IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
August 2023 Vol.:28, Issue:1

Evaluation of Anti-Osteoporotic Activity of Rasna Saptaka Kwatham – A Siddha Formulation Using Corticosteroid Induced Osteoporosis in Wistar Albino Rats

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ISSN 2349-7203
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Submitted: 25 July 2023
Accepted: 18 August 2023
Published: 30 August 2023

Keywords: Osteoporosis; Wistar rats; Serum calcium; Bone hardness; Rasna saptaka Kwatham

ABSTRACT

This study aimed to evaluate the Anti-osteoporotic activity of Rasna saptaka kwatham (RSK) in albino Wistar rats using a Glucocorticoid-induced osteoporosis model. Female Wistar rats were separated into five groups, each consisting of n=6. Osteoporosis was induced in all groups of rats, except for the normal control group, by administering dexamethasone at a dosage of 7mg/kg intramuscularly once a week for four consecutive weeks. The normal control group, labeled Group I, received a saline solution. Group II, designated as the disease control group, received the dexamethasone injection. Group III acted as the standard control and received oral treatment of sodium alendronate 0.2mg/kg from the 15th day to the 28th day. Group IV and V were treated with RSK at doses of 200mg/kg and 400mg/kg (p.o) respectively from the 15th to the 28th day. Following treatment, the anti-osteoporotic effect of RSK was evaluated by biochemical, biomechanical analysis, radiological observation & and Histopathology examination among the experimental groups. The serum levels of calcium, phosphate and Alkaline Phosphatase levels were evaluated in biochemical parameters. Bone weight, thickness, and hardness were evaluated in biomechanical parameters. The serum calcium, serum phosphorous levels, bone weight, thickness & and bone hardness were significantly increased 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. Radiological observations and Histopathological examination confirmed the positive effects of RSK on bone formation and density, revealing increased osteoblast activity and trabecular thickness in the treated groups.

INTRODUCTION

Osteoporosis is a bone disease that develops when Bone Mineral Density (BMD) and Bone Mass (BM) decrease. It is a systemic skeletal disorder and one of the major causes of fracture among the aged and adult population. This makes the bone weak and breaks more easily. Osteoporosis is a silent disease because it does not show any symptoms, and one may not know the disease until fracture. Osteoporosis is a major cause of fracture in Postmenopausal women and older men. [1]

According to statistics given by the World Health Organization (WHO), 30 percent of postmenopausal women suffer from osteoporosis. According to reports, osteoporosis affects approximately 61 million individuals in India, with women accounting for 80 percent of this population. The peak incidence of osteoporosis in India occurs 10–20 years earlier than in Western countries, which impinges harshly on health and economic resources. [2]

Osteoporosis is estimated to affect over 200 million people worldwide. Osteoporotic fractures will eventually occur in 1 in 5 males and 1 in 3 women over the age of 50. Hip fractures are predicted to become more common worldwide by 2050, rising by 240% for women and 310% for males.[3]

The main risk factor for osteoporosis is exhibited by age and sex, risk appears to be increased during childhood and adolescent period whereas risk decreases in mature life because of peak bone mass. But after the age of 45 years mainly in women risk again increases while in men risk is higher above the age of 60 years, Genetic factors also play an essential role in variation in bone density. A lifelong lack of calcium, estrogen, and vitamin D intake, and increased consumption of alcohol and nicotine play a major role in the occurrence of osteoporosis. Long-term use of some medications, such as prednisone, dexamethasone, methotrexate, and heparin, can seriously harm bones and eventually result in bone loss. Some endocrine and gastrointestinal disorders also contribute to the risk of osteoporosis.

Synthetic glucocorticoids (GCs) have been widely used for the treatment of autoimmune diseases, rheumatism, gastrointestinal diseases, tumors, and organ transplantation in clinical practice for decades. Although the therapeutic effects of GC have been fully confirmed, it inevitably produced many side effects by long-term use.

Glucocorticoid-induced osteoporosis (GIO) is one of the serious side effects that have become the most common secondary osteoporosis in adults. Due to rapid, dose-dependent

bone loss, adult patients with GIO are usually suffering from back pain, height loss, humpback, and even fracture. GC-induced fractures may cause disability, bringing a heavy economic burden to families and society. Trabecular bone is more commonly affected by bone loss than cortical bone. Localized changes in bone microarchitecture brought on by GCs can produce micro-lesions that weaken bones. It has been demonstrated that these localized changes in microarchitecture are connected to GC use. The formation/resorption balance is altered by GCs, which results in a preferred suppression of bone formation. Because GCs not only suppress bone formation but also speed up bone resorption, the loss of bone density is highest during the first few months of treatment. It has been established that this temporary increase in bone resorption is partly connected to the use of GCs, but it is also related to the underlying inflammatory condition, and it is well-known that inflammation encourages osteoclastic development. These particular effects account for why GC exposure is linked to a higher risk of fracture.[4]

Existing drugs used for osteoporosis are Bisphosphonates, Raloxifene, Teriparatide, body-building medications, lifestyle modifications and hormonal therapy which have some side effects such as gastrointestinal disturbance, peptic ulcers, nausea, dizziness, etc., to overcome these side effects herbal drugs are being explored. [5]

