR-142-3p level compared to control rats. Meanwhile all treated osteoporotic rats had a significant upregulation in miR-142-3p level compared to osteoporotic (untreated) group. On the other hand osteoporotic rats treated with CN had significant suppression in miR-142-3p level compared to control rats and osteoporotic rats treated with LS. While osteoporotic rats treated with LS loaded on CN had a significant upregulation in miR-142-3p level compared with the osteoporotic group treated with CN.

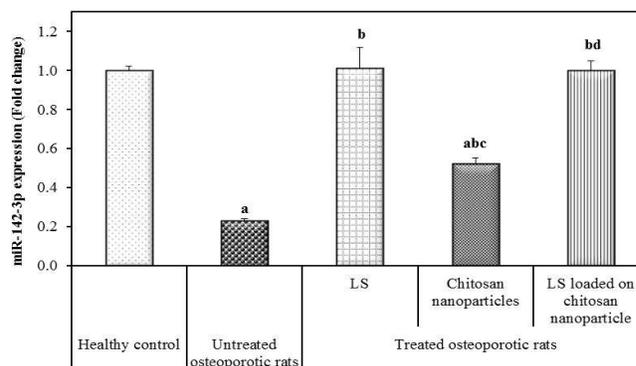


Figure (6): The changes in miR-23a and , miR-142-3p expressions (Fold change) in all studied groups) in all studied groups.

Data was expressed by using Mean \pm SE.

Pairwise comparison between each 2 groups was done using Post Hoc Test, (Tukey)

a: significant with healthy control group at $p \leq 0.05$

b: significant with untreated osteoporotic group at $p \leq 0.05$

c: significant with osteoporotic group treated with LS at $p \leq 0.05$

d: significant osteoporotic group treated with chitosan nanoparticles at $p \leq 0.05$

