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In-Vivo Evaluation of Anti-Osteoporotic Activity of *Manilkara zapota* Seed (MZS) Extract in Corticosteroid Induced Osteoporosis in Wistar Albino Rat

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Keywords: *Manilkara zapota* seed (MZS), Anti-osteoporosis, Glucocorticoid induced osteoporosis, osteoblast, osteoclast.

ABSTRACT

In-vivo evaluation of anti-osteoporotic activity of *Manilkara zapota* seed extract in corticosteroid induced osteoporosis in Wistar albino rat. Female Wistar albino rat was used as Glucocorticoid induced osteoporosis. The rats were separated into five groups, each consist of six animal per group(n=6). Osteoporosis was induced in all groups except control group of animal, and other group of animals receive dexamethasone at a dose of 7mg/kg intramuscularly once a week for four weeks. The normal control animal receives saline only throughout study, the disease control animal receives dexamethasone 7mg/kg once a week for four weeks, the standard control animal receives inducing agent and oral treatment of sodium alendronate 0.2m/kg from the 15th day to the 28th day. The treatment group of animals receive inducing agent and low dose of MZS (200mg/kg) and high dose of MZS (400mg/kg) from the 15th day to the 28th day. The anti-osteoporotic activity evaluated by biochemical parameters include serum calcium, serum phosphorus and serum alkaline phosphatase, and biomechanical parameters include bone hardness, length, weight, and thickness. And other observation includes radiological observation and histopathological examination. The serum calcium levels, serum phosphorus levels, bone weight, bone thickness and bone hardness were significantly increase in the treatment and standard groups ($P<0.01$& $P<0.0001$) while serum alkaline phosphatase levels decreased significantly ($P<0.0001$) compared with the disease control group. From Radiological observation and histopathological observation confirmed the positive favorable outcome effect of MZS on bone formation and increase trabecular thickness in the treatment groups.

1. INTRODUCTION

Osteoporosis characterized by decreased bone mass, porous bone, and structural degradation of bone. Bone thins as a result of the body losing too much bone and forming new bone more slowly. It is one of the main reasons why older adults and adults' fracture. It makes bone porous and so brittle that a small change in posture can result in a fracture. [(1)]

Currently there is an increasing incidence of hip fractures in the developed cities in asia. [(2)] Conservative estimates in a study suggest that 20% of women and about 10-15% of men are osteoporotic in India. Osteoporotic fractures in India occur commonly in both sexes. [(3)] Another highly conservative estimate by a group of experts suggested that 26 million Indians suffer from osteoporosis and this number has reached 36 million by 2013. [(4)]

Steroid significantly used as lifesaving drug, anti -inflammatory agent in modern therapy, compared to other drugs that cause osteoporosis, steroids play a vital role in causing severe osteoporosis. Additionally, it is used to treat medication allergies, asthma, auto immune disorders, hypersensitivity reactions such as atopic dermatitis, anaphylaxis, and blood transfusion therapy.

The most prevalent secondary osteoporosis in adults is called glucocorticoid-induced osteoporosis (GIO), which is one of the major side effects. Adult patients with GIO typically have back pain, height loss, humpback, and even fractures as a result of a fast, dose-dependent bone loss. GC-induced fractures may cause disability, bringing a heavy economic burden to families and society. [(5)]

Most commonly used model for osteoporosis is rat, because histomorphometry, biomechanics, and imaging methodologies have been well established. The appropriate animal models of GIO models are adult rats at the age of 6 months. It has been already found that significant decrease in BMD, bone strength, trabecular bone mass, and poor architecture in GC-treated rats. [(6)]

The cellular and molecular mechanisms which regulate the bone remodelling process in relation to oxidative stress (OS), inflammatory factors, and oestrogen deficiency. [(7)]

This study is based on anti-osteoporosis activity of seed extract of *Manilkara zapota* because it shows high antioxidant and free radical scavenging activities due to maximum number of phytochemicals. [(8)(9)]. It contains important phytoconstituent namely quercetin and D-

quercitol, from this quercetin have multiple action on bone including inhibit RANKL-mediated osteoclastogenesis, inhibit osteoblast apoptosis, inhibit oxidative stress, inhibit inflammatory response, promote antioxidant expression, improve absorption of calcium from small intestine and D-quercitol have anti-oxidant and anti-inflammatory activity.

