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ANTI-OSTEOPOROTIC ACTIVITY OF *CUCURBITA PEPO* AND LOW LEVEL LASER BEAM ON GLUCOCORTICOID INDUCED OSTEOPOROSIS IN RATS

G. Sindhura and K. P. Shivalinge Gowda *

Department of Pharmacology, PES College of Pharmacy, Bengaluru - 560050, Karnataka, India.

Keywords:

Dexamethasone, *Cucurbita pepo* seed powder, Low level laser beam, Urine parameters, serum parameters, Bone mechanical parameters

Correspondence to Author:
K. P. Shivalinge Gowda

Professor & HOD,
Department of Pharmacology,
PES College of Pharmacy,
Bengaluru - 560050, Karnataka,
India.

E-mail: shivalinge65@gmail.com

ABSTRACT: Objective: The main aim of the present study is to investigate the activity of the anti-osteoporotic activity of *Cucurbita pepo* seed powder (CPSP) and low level laser beam (LLLT) on glucocorticoid induced osteoporosis in rat model. **Materials and Methods:** Animals were randomly divided into six groups containing six rats each (n=6). Group I normal control, Group II dexamethasone induced osteoporotic control (7 mg/kg b.w i.m.), Group III received dexamethasone + LLLT irradiation (180 sec/day daily), Group IV received dexamethasone + CPSP (100 mg/kg b.w p.o), Group V received dexamethasone + sodium alendronate (3 mg/kg b.w p.o), Group VI dexamethasone + CPSP + LLLT. The treatment is made for 8 weeks. On the end of 4^{th} and 8^{th} week body weight were recorded, behavioral parameters, locomotar activity using actophotometer and anxiety levels using elevated plus maze (EPM) was studied. Urine parameters (calcium, phosphorous, creatinine), the blood was withdrawn from retro-orbital plexus and the serum was used for the estimation of ALP, calcium, phosphorous, creatinine. At the end of the 8^{th} week the bone mechanical parameters were measured. **Results:** CPSP as well as LLLT significantly decreased the increased body weight, improved the locomotar activity and decreased the anxiety levels. Significant reduction in the urine parameters and significant increase in the serum levels of calcium, phosphorous and creatinine was observed along with decreased ALP levels. Significant improvement in biomechanical parameters when compared to group II was observed. Group VI showed improved activity when compared to the singly administered groups proving its synergistic activity. **Conclusion:** From this study it can be concluded that, CPSP and LLLT has anti-osteoporotic activity and when given in combination exhibited synergistic activity.

INTRODUCTION: Osteoporosis is one of the metabolic bone disorder and major worldwide public health issue. It is characterized by loss of bone mineral density which occurs progressively along with micro architectural deterioration of bone tissue which ultimately enhances bone fragility thus there is an increasing susceptibility to fracture.

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Osteoporosis literally means "Porous Bone". The formation of bone is the continuous and balanced process, an imbalance between this balance results in osteoporosis. Fracture pertaining to osteoporosis may also cause disability and mortality [1].

Synthetic oral steroids were developed during 1940-1950's and are most potent anti-inflammatory and immunosuppressive agents which are used for the management of various conditions like rheumatoid arthritis, asthma, pulmonary disease, post transplantation immunotherapy, crohn's disease, systemic lupus erythematosus (SLE) and inflammatory bowel disease (IBD). Long term glucocorticoid therapy causes bone loss resulting in

condition called osteoporosis (Glucocorticoid induced osteoporosis or GIOP) [2]. The incidence of GIOP is indiscriminate of race, age and gender. Severe side effects are observed in 30-50% of patients. It is estimated that 3% of the population 50 years of age and older has used GCs, and this percentage rises to 5.2% among those 80 years of age and older [3]. The primary adverse effects of excess of glucocorticoid acts by decreasing the production of both osteoblast and osteocyte apaptosis and prolonging the life span of osteo-clasts. Glucocorticoids also decrease intestinal calcium absorption thus making further liable to osteoporosis [4].