Rat is the most commonly used model for osteoporosis, based on which, histomorphometry, biomechanics, and imaging methodologies have been well established. Adult rats at the age of 6 months were appropriate animal models for GIO models.[6] It has been already found that significant decrease in BMD, bone strength, trabecular bone mass, and poor architecture in GC-treated rats. Rasna saptaka kwatham is a polyherbal formulation. It contains 8 herbs including Zingiber officinale which is used as an adjuvant and herbs such as Alpinia galanga, Tinospora cordifolia, Boerhavia diffusa, Cassia fistula, Tribulus terrestris, Ricinus communis & Cedrus deodara. [7-13] Rasna saptaka kwatham is used for Amavata (Rheumatoid Arthritis) clinically. [14] However the ingredients tend to possess Antiosteoporotic activity which is not yet evaluated so far as per the literature review. So, the study aims to evaluate the Anti-osteoporotic activity of Rasna saptaka kwatham in albino Wistar rats using a Glucocorticoid-induced osteoporosis model.

MATERIALS AND METHODS

Procurement

The drug RSK was procured from a Siddha pharmacy and is manufactured by AVN Ayurveda Formulation Private Limited. The standard drug alendronate brand name Osteofos was procured from a retail pharmacy manufactured by Cipla Ltd. The inducing agent Dexamethasone was procured from a hospital pharmacy manufactured by Cipla Ltd.

Animal Procedure:

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/25.10.2016 and 06/AEL/IAEC/MMC, Date: 14.12.2022. For this investigation, 33 female Wistar albino rats were purchased from the Madras Medical College Animal House in Chennai, India. In a quarantine period, animals are kept apart from those already housed in the facility while their health as well as their microbiological condition are being assessed. The newly procured Wistar albino rats were quarantined for a period of one week to minimize the chance of introduction of pathogens into established animals and allowed to develop psychological, physiological and nutritional stabilization before their use. The animals were housed in a well-ventilated animal house which was maintained at a constant temperature and relative humidity of 55 to 60%. The animals were housed in spacious polypropylene cages and paddy husk was utilized as bedding material. The bed material was changed twice a week. The animals were maintained on standard pellets and purified water. The animals were provided with food ad libitum except during fasting. All animal cages used in the study had proper identification i.e., labels. Each animal in the cage was marked on the tail with picric acid for their appropriate identification.

Acute Toxicity

Since it is an herbal formulation a limit test has been performed with 3 animals at a dose of 2000mg/kg according to OECD guideline 423 (Acute oral toxicity) for about 14 days for dose selection. [15]

ANTI-OSTEOPOROTIC ACTIVITY

The experimental animals were grouped into 5 groups and each group had 6 animals each. Group 1 is a normal group administered with normal saline orally Group 2 the disease control group injected with Dexamethasone 7mg/kg in the intramuscular route once a week for 4 weeks. Group 3 is the standard control group given with Alendronate 0.2mg/kg p.o daily from day 15 to day 28. Group 4 is 200mg/kg of RSK from day 15 to day 28.

The standard drug is Alendronate (0.2mg/kg) and the test drug is Rasna Saptaka Kwatham (200 & 400mg/kg). The animals were treated with the standard protocol. Also, the rats were administered the standard and test drug with an oral gavage and the dexamethasone (7mg/kg) was given IM in the thigh muscles of the hind limb once a week for 4 weeks. [16-19]

EVALUATION OF ANTI-OSTEOPOROTIC ACTIVITY:

Body Weight Changes

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

Biochemical Parameters:

On the 29th day, the animals were anesthetized with isoflurane 2-3% and the blood was collected by cardiac puncture. The blood was collected in a rapid clot activator tube, the blood was centrifuged at 3000rpm for 15 mins and the serum was collected and stored at -20℃. The biochemical analysis for serum calcium, serum phosphorous and serum alkaline phosphatase levels were measured spectroscopically using an automatic analyzer. [16,17]

Biomechanical Parameters:

Then the animals were sacrificed with overdosage of isoflurane and the incisions were made in the lower abdominal position The hip bone was traced and both the femurs were removed and washed with saline and defatted using 5% nitric acid. The left bone was used for biomechanical analysis such as bone weight, bone length, bone hardness, and bone thickness. The bone weight was measured using a digital weighing balance, the bone length was measured using a ruler, the bone thickness was measured using a vernier caliper, and the bone hardness was measured using a hardness tester. The hardness of the bone was measured to determine the periosteal and endosteal arrangement of cortical bone by determining the

fracture point. The fracture point is the measure of the point at which the bone breaks when weight was applied. [16,17]

Radiological Observation:

Radiographs of all the animals were collected after the study using the table-top approach (44kVp and 2 mAs), with exposure times of 10 ms, and working distances of 1 m. [16]

Histopathological Examination:

The isolated femur bone was defatted over the course of 24 hours by being exposed to 5% nitric acid. Then, using an automated vacuum tissue processor, the bone samples were dehydrated. Dehydrated samples are then sectioned and embedded in paraffin wax. Hematoxylin and eosin (H&E), Masson trichome, and Alizarin were used to stain the sectioned bone, which was then examined under a light microscope. [20, 21]

Statistical Analysis:

All the values were expressed as mean ± SEM. Graph Pad Prism Software version 9.5.2 was used to statistically analyze the data using one-way ANOVA and Dunnett's multiple comparison test. P values were regarded as statistically significant if they were between 0.05 and 0.001.