% of change 1: from the control group

% of change 2: from the untreated osteoporotic group

3. Histopathological results:

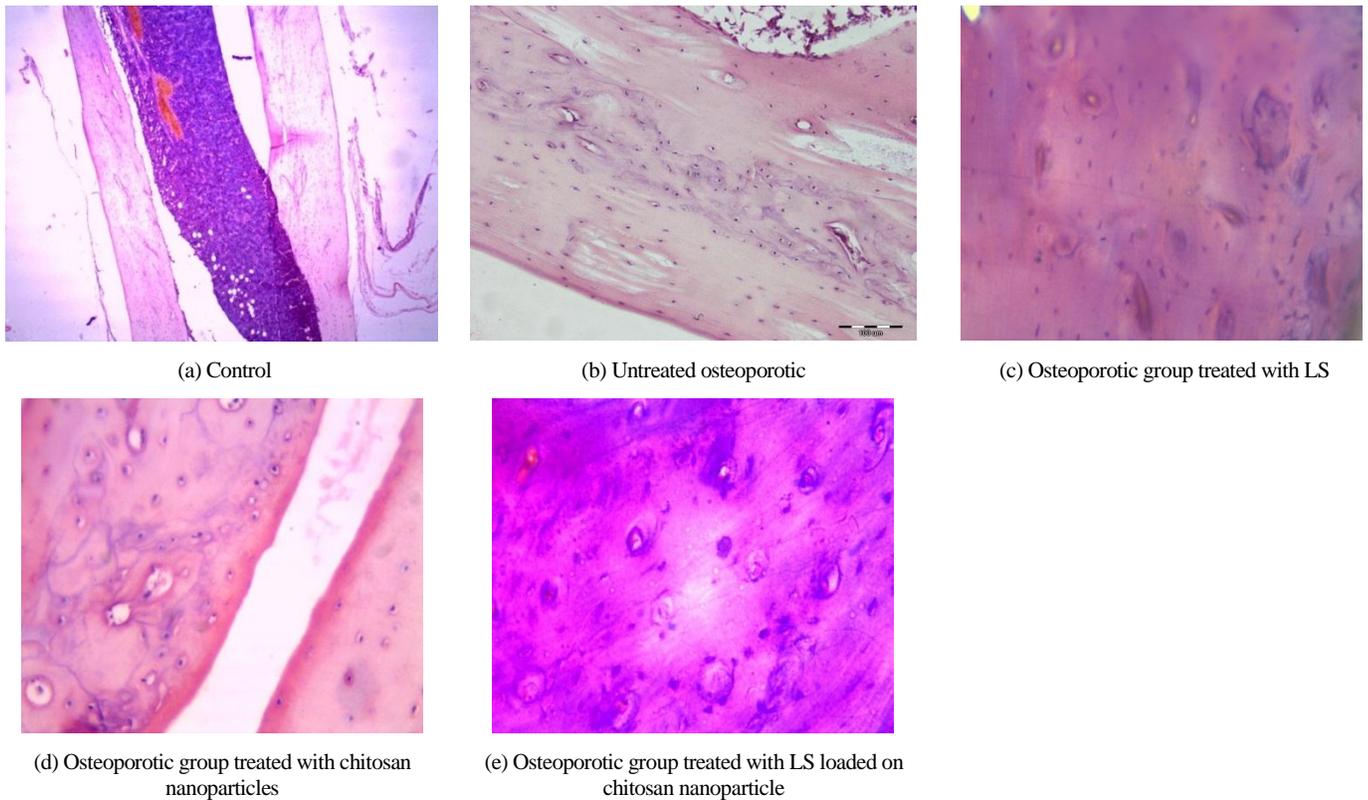
Hematoxylin and Eosin staining

Microscopical examination submitted slides of the control group as shown in figure (7a), groups treated with LS, CS and LS loaded on CS as shown in figure (7 c - e) reveals mature bone structures formed of trabecular compact bone tissue composed of lamellae, concentric rings of bone, surrounding a central channel. The lamellae are lined by osteoblasts and formed of bone matrix entangling osteocytic cells surround by lacunae. Fibrous vascular tissue interspersed between trabeculae are noted. Periosteal surface

are seen. The center of the bone structure is formed medullary cavity entangling bone marrow structure formed of different type of haemotopiotic cells. No evidence of osteoporotic change is noted in the examined biopsy. Normal tibial bony structures with no osteoporotic change.

While examination of the untreated osteoporotic group as shown in figure (2b) showed decreased eosinophilia of the bone matrix ($\downarrow\downarrow$ collagen) Multiple resorption cavities. Cement lines are also seen (indicate new bone formation). Vacuolated bone matrix, multiple resorption cavities and evident cement lines in untreated osteoporotic group.

Figure (7 a – e): Microscopical examination submitted slides of control, untreated osteoporotic group, Osteoporotic group treated with LS, chitosan nanoparticles and LS loaded on CN.



Alizarin staining:

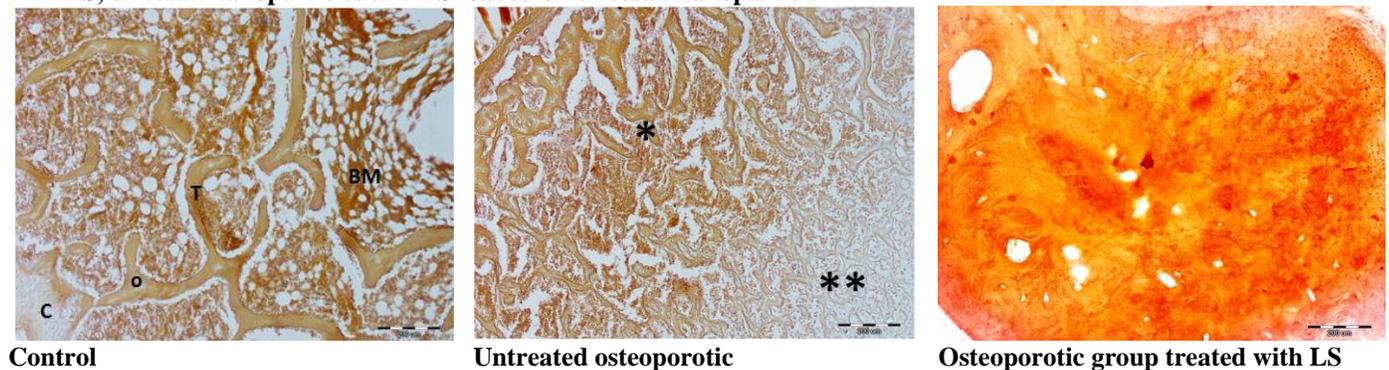
Cross section in the shaft of normal control rat femur as shown in figure (8a) showing homogenous average Alizarin (orange staining of osteoid matrix (o) forming the trabeculae (T) of cancellous bone. Note the negative staining of adjacent zone of cartilage (C) which lacks osteoid tissue. BM= bone marrow.