Many of the effective pharmacotherapies are available for both men and women which have been used in the treatment of osteoporosis. Calcium along with vitamin D, estrogen, selective estrogen receptor modulator (SREM's) which includes raloxifene, tamoxifene, calcitonin, biphosphonates (alendronate, risendronate) are approved for treatment of steroid induced osteoporosis (secondary osteoporosis) at the same time all these medicines has been associated with side effects such as hypercalcemia, hypercalciuria, risk of endometrial events, vaginal bleeding and GI ulcers.

The plant kingdom holds great potential to meet this need [5]. Many herbal medicines have been recommended for the treatment of osteoporosis. Treatment with herbal drugs is beneficial due to lesser side effects and low cost. Dietary factors play a key role in the prevention of various human diseases including bone disorders like osteoporosis. Herbal medicine is the oldest known medical healthcare and is being used since centuries by many cultures. In this study, the anti-osteoporotic activity of CPSP and LLLT locally available was investigated and compared with the standard anti-osteoporotic drug in rats.

MATERIALS AND METHODS:

Collection of Plant Material: Completely dried seeds of *Cucurbita pepo* were procured from online (amazon) in the month of August 2017. The plant was identified and authenticated by Dr. V Rama Rao, Botanist, of Regional Ayurvedic Research Institute for Metabolic Disorders. The voucher specimen was preserved in the Department of Pharmacology, PES College of Pharmacy, Bengaluru. The low level laser instrument was procured online (Amazon) in the month of September 2017.

Experimental Animals: Female Sprague dawely rats (180-200 g) were purchased from authenticated supplier Adita Biosys Private Limited, Tumakuru, Karnataka and were maintained in the animal house of PES College of Pharmacy, Bengaluru. All the animals were acclimatized for 10 days under standard husbandry conditions, *i.e*. the animals were housed in polypropylene cages maintained under controlled temperature at 23 ºC ± 2 ºC, relative humidity 45-55% and with 12h light: 12h dark cycle, temperature and humidity was recorded daily using thermometer and hydrometer mounted in animal house. The animals had free access to standard rat pellet along with water supplied *ad libitum* under strict hygienic conditions. Each experimental group had a separate set of animals and care was taken to ensure that animals used for one response were not employed elsewhere. Animals were habituated to laboratory conditions for 48 h prior to experimental protocol to minimize if any of non-specific stress.

The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) approval No- PESCP/IAEC/50/2016, Dated: 21/12/2016) and conducted according to CPCSEA guidelines, Government of India.

Powdering of *Cucurbita pepo* Seeds: The dried seeds of *Cucurbita pepo* were grounded into fine powder using electric blender to get uniform sized particles [6].

Preliminary Phytochemical Analysis: The pre-liminary phytochemical analysis was performed in order to test several chemical constituents present in the seeds of *Cucurbita pepo*.

Induction of Osteoporosis: Osteoporosis was induced by the administration of dexamethasone which is a glucocorticoid, 7 mg/kg b.w i.m. route once in a week for 4 weeks to all the groups except group I which served as normal control. The progression of osteoporosis was evaluated by observing their behavioral parameters like locomotar activity using actophotometer and also anxiety level using EPM.

The extent of osteoporosis was evaluated by measuring serum and urine calcium, phosphorous and creatinine levels using biomedical calcium and phosphorous kits [7, 8].

Pharmacological Activities: Animals were divided into six groups of six rats in each group. Group I (normal) received drinking water through-out the course till 8 weeks. Group II received dexamethasone (7 mg/kg) i.m. once in a week for 4 weeks. Group III received dexamethasone + LLLT (180 sec/day) [9] irradiation on right femur bone. Group IV received dexamethasone + CPSP (100 mg/kg) [10, 11] p.o. Group V received STD (sodium alendronate) 3 mg/kg p.o. [12, 13] as well as dexamethasone. Group VI received dexamethasone + CPSP + LLLT. The induction period was for first 4 weeks and the treatment period was for 8 weeks. The body weight was measured on the first day of the study period, at the end of 4th week and after the study period (8th week) using digital weighing balance [14]. Locomotar activity and anxiety levels were evaluated for all the groups at the end of 4th week and 8th week respectively [15, 16].