RESULTS AND DISCUSSION

***In-Vivo* Acute Toxicity Studies:**

Acute Oral toxicity is performed as per OECD guideline 423. [15] The results of the Acute toxicity studies revealed no indications of morbidity or mortality. The subjects exposed to the test substance did not exhibit any adverse health effects or signs of illness that could be attributed 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. Hence 1/5th and 1/10th dose of 2000mg/kg was selected as low and high dose respectively.

In-Vivo Anti-Osteoporotic Activity: [16-19]

Effect of Rasna Saptaka Kwatham on body weight changes in corticosteroid-induced osteoporosis rat model:

Throughout the investigation, the body weight of the normal rats increased. The body weight of the disease-control rats decreased. The disease-control rats showed a decrease in body weight. There was an increase in body weight in treated rats with the respective doses of Rasna saptaka kwatham [(200mg/kg) and (400mg/kg) of RSK].

Table 1. Body Weight Changes.

S. No	Groups	Day 0	Day 7	Day 14	Day 21	Day 28
1	**Control**	160.33±107	160.53±4.8	162.17±7.1	162.8±8.4	163.33±8.2
2	**Disease control**	123.5±2.4	123.17±4.3	111.5±5.5	104.83±3.8	103.83±5.1
3	**Standard control**	150±2.4	152.83±3.0	152.67±7.3	154.17±8.3	154.33±6.7
4	**200mg/kg of RSK**	126.83±3.1	127.67±3.8	140±7.6	150.17±7.1	150.33±7.3
5	**400mg/kg RSK**	112.33±2.6	117±2.4	143.83±10.1	147.17±8.6	150.67±3.9

All the values are expressed as Mean± SEM.(n=6)

Effect of Rasna Saptaka Kwatham on biochemical parameters on corticosteroid-induced osteoporosis rat model:

Dexamethasone-treated rats showed decreased serum calcium levels on the 29th day compared to that of normal control rats which demonstrated that glucocorticoid affects the intestinal absorption of calcium and decreased osteoblastic activity. On treatment with sodium alendronate (0.2mg/kg) and Rasna saptaka kwatham at the dose of 200mg/kg and 400 mg/kg showed significant changes in serum calcium levels. Treatment with the standard group showed increased levels of calcium significantly ($P < 0.001$). It also showed a

significant (P < 0.01and P<0.001) increase in the level of serum calcium in the low and high-dose treated group when compared with disease control rats as shown in Table 2.

Similarly, serum phosphorus decreased in dexamethasone-treated rats when compared with the normal control group of rats. When treated with sodium alendronate there was a significant (P < 0.0001) increase in serum phosphorus levels. Rasna saptaka kwatham showed a significant increase in the serum phosphorus level compared with disease control rats as shown in Table 2. These results confirmed that glucocorticoids decrease the serum mineral level and increase the urine calcium loss in glucocorticoid-induced osteoporosis. Rasna saptaka kwatham restores the bone mineral contents.

Alkaline phosphatase is an important biochemical marker of bone turnover. In humans, alkaline phosphatase is present in almost all the tissue throughout the entire biological system, but it is practically concentrated in the liver, bone, bile duct, and intestinal mucosa. ALP is an indicator of bone activity and is used to monitor metabolic bone disease. The serum ALP level was found to be significantly (P < 0.0001) increased in osteoporotic rats compared with the normal control rats. The increased bone turnover and fracture risk were manifested by ALP activity. Also, from the results alkaline phosphatase was seen to be significantly (P < 0.01 and P < 0.001) reduced in rats treated with Rasna saptaka kwatham at both the doses (200mg/kg and 400 mg/kg respectively) when compared to disease control rats as shown in Table 2. Such a similar effect was seen in sodium alendronate-treated rats.

Table 2. Levels of Serum Biochemical Parameters

S.No	Groups	Calcium Level mg/dl	Phosphorous Level mg/dl	Alkaline Phosphatase Level IU/L
1	Normal	11.80±0.88	8.47±0.24	251.3±14.8
2	Disease Control	3.61±0.1[a]	5.10±0.27[a]	890.6±7.8[a]
3	Standard control	10.89±0.39[b]	8.83±0.16[c]	151.6±2.3[d]
4	200mg/Kg of RSK	10.41±0.18[b]	8.49±0.13[d]	332.6±16.9[c]
5	400mg/Kg of RSK	10.65±0.36[b]	8.65±0.41[d]	213±5.8[d]

a P < 0.0001 compared with normal control.

b P < 0.01 compared with disease control.

c P< 0.001 compared with disease control.

d P< 0.0001 compared with disease control

Effect of Rasna Saptaka Kwatham on biomechanical parameters on corticosteroid-induced osteoporosis rat model:

The anti-osteoporotic effect of the formulation was also evaluated in the biomechanical parameters like bone weight, length, thickness, and bone-breaking strength shown in Figure 1. Weight and length of femoral bone were decreased (P < 0.0001) in the disease control group compared with the normal control group. Following administration of sodium alendronate (0.2mg/kg) showed a significant (P < 0.0001) increase in bone weight compared with the disease control group of rats. Rasna saptaka kwatham at the dose of 200mg/kg and 400mg/kg showed a significant (P < 0.001) increase in bone weight when compared to the disease control group as shown in rats, whereas there were no significant changes in the length of the femoral bone was observed when treated with sodium alendronate (0.2mg/kg) and Rasna saptaka kwatham at the dose of 200mg/kg, and 400mg/kg as shown in Figure 1. Hence there were no changes in bone length. The thickness of the femoral bone was decreased (P < 0.001) significantly in the disease control group when compared with the normal control group as per Figure 1. Administration of Rasna saptaka kwatham (200mg/kg and 400mg/kg) and alendronate treated group showed improvement in the thickness when compared to the disease control group.

The major impact of glucocorticoid-induced osteoporosis is an equal increase in fracture risk at both trabecular and cortical sites. The disease control group showed a significant (P < 0.0001) decrease in hardness when compared to the Normal control rats as shown in Figure 1. Whereas treatment with Rasna saptaka kwatham (low dose 200mg/kg and high dose 400 mg/kg) group showed an increase in hardness of bone when compared to the disease control group. Alendronate-treated rats also showed a similar effect in osteoporotic rats. Bone biomechanical parameters have proven that fractures are associated with steroid-induced osteoporosis. The Rasna saptaka kwatham has been effective in improving the biomechanical properties of bone.