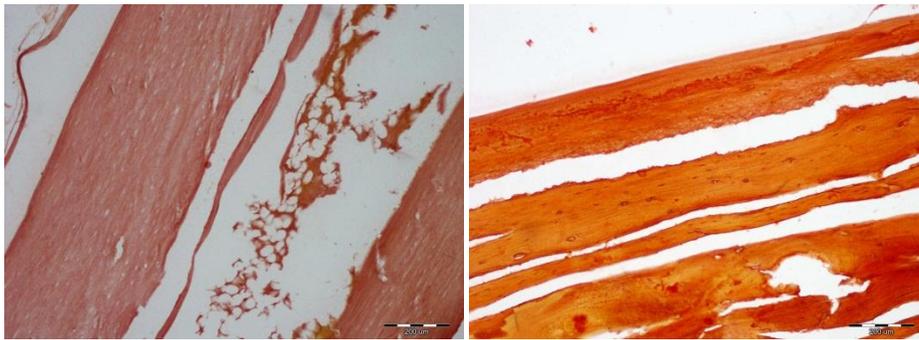
Cross section of rat femur from untreated osteoporotic group as shown in figure (8b) showing faint Alizarin (yellowish)

staining of bone matrix forming the trabeculae of cancellous bone as compared to control group.

Cross section of rat femur from osteoporotic group treated with chitosan nanoparticles showing Alizarin (reddish) staining of bone matrix showing stronger reaction with the matrix as compared to osteoporotic untreated rats, as shown in figure (8c - e)

Fig. (8 a – e): Alizarin staining submitted slides of control, untreated osteoporotic group, Osteoporotic group treated with LS, chitosan nanoparticles and LS loaded on chitosan nanoparticle.





Osteoporotic group treated with chitosan nanoparticles

Osteoporotic group treated with LS loaded on chitosan nanoparticle

Correlation studies

The statistical analysis in osteoporotic rats using Pearson correlation reveals the following;

Serum P levels had a positive correlation with both the serum calcium levels ($r=0.320$, $p=0.044$, fig (9.a)) and miR142-3p expression ($r=0.343$, $p=0.030$, fig (9.b)).