Biochemical Analysis: At the end of the 4th week as well as at the end of the study period (8th week), rats were transferred to the individual polyethylene funnel. After placing the rats in the funnel, a plastic lid with ventilation holes punched into it was placed over the top of the funnel. The apparatus was placed in an appropriate holder and a collection beaker was placed beneath it. After a stabilization period of 14 h, the spontaneously voided urine samples were collected and was used for the estimation of calcium, creatinine and phosphorous. The blood was withdrawn from the individual animals of all the 6 groups by retro-orbital puncture under light ketamine anesthesia (40 mg/kg i.p). The blood sample which was collected in the eppendorfs tube was kept in upright position for approximately 10-15 min to facilitate clotting. The sample was centrifuged at 3000 rpm for 15 min. The separated serum was used for the estimations of ALP, calcium, creatinine, phosphorous.

Biomechanical Parameters: At the end of the 8thweek of study, all the animals were sacrificed by injecting overdose of pentobarbitone anesthesia intraperitoneally (150 mg/kg b.w.) and the right femur bone were removed by disarticulating them at coxofemoral and femoral tibial joints by using scalpel blade. The adjacent soft tissues were removed by gentle stripping. It was made sure that the femur was freed from any tendon, muscle or cartilage. Mainly three parts of the femur was obtained- i) the proximal part containing femur head, femoral neck and proximal diaphysis ii) the diaphysis iii) the distal epiphysis [17]. Bone length (distance between greater trochanter and medial condyle) was measured using ruler, bone thickness at the femoral midshaft was also measured using digital calipers. Then the bones were kept in an evacuated oven and dried at 100 ºC and weights of the dried bones were determined by using a digital weighing balance [18]. The bone mechanical strength was determined by placing the right femur bone in digital hardness tester compress until it gets fractured and the reading was recorded in Newton's (kg/cm^2) [19].

Histopathological Investigations: The dissected samples of the right femur bone from each group of osteoporotic animals were collected in 10% formalin saline solution and stained with hematoxylin and eosin for the preparation of section using a microtome and histopathological studies were carried out.

Statistical Analysis: All the values were expressed as mean ± SEM. Statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Dunnett compare all column versus control column using Graph Pad Prism version 5.0. *P<0.05, **P<0.01, **P<0.001 was considered as significant compared to disease control.

RESULTS:

Phytochemical Investigations: The preliminary phytochemical analysis was performed in order to test several chemical constituents present in the seeds of *Cucurbita pepo*. The phytochemical screening revealed the presence of carbohydrates, glycosides, traces of proteins, mucilage, flavonoids, tannins, phenolic compounds, steroids and triterpinoids.

Effect of CPSP and LLLT on % Change in Body Weight on 4th Week: Initially the animals of all the groups were more or less of the similar body

weight. When the body weight was measured on 4th week, Group II rats showed 17.56% increase in body weight after being administered with dexamethasone (glucocorticoid) as compared with the normal group (Group I). Upon irradiation with LLLT, group III rats showed 11.2% increase in body weight and upon administration of CPSP (Group IV), alendronate sodium (Group V) and CPSP in combination with LLLT (Group VI) showed 4.32%, 6.1% and 6.05% increase respectively when compared with the Group II animals treated only with dexamethasone.