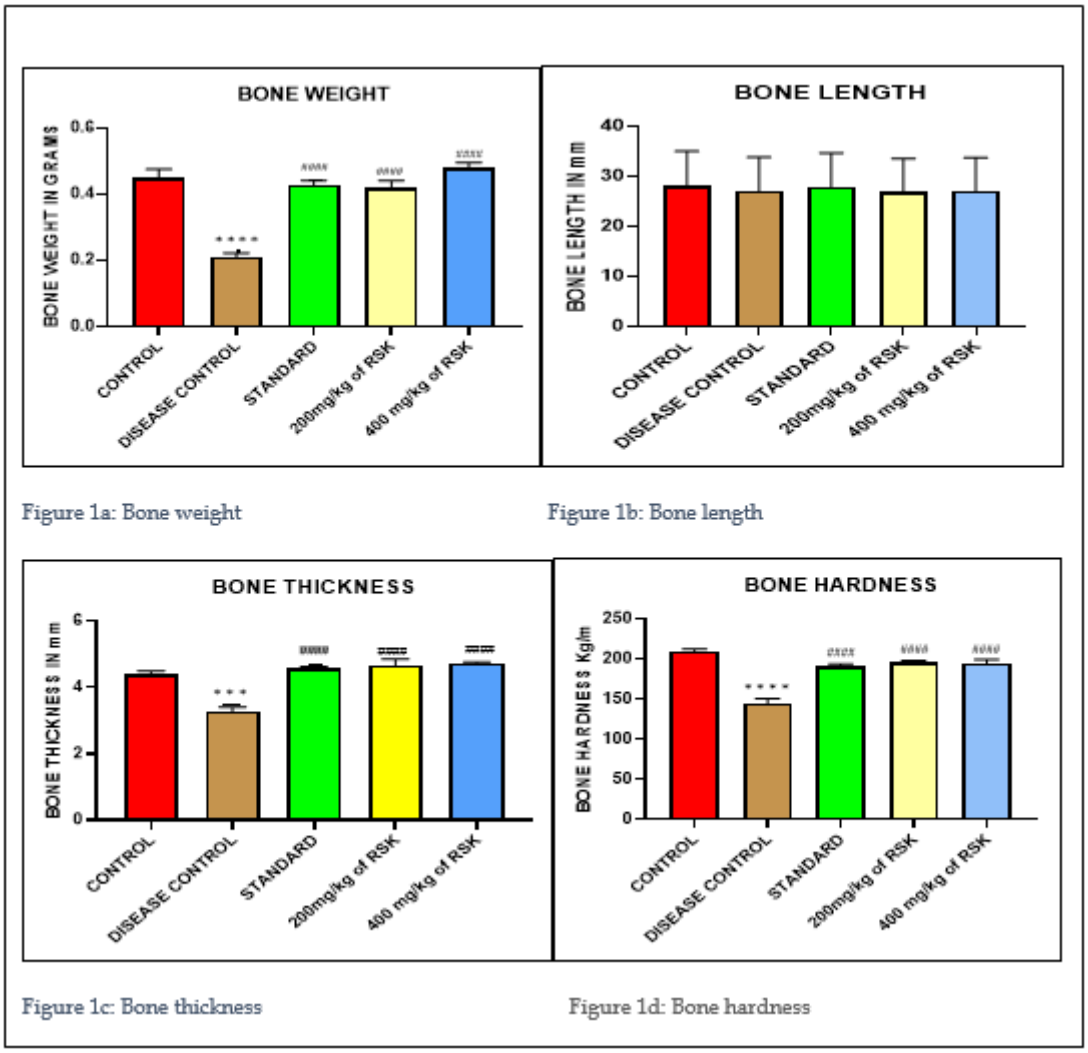

Figure 1a: Bone weight

Figure 1b: Bone length

Figure 1c: Bone thickness

Figure 1d: Bone hardness

Figure 1. Effect of RSK on biomechanical parameters. All the Values are plotted as mean ± SEM (n=6). Analyzed by One-way analysis of variance (ANOVA) followed by multiple comparisons Dunnet's t-test. #### P < 0.0001 compared with disease control; ****P < 0.0001 compared with normal control.

Radiographic Observation:

Radiographic observation showed an osteoporotic fracture in the disease control group Figure 2. Cortical thinning as well as a complete radiolucent view confirmed the osteoporosis by radiological findings. Treatment with alendronate and the test drug showed marked improvement in bone formation, and thickening of cortical bone which confirmed that the test drug has an effect in reducing porous bone and improving bone formation by radiological observation.

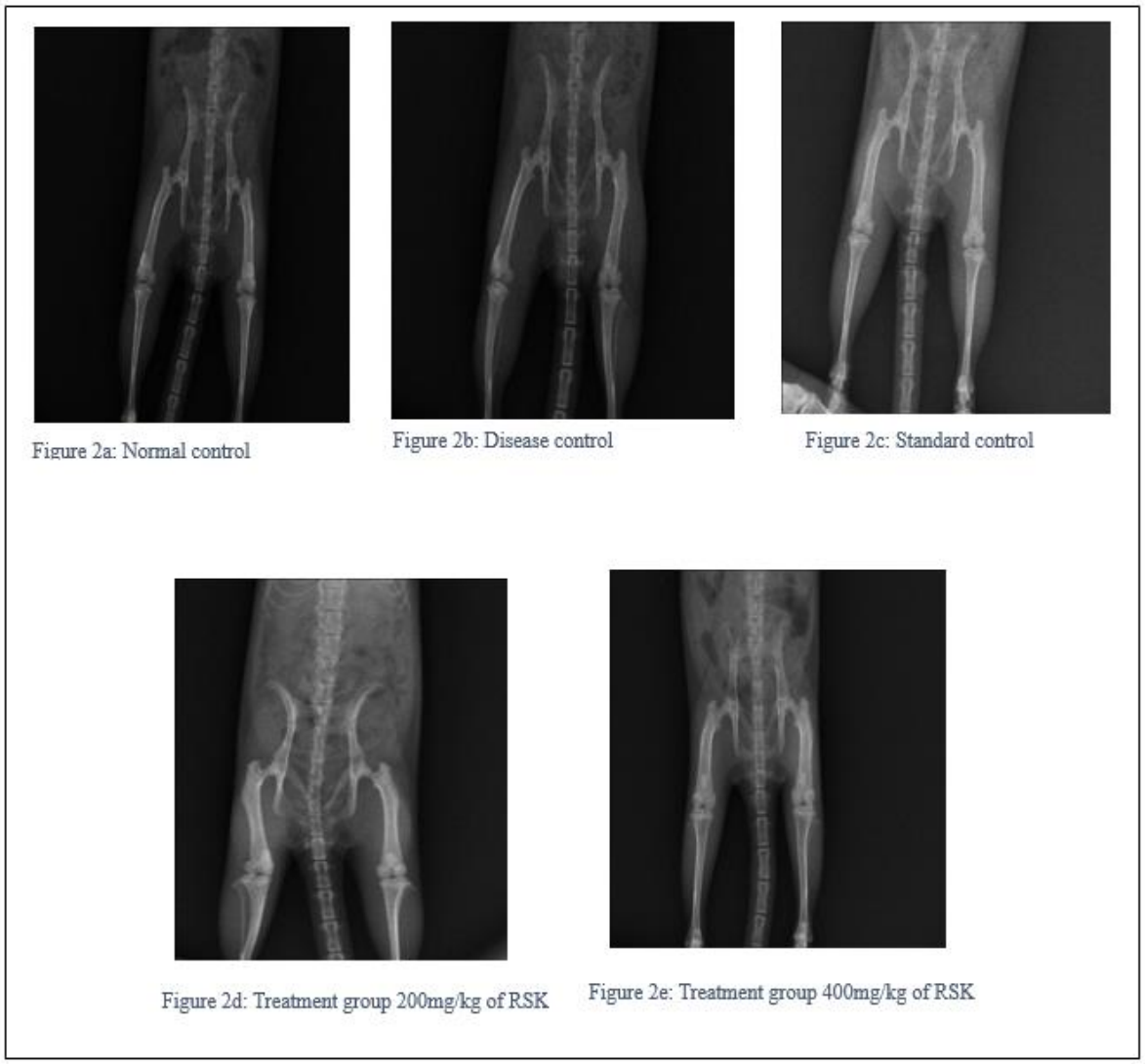

Figure 2. Effect of RSK on biomechanical parameters.