The plant has wide application of various parts such as leaves, fruit and seed. In addition, bioactivities such as antioxidant, antimicrobial, antidiabetic, hypocholesterolaemia, antinociceptive, anti-inflammatory, antidiarrheal, anthelmintic, anti-arthritis, xanthine oxidase inhibitory activity and feeding deterrent activity of the plant established. [10]

So, the study based on anti-osteoporotic activity of MZS extract in-vivo in corticosteroid induced osteoporosis using wistar albino rat.

2. MATERIALS AND METHOD

2.1 Collection and extraction of plant material

The fruit of *Manilkara zapota* were purchased from local market and the seed was isolated from the fruit. Extraction with the help of acetone solvent.

2.2 Experimental Animal

The present study was conducted after obtaining approval from the Institutional Animal Ethics Committee and this protocol met the requirements of national guidelines of CPCSEA/IAEC approval no: 1917/GO/ReBi/S/16/CPCSEA/20.09.2021 and 06/AEL/IAEC/MMC, Date:26/12/2023.

Female Wistar albino rats were used for this study was procured from Animal house, Madras Medical College, Chennai, India.

2.3 Acute Toxicity Study [11]

The main objective of acute toxicity assessment is to use both quantitative and qualitative analysis to evaluate the level of toxicity. Three healthy adult Wistar albino rats weighing between 150-250gm was selected for the study. For all the three animals' food, but not water was withheld overnight prior to dosing. Hence a limit test one dose level of 2000mg/kg b.w was conducted in all the three animals as per the OECD guidelines 423.

2.4 Experimental procedure [12][13][14]

Thirty healthy female wistar albino rats were weighed and grouped into six groups (n=6). Group I kept as normal control received normal saline orally. Group II was treated with dexamethasone 7mg/kg in the intramuscular route once a week for 4 weeks. Group III kept as standard control group was treated with dexamethasone 7mg/kg once a week for 4 weeks intramuscularly and standard drug alendronate 0.2 mg/kg p.o daily from day 15 to 28. Group IV and Group V were considered as treatment groups that received *Manilkara zapota seed* (MZS)extract at a dose of 200 mg/kg and 400 mg/kg orally from day 15 to 28 along with dexamethasone 7mg/kg once a week for 4 weeks intramuscularly.

2.5 Evaluation Parameters

2.5.1. Body weight changes

The body weight was measured every week till the end of the study and the body weight changes were observed.

2.5.2. Biochemical Parameters

Serum alkaline phosphatase and serum inorganic minerals were among the parameters that were evaluated. After giving the animals, a 2-3% isoflurane anaesthesia, the animals' cardiac puncture to get blood using a fine capillary tube. After the blood was drawn, it was centrifuged for 15 minutes at 3000 rpm to extract serum from blood, and it was then kept cold at -20°C.The serum was placed for further tests.

2.5.3. Biomechanical Analysis

After euthanizing the animal by overdose of isoflurane, the animal was placed on a dissecting board and the hip ball and socket joint was accessed by invasion and retraction. Then femoral bone was kept in 10% neutralized buffer formalin solution after it had been dislocated and the surrounding tissues removed. The femur was then subjected to following analysis. bone weight, bone length, bone thickness and bone hardness.

Each bone's weight was determined with a digital balance. Length was assessed using a ruler, while thickness at the epiphyseal growth plate region was measured with a Vernier Caliper. The hardness was measured by determining the fracture point. The fracture point is measure of the point at which the bone breaks when weight was applied. In this study hardness tester

was used. This bone breaking strength is indicated in (kg/m) N as unit.

2.5.4. Radiological Observation

At the end of the study, radiographs were taken using table top procedure (44kVp and 2 mAs) with exposure time of 10ms and working distance of 1m in all animals.