TABLE 1: % CHANGE IN THE BODY WEIGHTS OF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 4th WEEK

Group (n=6)	Treatment	Body weight (g) Initial	Body weight (g) 4th week	Body weight (g) % change
I	Normal control	202.33 ± 3.36	214.36 ± 16.52	5.96% increase
II	Dexamethasone	203.00 ± 4.02	238.66 ± 48.65	17.56% increase
III	Dexamethasone + LLLT	198.00 ± 3.44	220.34 ± 26.41	11.2% increase
IV	Dexamethasone + CPSP	218.66 ± 6.61	228.12 ± 32.42	4.32% increase
V	Dexamethasone + Alendronate sodium	196.00 ± 4.38	208.14 ± 32.42	6.1% increase
VI	Dexamethasone + LLLT + CPSP	200.33 ± 3.72	212.46 ± 19.02	6.05% increase

Effect of CPSP and LLLT on % Change in Body Weight on 8th week: Similarly the body weight was measured at the end of the study (8th week) and the % change in the body weight was measured from its 4th week to the 8th week were similar kind of change in the body weight was observed. Group II rats showed a further increase in 7.57% body weight when compared with the normal group. Upon irradiation with LLLT, Group III rats showed 3.66% increase in body weight and upon administration of CPSP (Group IV), alendronate sodium (Group V) and CPSP in combination with LLLT (Group VI) showed % decrease of 3.43%, 2.18% and 2.79% respectively when compared with the group II animals treated only with dexamethasone. Further, it was found that the more significant % decrease in body weight in Group III, IV, V and IV was observed when compared with 4th week.

TABLE 2: % CHANGE IN THE BODY WEIGHTS OF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 8th WEEK

Group (n=6)	Treatment	Body weight (g) 4th week	Body weight (g) 8th week	Body weight (g) % change
I	Normal control	214.36 ± 16.52	224.98 ± 19.64	4.95% increase
II	Dexamethasone	238.66 ± 48.65	256.73 ± 12.61	7.57% increase
III	Dexamethasone + LLLT	220.34 ± 26.41	228.42 ± 13.81	3.66% increase
IV	Dexamethasone + CPSP	228.12 ± 32.42	220.28 ± 31.41	3.43% decrease
V	Dexamethasone + Alendronate sodium	208.14 ± 32.42	212.69 ± 10.12	2.18% decrease
VI	Dexamethasone + LLLT + CPSP	212.46 ± 19.02	218.39 ± 26.44	2.79% decrease

Effect of CPSP and LLLT on Number of Movements on 4th Week: When assessed on 4th week, group II animals showed a significant decrease in the number of movements (55.83 ± 2.42^{***a}) when compared to the normal group of animals. Group III animals have shown an insignificant increase (60.33 ± 4.20) in number of movements when compared with group II animals.

A significant increase in the number of movements (73.16 ± 1.55^{**b}) was observed in group IV animals treated with CPSP. Group V (136.83 ± 2.68^{***b}) and group IV (91.5 ± 3.61^{***b}) animals also showed a significant increase in the number of movement when compared with the group II animals.

TABLE 3: NUMBER OF MOVEMENTS (IN 10MIN) OF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 4th WEEK

Group (n= 6)	Treatment	Number of movements (for 10 min)
I	Normal control	170.00 ± 4.48
II	Dexamethasone	55.83 ± 2.42^{***a}
III	Dexamethasone + LLLT	60.33 ± 4.20
IV	Dexamethasone + CPSP	73.16 ± 1.55^{**b}
V	Dexamethasone + Alendronate sodium	136.83 ± 2.68^{***b}
VI	Dexamethasone + LLLT + CPSP	91.5 ± 3.61^{***b}

Effect of CPSP and LLLT % Reduction in Locomotar Activity on 4th Week: After assessing the number of movements, % reduction in

locomotar activity was calculated. On 4th week group II animals showed 67.15% reduction in locomotar activity when compared to normal group. Group III animals showed 64.51% reduction in locomotar activity. Group IV, V and group VI showed 56.96%, 46.17% and 19.51% reduction in locomotar activity.