Histopathological Examination:

The histopathological examination was performed in various groups using H&E stain, Alizarin stain and Masson trichome stain and is shown in Figures 3, 4 and 5 respectively.

In the hematoxylin and eosin stain control group animals showed normal architecture and normal bone compactness as shown in Figure 3. The rats in the steroid-induced model group showed thinning of trabeculae, an increase in osteoclast number, and decreased osteoblast number. The alendronate and RSK group exhibited significant restorative progress with increased osteoblast and trabecular thickness. When comparing the high dose of Rasna saptaka kwatham with alendronate showed a resemblance to the alendronate group with more osteoblastic numbers.

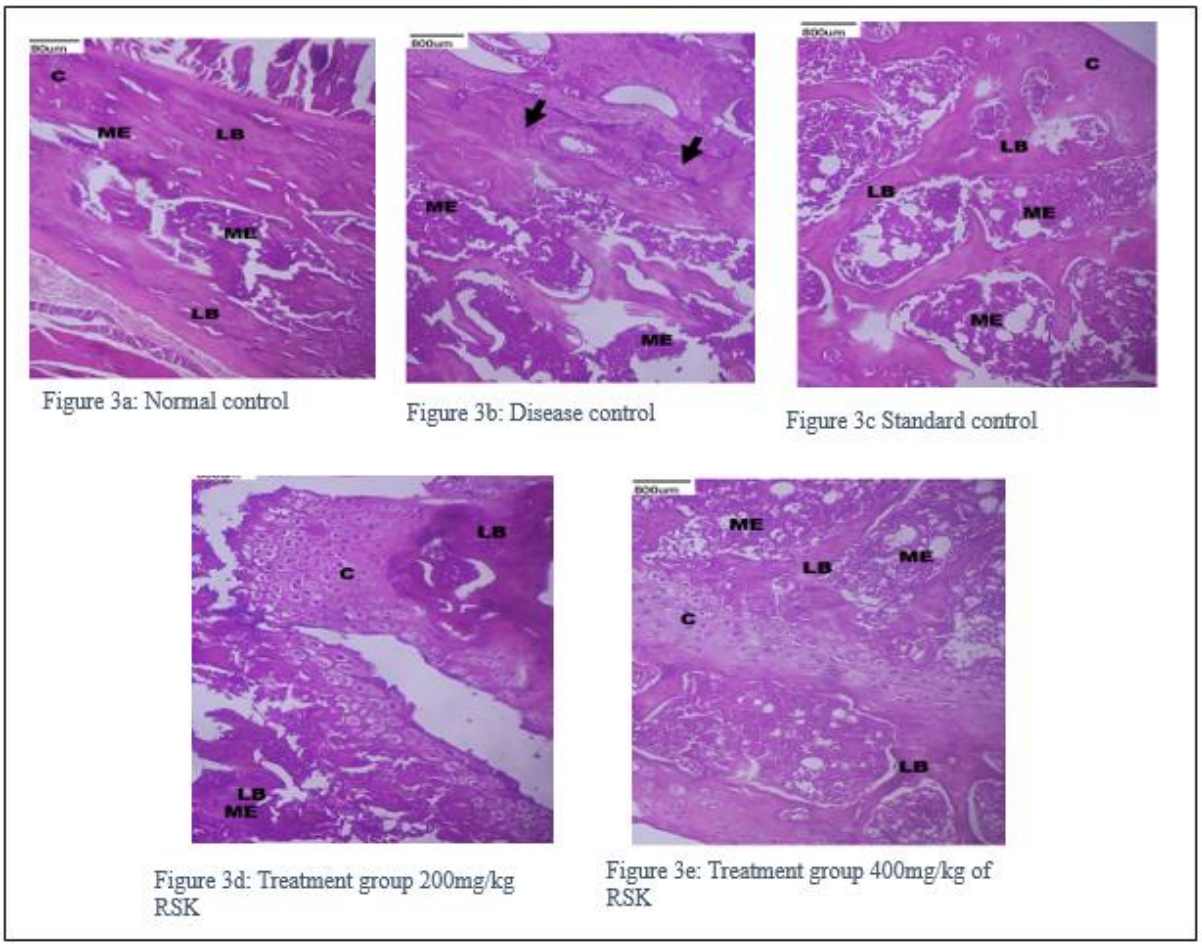

Figure 3. Histopathological examination using H&E stain. LB-Lamellar bone, ME-Marrow Elements, C-cartilaginous elements & Black arrow-necrotic irregular lamellar bone.

The application of Alizarin stain showed more favorable results in the treatment group and standard group compared to the diseased group Figure 4. In the treatment group and standard group, Alizarin stain exhibits enhanced visualization and characterization of mineralized bone, allowing for a comprehensive evaluation of bone integrity and mineralization. Conversely, in the diseased group, the stained outcome may be less pronounced, potentially indicating compromised bone structure and mineralization. These findings suggested that Alizarin stain when applied in the treatment group, provides superior results, indicating positive therapeutic effects on bone tissue and mineralization.