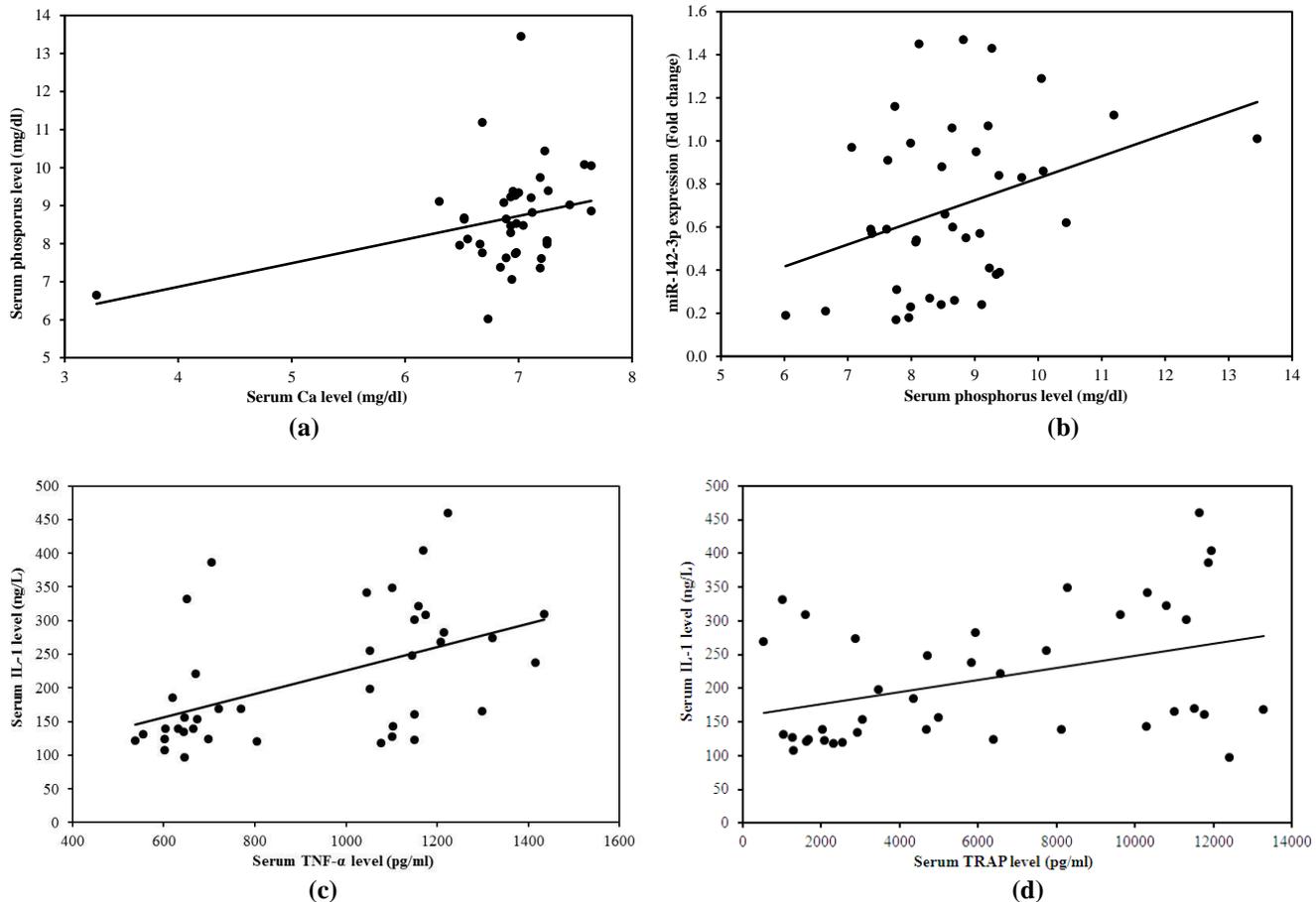
Serum IL-1 levels had a positive correlation with both serum TNF- α ($r=0.504$, $p=0.001$, fig(9.c)) and serum TRAP ($r=0.383$, $p=0.015$, fig(9.d)).

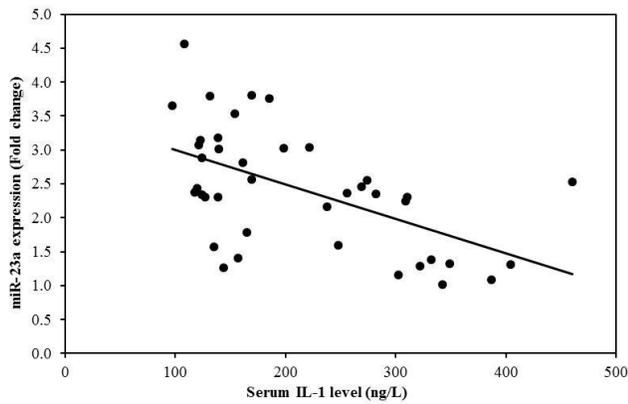
miR23a level had a negative correlation with both serum IL-1 ($r=0.555$, $p<0.001$, fig(9.e)), serum TNF- α ($r=0.407$,

$p=0.009$, fig(9.f)) and miR142-3p ($r=0.590$, $p<0.001$, fig(9.g)).

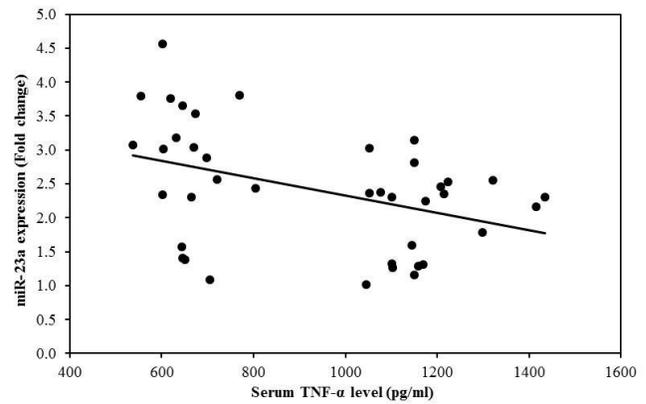
miR142-3p level had a positive correlation with both serum IL-1 ($r=0.676$, $p<0.001$, fig(9h)) and serum TNF- α ($r=0.675$, $p<0.001$, fig(9.i)).