TABLE 4: % REDUCTIONOF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 8th WEEK

Group (n= 6)	Treatment	Number of movements (for 10 min)	% Reduction
I	Normal control	170.00 ± 4.48	0%
II	Dexamethasone	55.83 ± 2.42	67.15%
III	Dexamethasone + LLLT	60.33 ± 4.20	64.51%
IV	Dexamethasone + CPSP	73.16 ± 1.55	56.96%
V	Dexamethasone + Alendronate sodium	136.83 ± 2.68	46.17%
VI	Dexamethasone + LLLT + CPSP	91.5 ± 3.61	19.51%

Effect of CPSP and LLLT on Number of Movements on 8th Week: When assessed at 8th week, group II animals showed a significant decrease in the number of movements (55.66 ± 2.85^{***a}) when compared to the normal group of animals. Group III animals have shown an insignificant increase (57.16 ± 3.23) in number of movements when compared with group II animals.

TABLE 5: NUMBER OF MOVEMENTS (IN 10MIN) OF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 8th WEEK

Group (n= 6)	Treatment	Number of movements (for 10min)
I	Normal control	168.00 ± 2.28
II	Dexamethasone	55.66 ± 2.85^{***a}
III	Dexamethasone + LLLT	57.16 ± 3.23
IV	Dexamethasone + CPSP	106.66 ± 3.14^{***b}
V	Dexamethasone + Alendronate sodium	113.66 ± 3.00^{***b}
VI	Dexamethasone + LLLT + CPSP	157.16 ± 3.69^{***b}

A significant increase in the number of movements (106.66 ± 3.14^{***b}) was observed in group IV animals treated with CPSP. Group V (113.66 ± 3.00^{***b}) and group IV (157.16 ± 3.69^{***b}) animals also showed a significant increase in the number of movement when compared with the group II animals. Further when the results were compared with the 4th week it was found that number of movements were significantly improved more in 8th week in Group IV, V and VI. Whereas, no much improvement was found in Group II from 4th week to 8th week.

Effect of CPSP and LLLT % Reduction in Locomotar Activity on 8th Week: On 8th week group II animals showed 66.86% reduction in locomotar activity when compared to normal group. Group III animals showed reduction 65.97% in locomotar activity. Group IV, V and group VI showed 36.5%, 6.45% and 32.34% reduction in locomotar activity. It was observed that the when compared with 4th week, % reduction in locomotar activity reduced more significantly in the groups IV, V, VI respectively.

TABLE 6: % REDUCTION OF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 8th WEEK

Group (n= 6)	Treatment	Number of movements (for 10min)	% Reduction
I	Normal control	168.00 ± 2.28	0%
II	Dexamethasone	55.66 ± 2.85	66.86%
III	Dexamethasone + LLLT	57.16 ± 3.23	65.97%
IV	Dexamethasone + CPSP	106.66 ± 3.14	36.5%
V	Dexamethasone + Alendronate sodium	113.66 ± 3.00	6.45%
VI	Dexamethasone + LLLT + CPSP	157.16 ± 3.69	32.34%

Effect of CPSP and LLLT on Behavioral Assessment by Elevated Plus Maze (EPM) on 4th Week: At 4th week, Group II rats showed less number of entries (3.66 ± 0.36^{***a}) and also the time spent (50.33 ± 2.86^{***a}) on open arm was significantly shorter when compared with that of the normal group of rats, whereas the number of entries (7.00 ± 0.63^{***a}) and time spent (253.5 ± 5.49^{***a}) in closed arm was significantly longer when compared with normal group. The LLLT irradiated rats insignificantly increased the number of entries (3.83 ± 0.33) and time spent (55.66 ±

2.49) open arm and there was no change in the number of entries in the closed arm (7.16 ± 0.52) but the time spent in the closed arm (240.16 ± 4.35) was found to be decreased insignificantly when compared to group II animals. Group IV rats treated with CPSP significantly increased the number of entries (5.00 ± 1.41^{*b}) and time spent (62.16 ± 3.51^{*b}) in the open arm and insignificantly decreased the number of entries (5.66 ± 0.36) and significantly decreased the time spent (234.33 ± 3.17^{**b}) in the closed arm. Group V & VI animals showed significant increase in the number of entries (5.66 ± 0.46^{**b} & 5.33 ± 0.61^{**b}) and time spent in the open arm (77.00 ± 3.27^{***b} & 73.16 ± 2.12^{***b}) and a significant decrease in the number of entries (5.16 ± 0.52^{*b} & 5.33 ± 0.36^{*b}) and time spent (216.83 ± 3.02^{***b} & 234.83 ± 3.17^{***b}) in the closed arm.