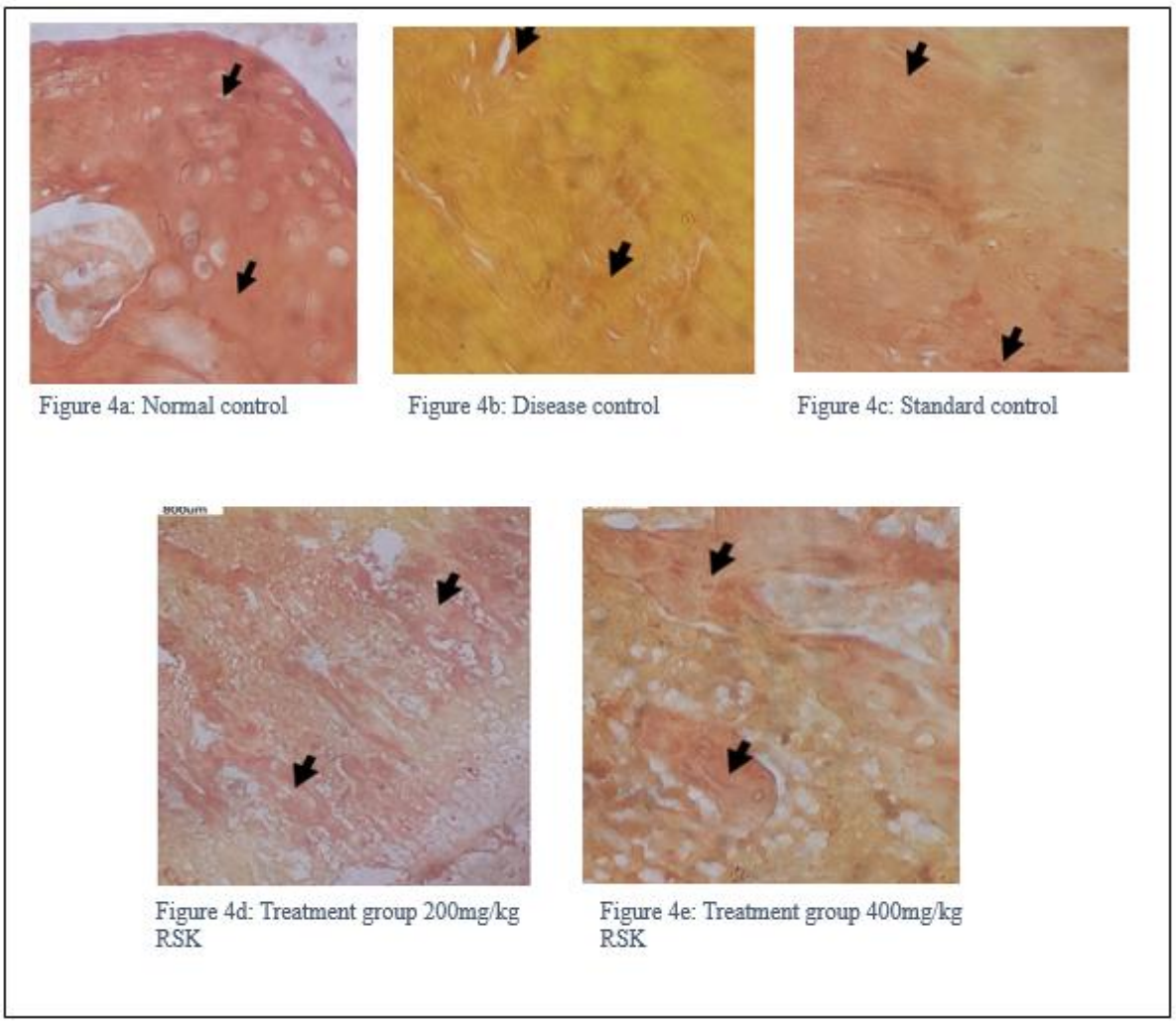

Figure 4. Histopathological examination using Alizarin stain. Red staining is indicated by a Black arrow.

Masson's trichrome stain in the treatment groups, particularly with 1200 mg/kg and 400 mg/kg of RSK and the standard group, exhibits better staining outcomes compared to the disease control group Figure 5. The stain effectively differentiated and highlighted the tissue components, providing enhanced visualization and characterization. In the treatment groups, the stain showcased improved visualization of collagen fibers, normal cartilage, normal marrow elements, osteoid deposition, and fibrous tissue, facilitating a more comprehensive assessment of bone histopathology.