2.5.5. Histopathological Examination

The isolated femur bone was defatted by immersion in 5% nitric acid for 24 hours. Following this, the bone samples were dehydrated using an automated vacuum tissue processor. The dehydrated samples were subsequently embedded in paraffin wax and sectioned. These sections of bone were then stained with hematoxylin and eosin (H&E) before being observed under a light microscope.

2.6. Statistical Analysis

All the values are expressed as mean ± SEM. The data are statistically analyzed by means of one-way ANOVA followed by Dunnett's multiple comparison test using Graph Pad Prism Software version 10.2.2. One-way ANOVA was used to correlate the statistical difference between the variables. P values ($P<0.05$, $P<0.01$, $P<0.001$, $P<0.0001$) was considered as statistically significant.

3. Result

3.1. Acute Toxicity Studies

Since there was no sign of adverse effect and mortality on observing the animals for 14days after the administration of Manilkara zapota seed extract at the dose of 2000mg/Kg, $1/10^{th}$ (200mg/kg) and $1/5^{th}$ (400mg/kg) dose was selected for the low and high dose treatment groups respectively.

3.2. Body Weight Changes

The body weight of all animals was taken for every week throughout the study and is the weight of the animals there was a marginal change in body weight throughout the experiment with normal rats. The disease control rats showed decrease in body weight. There was a progressive increase in the weight of rats which were treated with 200mg/kg and 400mg/kg of MZS.

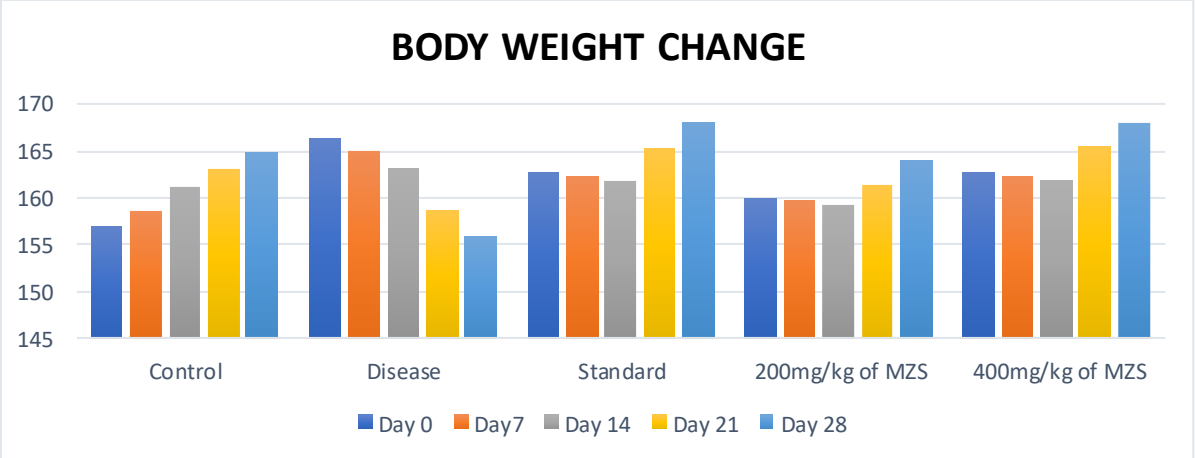


Figure 1: Effect of MZS extract on body weight changes

3.3. Biochemical Parameters

On 29th day the blood samples were collected from experimental animal. The biochemical parameters are measured on serum of the experimental animal include serum calcium, serum phosphorus and Serum alkaline phosphatase.

Table 1: Effect of MZS extract on Biochemical parameters

Groups	Serum calcium level mg/dl	Serum phosphorous level mg/dl	Serum Alkaline Phosphatase level IU/L
Control	9.00±0.09	8.44±0.09	144.50±4.50
Disease control	6.27±0.25$^{###}$	4.49±0.13$^{####}$	250.29±7.02$^{####}$
Standard control	8.11±0.11****	7.43±0.10****	144.95±6.09****
200mg/kg of MZS	7.25±0.24**	5.97±0.04****	170.32±2.20****
400mg/kg MZS	8.36±0.15****	7.06±0.06****	149.23±4.28****

All the Values are expressed as mean ± SEM (n=6).