Fig. (9 a – e): Correlation studies in osteoporotic rats using Pearson correlation

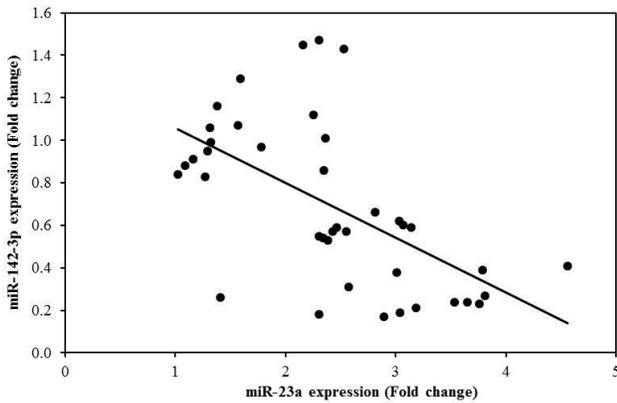




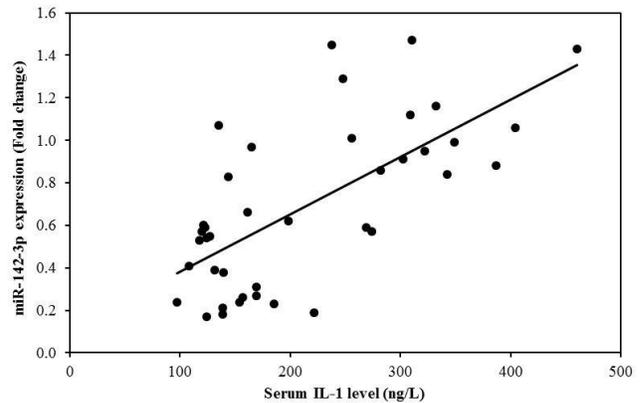
(e)



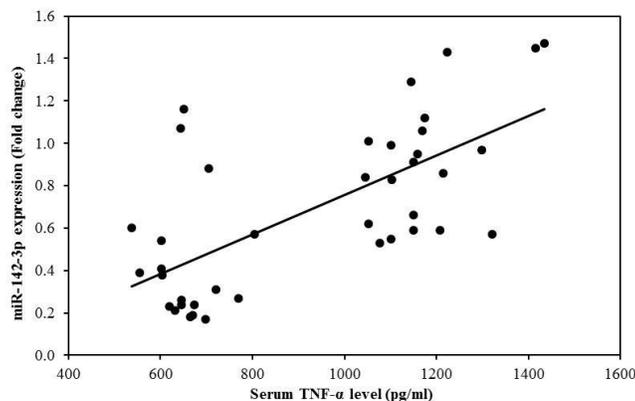
(f)



(g)



(h)



(i)

4. Discussion

The hallmarks of osteoporosis include the loss of bone mass and decline of bone tissue causing bone weakness and, therefore, fractures incidence ([Sambrook & Cooper, 2006](#)). Numerous endocrine, metabolic, and environmental variables have been linked to osteoporosis. Clinical and molecular data indicates that inflammation significantly affects bone turnover, contributing to the development of osteoporosis. many pro-inflammatory cytokines are involved in the control of osteoblast and osteoclasts and an activate immune profile is implicated as major risk factor in the incidence of the disease ([Ginaldi et al., 2005](#)).