TABLE 7: EFFECTS ON BEHAVIORAL ASSESSMENT BY ELEVATED PLUS MAZE (EPM) OF NORMAL, CPSP TREATED, LLLT TREATED, CPSP+LLLT TREATED, OSTEOPOROTIC RATS AT 4th WEEK

Group (n= 6)	Treatment	Open arm		Closed arm	
		No of entries	Time spent (sec)	No of entries	Time spent (sec)
I	Normal control	6.83 ± 0.33	86.00 ± 3.38	3.66 ± 0.36	196.66 ± 3.04
II	Dexamethasone	3.66 ± 0.36^{***a}	50.33 ± 2.86^{***a}	7.00 ± 0.63^{***a}	253.5 ± 5.49^{***a}
III	Dexamethasone + LLLT	3.83 ± 0.33	55.66 ± 2.49	7.16 ± 0.52	240.16 ± 4.35
IV	Dexamethasone + CPSP	5.00 ± 1.41^{*b}	62.16 ± 3.51^{*b}	5.66 ± 0.36	234.33 ± 3.17^{**b}
V	Dexamethasone + Alendronate sodium	5.66 ± 0.46^{**b}	77.00 ± 3.27^{***b}	5.16 ± 0.52^{*b}	216.83 ± 3.02^{***b}
VI	Dexamethasone + LLLT + CPSP	5.33 ± 0.61^{**b}	73.16 ± 2.12^{***b}	5.33 ± 0.36^{*b}	234.83 ± 3.17^{***b}

Effect of CPSP and LLLT on Behavioral Assessment by Elevated Plus Maze (EPM) on 8th Week: Also when evaluated on 8th week, similar improvement was shown. Group II rats continued to showed less number of entries (2.83 ± 0.33^{***a}) and also the time spent (45.00 ± 2.40^{***a}) on open arm was significantly shorter when compared with that of the normal group of rats, whereas the number of entries (6.83 ± 0.33^{***a}) and time spent (260.83 ± 3.50^{***a}) in closed arm was significantly longer when compared with normal group. The LLLT irradiated rats insignificantly increased the number of entries (4.33 ± 0.36) and significantly increased the time spent (58.33 ± 3.76^{**b}) open arm and there was no change in the number of entries in the closed arm (6.83 ± 0.33) but the time spent in the closed arm (239.83 ± 5.50^{**b}) was found to be decreased significantly when compared to group II animals. Group IV rats treated with CPSP showed insignificant increase in the number of entries (5.66 ± 0.23) and significant increase in time spent (65.16 ± 2.74^{***b}) in the open arm and insignificantly decreased the number of entries (5.00 ± 0.4) and significantly decreased the time spent (232.50 ± 4.39^{***b}) in the closed arm. Group V & VI animals showed significant increase in the number of entries (6.16 ± 0.33^{*b} & 5.50 ± 0.46^{*b}) and time spent in the open arm (86.16 ± 3.58^{***b} & 74.66 ± 2.50^{***b}) and a significant decrease in the number of entries (4.50 ± 0.36^{*b} & 4.66±3.66^{*b}) and time spent (201.00 ± 4.22^{***b} & 232.83 ± 4.10^{***b}) in the closed arm. However, the values were independent from that of the 4th week.