Analyzed by One-way analysis of variance (ANOVA) followed by multiple comparison Dunnet 't' test.

####P < 0.0001 compared with normal control; **P < 0.01, ****P < 0.0001 compared with disease control.

As shown in the **table1,** the Serum calcium and Serum phosphorus of disease control was reduced significantly (P<0.0001) as compared with the normal group. In the standard group

the serum calcium and serum phosphorus level were increased significantly (P<0.01, P<0.0001) as compared with the disease control group.The treatment group such as low dose 200mg/kg and high dose 400mg/kg has significantly (P<0.0001) increased serum calcium levels as compared with disease control.

As shown in the **table1**, the serum ALP level of disease control was increased significantly (P<0.0001) as compared with the normal control group. In the standard group the serum ALP level was decreased significantly (P<0.0001) as compared with the disease control group. The treatment group such as low dose 200mg/kg and high dose 400mg/kg has significantly (P<0.0001) decreased serum ALP levels as compared with disease control.

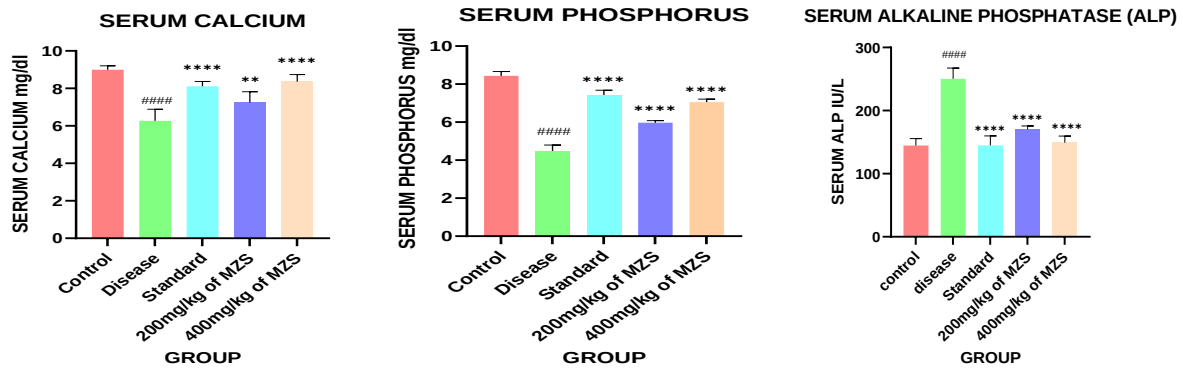


Figure 2: Graphical representation of serum calcium, phosphorous, alkaline phosphatase in various groups

3.4. BIOMECHANICAL PARAMETERS

At the end of the study the animals were sacrificed femur bone was isolated for estimation of biomechanical parameters. Biomechanical parameters include, bone weight, bone length, bone thickness, bone hardness.

Table 2: Effect of MZS extract on Biochemical parameters

Groups	Bone weight in gram	Bone length in mm	Bone thickness in mm	Bone hardness in(kg/m)N
Control	0.42±0.004	33.35±0.17	4.13±0.05	200.5±1.06
Disease control	0.26±0.007####	31.55±0.14	3.38±0.05####	153±0.97####
Standard control	0.39±0.006****	32.44±0.06	3.9±0.04****	171.5±0.89****
200mg/kg of MZS	0.30±0.01***	31.44±0.14	3.65±0.06**	159±0.58***
400mg/kg MZS	0.33±0.004****	31.51±0.04	3.75±0.04***	161.3±0.49****

Analyzed by One-way analysis of variance (ANOVA) followed by multiple comparison Dunnet 't' test.

P < 0.0001 compared with normal control; **P < 0.01, ***P < 0.001,****P < 0.0001 compared with disease control.

As shown in the **table2**, the bone weight, bone thickness, bone hardness of disease control was reduced significantly (P<0.0001) as compared with the normal group. In the standard group the bone weight is increased significantly (P<0.0001) as compared with the disease control group. The treatment group such as low dose 200mg/kg and high dose 400mg/kg has significantly (P<0.0001, P<0.001, P<0.01) increased bone weight as compared with disease control.