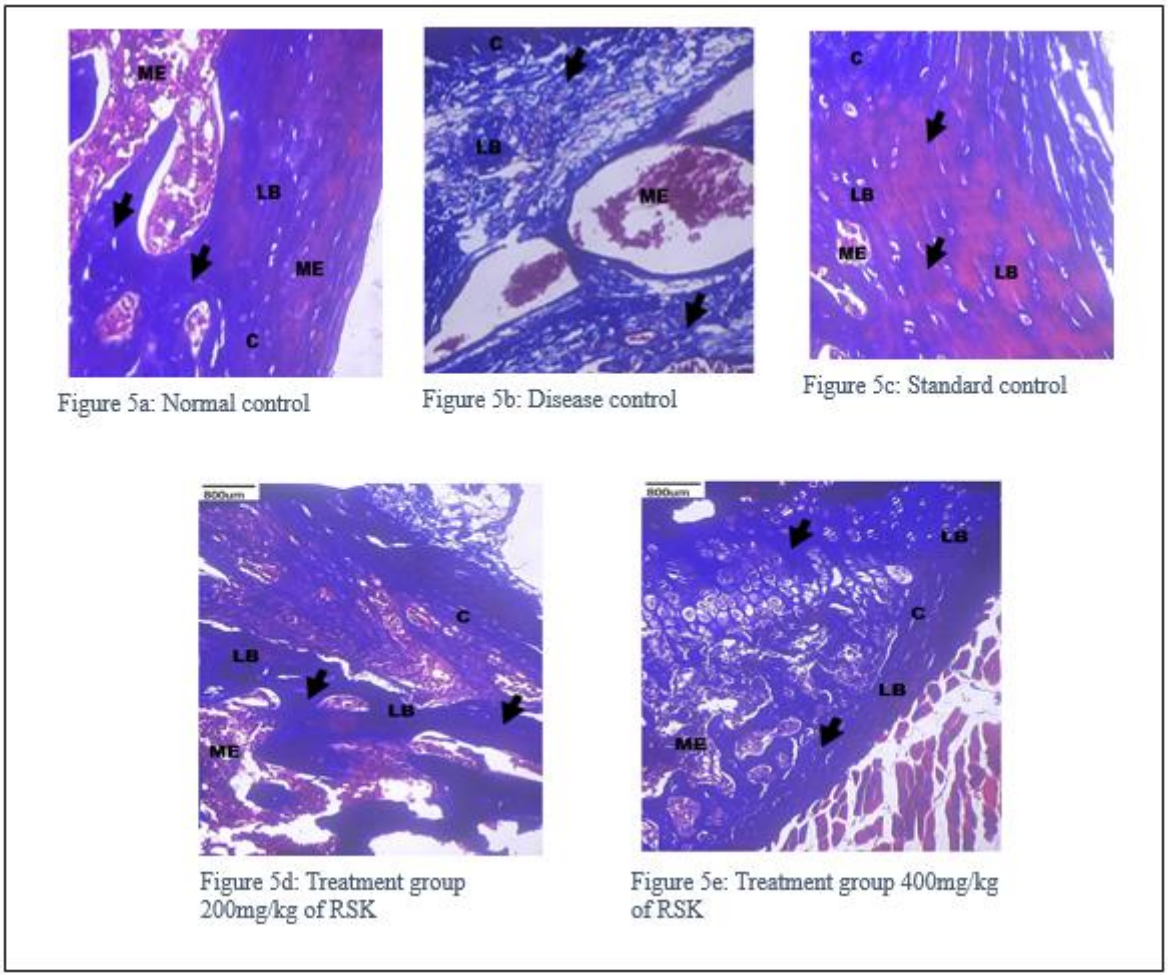

Figure 5a: Normal control

Figure 5b: Disease control

Figure 5c: Standard control

Figure 5d: Treatment group 200mg/kg of RSK

Figure 5e: Treatment group 400mg/kg of RSK

Figure 5. Histopathological examination using Masson trichome stain. LB-Lamellar bone, ME-Marrow Elements, C-cartilaginous elements & Black arrow-necrotic irregular lamellar bone.

Therefore, the above histopathological examination of the treated group of rats showed that the Rasna saptaka kwatham was active in bone formation and had beneficial effects in the treatment of osteoporosis. The discussion showed that the Rasna saptaka kwatham had a better ameliorative effect on steroid-induced osteoporosis.

CONCLUSION

From the study, it is concluded that the RSK possesses a beneficial effect against steroid-induced osteoporosis proved by the valid data obtained from the in-vitro and in-vivo evaluation which includes antioxidant potential, serum markers, biomechanical properties, radiological observation, and histopathological examination. The present study provided basic evidence that the RSK has a beneficial effect on the treatment of osteoporosis as it was

confirmed by biochemical, biomechanical, radiological, and histopathological studies. Further studies are required to elucidate the molecular mechanism of action and its therapeutic potential in the treatment of osteoporosis. It can also be confirmed using ovariectomy-induced osteoporosis. Further clinical trials can be performed to provide insight into including RSK in the treatment of osteoporosis.

ACKNOWLEDGEMENT

I would like to express my heartfelt gratitude to God for guiding and supporting me throughout the completion of my project. I am immensely grateful to my parents, Mr. Gopi Chandran and Mrs. Vedvalli, for their unwavering love, unwavering support, and unwavering belief in me. I owe my deep gratitude to my project guide Dr. M. Sakthi Abirami, M. Pharm., Ph.D., Assistant Professor, Department of Pharmacology, Madras Medical College, Chennai 03. I would like to express my heartfelt gratitude to my guide for the unwavering support and guidance provided throughout my project. I wholeheartedly extend my gratitude to my friends and Juniors for their support and kind help during my project work at every stage.

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