In our study serum Ca levels revealed that untreated osteoporotic rats had a significant decline in serum Ca level compared to control rats. While all other treated animals had

a significant elevation compared to osteoporotic (untreated) rats and osteoporotic rats treated with LS had an relative elevation compared to untreated osteoporotic rats. This significant decline of serum Ca levels of osteoporotic untreated compared to control rats could be as a result of many mechanisms. Firstly glucocorticoids (in our study we used methylprednisolone which is a synthetic glucocorticoid) inactivate intestinal Ca absorption. Secondly glucocorticoids enhance urinary Ca excretion (by deactivating the renal tubular Ca reabsorption), causing the decline in bone mass ([Kuchuk et al., 2014](#)). Thirdly the low serum Ca activates the secretion of parathyroid hormone (PTH) and its increased level causes secondary hyperparathyroidism; that activates bone remodeling causing loss of bone and increased fractures incidence ([Nieves, 2003](#)).

In our study both groups treated with CN and LS loaded on CN showed significant elevation in Ca level compared with the untreated osteoporotic rats. While the rats treated with LS showed relative elevation in Ca compared to osteoporotic untreated rats. LS contains a high content of omega-3 fatty acids, that increases Ca absorption in the small intestine from the enterocytes. These enterocytes are exposed to eicosapentaenoic acid, docosahexaenoic acid, or arachidonic acid. Interestingly, docosahexaenoic acid is to increase the basal Ca²⁺ absorption (Coetzee *et al.*, 2007).

Serum P levels in our study revealed that, in comparison to control rats, the osteoporotic (untreated) rats had a relative drop in serum P levels. There are various potential mechanisms that could account for these observations. First, it has been demonstrated that glucocorticoids (GCs) increase in urine P excretion cannot fully account for the decrease in serum P associated with GCs therapy (Borowitz & Granrud, 1992). Second, it has been demonstrated that GCs raise the excretion of renal P (Borowitz & Granrud, 1992). The proximal tubule, which has GCs receptors, is primarily where GCs work to reduce phosphate reabsorption. Furthermore, it has been demonstrated that in isolated brush-border membrane vesicles, GCs administered *in vivo* into rats reduce sodium phosphate co-transport (Noronha-Blob & Sacktor, 1986). Rather than a shift in phosphate affinity, the inhibition of phosphate absorption by the membrane vesicles is caused by a decrease in the maximal velocity of transport (Noronha-Blob & Sacktor, 1986). In our study the data showed no significant difference in the activity of serum ALP between osteoporotic groups (treated and untreated) and control group. These findings in this study may be due to ALP can be drained from osteoblast which is rich with ALP, also it found in plasma membrane of the cell in the liver, intestine, and placenta, all of which may contribute to the total amount of ALP (Ali, 2018).

In our study all treated rats had non-significant elevation in P level compared to both the control and osteoporotic untreated rats. Also the osteoporotic rats treated with LS loaded on CN had a significant decline in ALP activity and increase in TRAP compared to osteoporotic rats treated with LS. The positive effect on bone density of LS is due to its high Ca content, and on its ability to elevate alpha linolenic acid docosahexaenoic acid and eicosapentaenoic acid that were shown to have beneficial effects on bone (Elshal *et al.*, 2013).

In our study the results of serum TNF- α level declared that all osteoporotic groups (treated and untreated) had a significant elevation compared to control rats. Meanwhile the results of serum IL-1 level revealed that all osteoporotic groups (treated and untreated) had a significant elevation compared with control rats.

Osteoporotic rats treated with CN had a significant decrease in TNF- α and IL-1 compared to osteoporotic rats treated with LS. On the other hand the osteoporotic rats treated with LS loaded on CN had significant elevation in TNF- α and IL-1 compared to osteoporotic untreated rats.

The decrease in TNF- α and IL-1 due to CN treatment can be explained as CN have significant biological activities such as antioxidant and anti-inflammatory, antitumoral, and

antibacterial activities and also improve immune function (Mosa *et al.*, 2021).

This increase in TNF- α and IL-1 may be due to many mechanisms. First, a variety of pathways that cause inflammatory bone loss are directly opposed by GCs. Among them, their direct inhibition of osteoclastogenesis and osteoclast activation appears to be important in mediating their bone sparing actions in chronic inflammatory joint deterioration, as does their suppression of pro-inflammatory markers such Receptor activator of Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) ligand (RANKL) TNF- α , and IL-6 (Fenton *et al.*, 2019).