TABLE 8: EFFECTS ON BEHAVIORAL ASSESSMENT BY ELEVATED PLUS MAZE (EPM) OF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 8th WEEK

Group (n= 6)	Treatment	Open arm		Closed arm	
		No of entries	Time spent	No of entries	Time spent
I	Normal control	6.16 ± 0.33	92.16 ± 1.96	4.5 ± 0.37	197 ± 3.61
II	Dexamethasone	2.83 ± 0.33^{***a}	45.00 ± 2.40^{***a}	6.83 ± 0.33^{***a}	260.83 ± 3.50^{***a}
III	Dexamethasone + LLLT	4.33 ± 0.36	58.33 ± 3.76^{**b}	6.83 ± 0.33	239.83 ± 5.50^{**b}
IV	Dexamethasone + CPSP	5.66 ± 0.23	65.16 ± 2.74^{***b}	5.00 ± 0.4	232.50 ± 4.39^{***b}
V	Dexamethasone + Alendronate sodium	6.16 ± 0.33^{*b}	86.16 ± 3.58^{***b}	4.50 ± 0.36^{*b}	201.00 ± 4.22^{***b}
VI	Dexamethasone + LLLT + CPSP	5.50 ± 0.46^{*b}	74.66 ± 2.50^{***b}	4.66 ± 3.66^{*b}	232.83 ± 4.10^{***b}

Effect of CPSP and LLLT on Urine and Serum Levels of Calcium, Creatinine and Phosphorous on 4th Week: Group II rats treated with dexamethasone have shown a significant increase in excretion of calcium (19.89 ± 1.32^{***a}), creatinine (7.24 ± 0.04^{***a}), phosphorous (7.24 ± 0.04^{***a}) when compared with the normal group of animals. Group III animals irradiated with LLLT showed an insignificant decrease in the excretion of calcium (17.17 ± 0.33), creatinine (22.02 ± 0.74),

phosphorous (6.49 ± 0.10^{***b}) when compared to the group II animals. Group IV animals treated with CPSP demonstrated a significant decrease in the excretion of calcium (15.32 ± 1.76^{*b}), creatinine (15.47 ± 0.83^{***b}), phosphorous (5.02 ± 0.05^{***b}) in urine when compared to group II. A significant decrease in urinary excretion of calcium, creatinine, phosphorous (11.96 ± 1.45^{***b}, 16.07 ± 0.91^{***b}, 4.38 ± 0.09^{***b} & 12.30 ± 1.50^{***b}, 16.05 ± 1.25^{***b}, 4.38 ± 0.09^{***b}) was observed in group V & VI respectively. There is a significant decrease in serum levels of calcium (7.56 ± 0.42^{***a}), creatinine (0.20 ± 0.29^{***a}), phosphorous (7.17 ± 0.07^{***a}) in group II treated with dexamethasone when compared with the normal group of animals.

Group III animals irradiated with LLLT showed an insignificant increase in the serum calcium (10.66 ± 0.53), significant increase in creatinine (0.64 ± 0.05^{***b}), phosphorous (8.19 ± 0.12^{***b}) when compared to the group II animals. Group IV animals treated with CPSP demonstrated a significant increase in the calcium (13.31 ± 1.27^{***b}), creatinine (0.78 ± 0.04^{***b}), phosphorous (9.93 ± 0.20^{***b}) in serum when compared to group II. A significant increase in serum calcium, creatinine, phosphorous (16.02 ± 0.88^{***b}, 0.70 ± 0.02^{***b}, 10.47 ± 0.06^{***b}& 14.29 ± 0.96^{***b}, 0.99 ± 0.03^{***b}, 10.27 ± 0.14^{***b}) was observed in group V & VI respectively.

TABLE 9: URINE AND SERUM LEVELS OF CALCIUM, CREATININE AND PHOSPHOROUS OF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 4th WEEK