As shown in the **table2,** In the standard group the bone length has no significant changes as compared with the disease control group. The treatment group such as low dose 200mg/kg and high dose 400mg/kg of MZS also has no significant changes in bone length as compared with disease control.

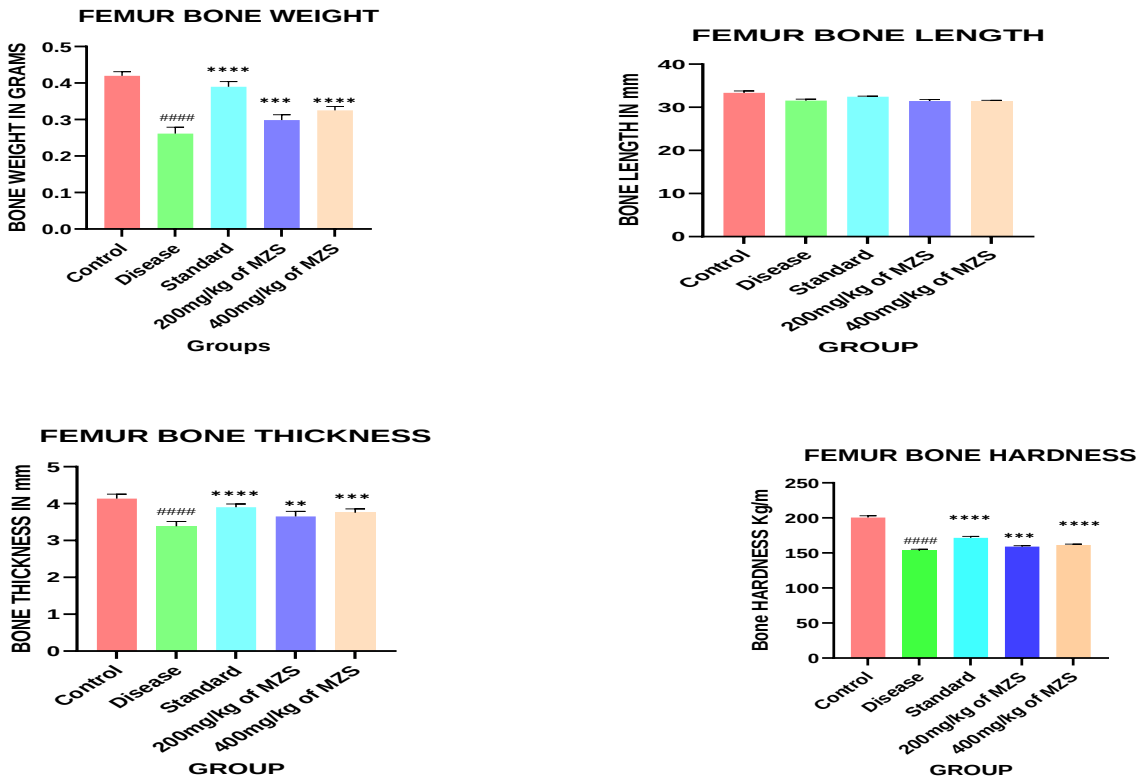


Figure 3: Graphical representation of Bone weight, Bone length, Bone thickness, Bone hardness in various groups

3.5. Radiological Observation

The radiological observation of the control, disease control, standard, low dose MZS, High dose MZS animals is shown in the below:

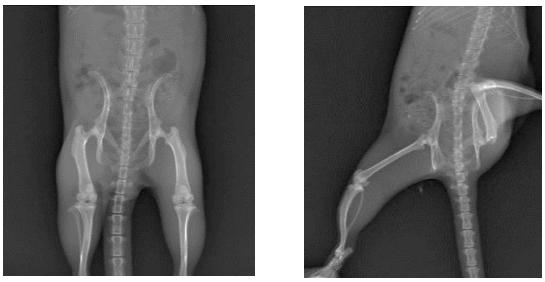


Figure 4: Normal control Animal

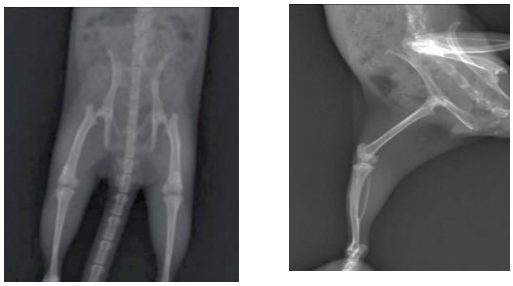
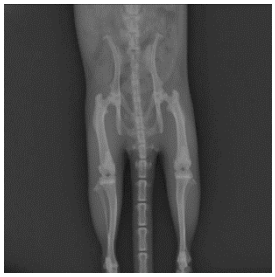


Figure 5: Disease Control Animal



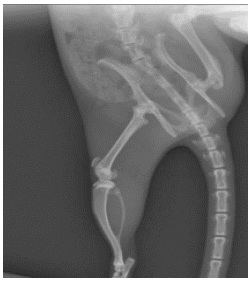
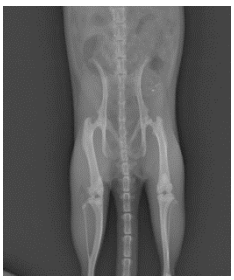


Figure 6: Standard Animal



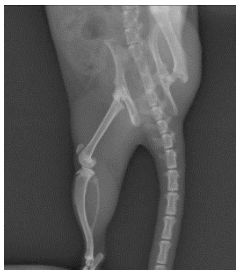


Figure 7: Treatment Animal (Low Dose Of MZS)

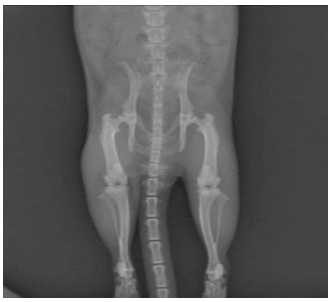


Figure 8: Treatment Animal (High Dose of MZS)

At the conclusion of the study, X-rays were conducted. In the normal control group, rats exhibited typical bone architecture characterized by a thick and radiopaque cortex with normal density in the marrow cavity. Conversely, the disease control group displayed a wholly radiolucent perspective, featuring a thin cortex and abnormal density in the marrow cavity. However, in the other group, X-ray imaging revealed an amelioration in bone structure.

3.6. Histopathological Examination

The histopathological examination of different groups using haematoxylin and eosin is shown below:

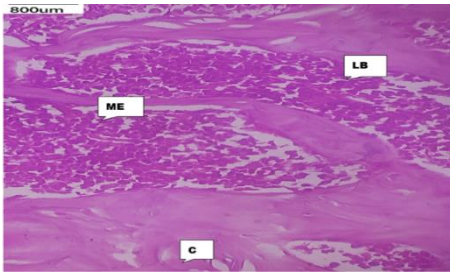


FIGURE 9: CONTROL GROUP

Sections showed normal lamellar bone (LB) with marrow elements (ME), normal cartilaginous elements(C).

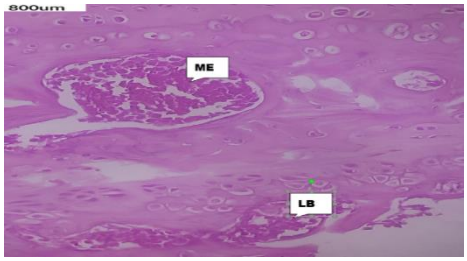


FIGURE 10: DISEASE CONTROL GROUP

Sections showed necrotic irregular lamellar bone (LB) Marrow elements (ME) were appeared normal.