Second, bone remodeling could be impacted by protein glycation (Hein *et al.*, 2006; Semba *et al.*, 2010). Advanced glycation end products (AGEs) are produced and accumulate as a result of nonenzymatic glycation reactions. These products alter protein structure, trigger cellular reactions through AGE-specific receptors, and produce reactive oxygen intermediates, all of which can lead to tissue damage (Semba *et al.*, 2010). In aging, AGEs are produced as a natural and physiological byproduct but their excessive production and accumulation are observed in osteoporosis (Franke *et al.*, 2007).

Third, gastrointestinal (GI) adverse effects connected to GCs use include peptic ulcer disease (PUD), GI bleeding, and pancreatitis (Caplan *et al.*, 2017), it was demonstrated that ulcerogenic factors can elevate certain inflammatory cytokines in gastric mucosa (Watanabe *et al.*, 2002).

In this study the results of serum tartrate resistant acid phosphatase (TRAP) revealed that all osteoporotic groups (treated and untreated) had a significant elevation compared to control rats. These observations may be due to firstly during inflammation, TRAP (5a and 5b) isoforms are found in monocytes, and TRAP-5b is correlated with osteolytic responses, bone resorption, and bony metastases (Halleen *et al.*, 2006; Janckila *et al.*, 2007), so serum TRAP 5b is a highly useful marker to follow antiresorptive treatment whereas total serum TRAP cannot be used for that purpose (Halleen *et al.*, 2000). Secondly, (TRAP5a), a product of activated macrophage, is proposed to be a biomarker for systemic inflammation (Chen *et al.*, 2015). This may be the reason of the significant elevation in total TRAP due to increase in TRAP 5a in the inflammation caused by osteoporosis.

Meanwhile both the osteoporotic groups (treated with 400 mg/kg b.wt/day LS and LS loaded on CS nanoparticle) had a significant suppression in miR-23a level compared to untreated osteoporotic group. These findings are in line with that miR-23a decreased the osteogenic regulator called Runt-related transcription factor 2 (Runx2) causing decrease in osteoblastic differentiation (Zhang *et al.*, 2012).

This study declared that the untreated osteoporotic group had a significant suppression in miR-142-3p level compared to control rats. Meanwhile all treated osteoporotic animals had significant upregulation in miR-142-3p level compared to osteoporotic (untreated) rats. These results highlight the role that miR 142 3p plays in promoting osteoblast differentiation by enhancing precursor differentiation into osteoblasts through modulation of the Wnt signaling pathway. By upregulating osteoprotegerin (OPG) expression and

downregulating RANKL expression in osteoblasts, this signaling pathway also inhibits bone resorption.

All these findings were confirmed by the histological examination which showed that the untreated osteoporotic rats showed decreased eosinophilia of the bone matrix (↓↓ collagen), multiple resorption cavities, cement lines are also seen (indicate new bone formation), vacuolated bone matrix, osteoclasts were observed within the resorption cavities, then all these histological responses were ameliorated in the osteoporotic groups treated with LS, CN and LS loaded on CN. Meanwhile, Alizarin red S staining was applied for assessing the degree of mineralisation. Alizarin red S staining interacts with calcium, that precipitates forming calcified nodules during the bone matrix deposition process (Fouad-Elhady et al., 2020). The findings of the current studies showed that the osteoporotic rats treated with LS, CN and LS loaded on CN showed strong intensity of alizarin red S staining versus that of the untreated osteoporotic rats.

5. Conclusion

From this study we concluded that:

- *Lepidium sativum* L. ethanolic extract has powerful therapeutic effects in the osteoporotic rat model.
- Compared to the LS and CN treatment groups, therapy with LS loaded on CN for a prolonged period of time (12 weeks) greatly enhanced the biochemical bone indices and restored microarchitecture of the femurs and vertebral bones of the glucocorticoid-induced osteoporotic rats. In cases of osteoporotic illnesses, loading LS on CN may offer a novel therapeutic approach for halting bone loss and improving bone mass and quality. LS is a promising therapeutic agent for the therapy of osteoporosis because of all these benefits in addition to its safety and lack of side effects.

Recommendations

- More investigation is required to elucidate the particular component of LS that is responsible for the anti-osteoporotic effect documented with the treatment.
- MicroRNAs are great biomarkers for numerous diseases, such as osteoporosis.

6. References

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