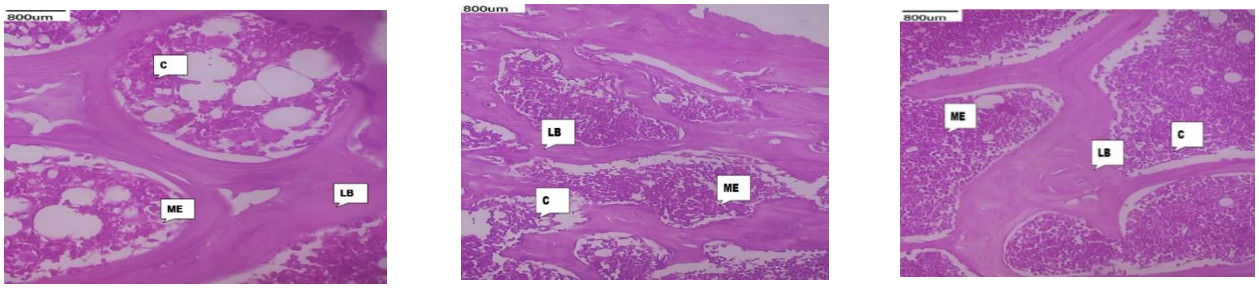


FIGURE 11: STANDARD GROUP, 200MG/KG OF MZS, 400MG/KG OF MZS

Sections showed normal lamellar bone (LB) with marrow elements (ME), normal cartilaginous elements(C).

4. Discussion

The Acute toxicity studies found no evidence of adverse reaction or death. Those exposed to the test substance showed no harmful health effects or signs of illness linked to toxicity. These findings indicate that, at the tested dose levels, the substance does not pose an immediate threat to the well-being or survival of the subjects. Therefore, doses equivalent to one-fifth and one-tenth of 2000mg/kg were chosen as the high and low doses, respectively.

Throughout the study duration, the body weight of the animals across all groups was meticulously documented. Notably, there was a very slight increase body weight in treated rats with the respective doses of *Manilkara zapota* seed extract. [low dose (200mg/kg) and high dose (400mg/kg)].

Rats treated with dexamethasone had lower blood calcium and phosphorus levels on the 29th day compared to normal control rats. However, when treated with sodium alendronate (0.2mg/kg) or *Manilkara zapota* seed extract at doses of 200mg/kg and 400 mg/kg, significant changes in blood calcium and phosphorus levels were observed.

Alkaline phosphatase (ALP) serves as a key marker for bone turnover, reflecting bone activity and metabolic health. In the disease group rats, serum ALP levels were significantly higher compared to normal control rats. However, rats treated with *Manilkara zapota* seed extract at doses of 200mg/kg and 400 mg/kg, as well as those treated with sodium alendronate, showed significant reductions in ALP levels compared to the disease control rats.

However, administration of sodium alendronate (0.2mg/kg) resulted in a significant increase in bone weight, thickness and hardness compared to the disease control rats. Similarly, treatment with *Manilkara zapota* seed extract at doses of 200mg/kg and 400mg/kg led to significant increases in bone weight, thickness and hardness compared to the disease control group. Notably, there were no significant changes observed in bone length, indicating stability in bone size across the experimental groups.

Cortical thinning as well as complete radiolucent view confirmed the osteoporosis by radiological findings. Treatment with alendronate and test drug showed mild to marked improvement in bone formation, thickening of cortical bone which confirmed that test drug has effect in reducing porous bone and improving the bone formation by radiological observation.

The histopathological examination confirmed our findings. We looked at sections of the femur bone to see if there were any changes on a microscopic level. In the control group, the animals had normal bone structure and density. However, in the disease control group where we induced steroid effects, we observed a thinning of the trabeculae.

In contrast, the group treated with alendronate showed significant improvement in restoring trabecular thickness. when we compared the group treated with a high dose of *Manilkara zapota* seed extract to the alendronate group, we found that it closely resembled the alendronate group in terms of increased trabecular thickness, suggesting a similar therapeutic effect.

Therefore, from the above histopathological examination of the treated group of rats showed

that the extract of *Manilkara zapota* seed is active in bone formation and has beneficial effects in the treatment of osteoporosis.

5. Conclusion

The present study provided basic evidence that the *Manilkara zapota* seed extract beneficial effect against steroid induced osteoporosis proved by the valid data obtained from the in-vivo evaluation which includes serum markers, biomechanical properties, radiological observation and histopathological examination. Further studies are required to elucidate the molecular mechanism of action of phytoconstituents present in the seed extract and its therapeutic potential in the treatment of osteoporosis.

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Conflict of interest statement: The authors declared no conflict of interest.

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