Group	Serum			Urine		
	Ca^{2+} (mg/dl)	Po_4^- (mmol/dl)	Creatinine (mg/dl)	Ca^{2+} (mg/dl)	Po_4^- (mmol/dl)	Creatinine (mg/dl)
Normal control	16.55 ± 1.06	11.13 ± 0.18	0.98 ± 0.06	9.33 ± 0.43	4.07 ± 0.13	18.41 ± 0.88
Dexamethasone	7.56 ± 0.42^{***a}	7.17 ± 0.07^{***a}	0.20 ± 0.29^{***a}	19.89 ± 1.32^{***a}	7.24 ± 0.04^{***a}	24.87 ± 1.98^{***a}
Dexamethasone + LLLT	10.66 ± 0.53	8.19 ± 0.12^{***b}	0.64 ± 0.05^{***b}	17.17 ± 0.33	6.49 ± 0.10^{***b}	22.02 ± 0.74
Dexamethasone + CPSP	13.31 ± 1.27^{***b}	9.93 ± 0.20^{***b}	0.78 ± 0.04^{***b}	15.32 ± 1.76^{*b}	5.02 ± 0.05^{***b}	15.47 ± 0.83^{***b}
Dexamethasone + Alendronate sodium	16.02 ± 0.88^{***b}	10.47 ± 0.06^{***b}	0.70 ± 0.02^{***b}	11.96 ± 1.45^{***b}	4.47 ± 0.07^{***b}	16.07 ± 0.91^{***b}
Dexamethasone + LLLT + CPSP	14.29 ± 0.96^{***b}	10.27 ± 0.14^{***b}	0.99 ± 0.03^{***b}	12.30 ± 1.50^{***b}	4.38 ± 0.09^{***b}	16.05 ± 1.25^{***b}

Effect of CPSP and LLLT on Urine and Serum Levels of Calcium, Creatinine and Phosphorous on 8th Week: When measured on 8th week similar effects as that of 4th week were observed. There was a significant increase in excretion of calcium (23.03 ± 1.63^{***a}), creatinine (24.76 ± 1.16^{***a}), phosphorous (9.43 ± 0.10^{***a}) in group II treated with dexamethasone when compared with the normal group of animals. Group III animals irradiated with LLLT showed a significant decrease in the excretion of calcium (18.04 ± 0.45^{***b}), creatinine (20.61 ± 0.51^{***b}), phosphorous (9.43 ± 0.10^{***a}) when compared to the group II animals. Group IV animals treated with CPSP demonstrated a significant decrease in the excretion of calcium (13.34 ± 0.87^{***b}), creatinine (13.37 ± 0.45^{***b}), phosphorous (4.82 ± 0.05^{***b}) in urine when compared to group II.

A significant decrease (10.81 ± 0.85^{***b}, 15.51 ± 0.99^{***b}, 3.77 ± 0.09^{***b} & 12.13 ± 0.61^{***b}, 11.26 ± 0.47^{***b}, 4.38 ± 0.09^{***b}) in urinary excretion of calcium was observed in group V & VI respectively. It was observed that when compared with 4th week, there was a further decrease in urine calcium excretion on 8th week in group IV, V & VI. In group III no such effects were observed (no decrease in 8th week when compared with 4th week).

When measured on 8th week similar kind of effect was observed. There was a significant decrease in serum levels of calcium (5.74 ± 0.64^{***a}), creatinine (0.15 ± 0.01^{***a}), phosphorous (5.65 ± 0.08^{***a}) in group II treated with dexamethasone when compared with the normal group of animals. Group III animals irradiated with LLLT showed a significant increase in the serum calcium (12.20 ± 0.54^{***b}), creatinine (0.43 ± 0.03^{***b}), phosphorous (8.36 ± 0.05^{***b}) when compared to the group II animals. Group IV animals treated with CPSP demonstrated a significant increase in the calcium (13.92 ± 1.76^{***b}), creatinine (0.46 ± 0.03^{***b}), phosphorous (10.36 ± 0.21^{***b}) in serum when compared to group II. A significant increase in serum calcium, creatinine, phosphorous (16.38 ±

0.55^{***b}, $0.69 \pm 0.07^{***b}$, $12.93 \pm 0.11^{***b}$ & $14.72 \pm 1.12^{***b}$, $0.95 \pm 0.01^{***b}$, $11.93 \pm 0.13^{***b}$) was observed in group V & VI respectively. It was observed that when compared with 4th week, there was a further increase in serum calcium levels on 8th week which almost returned to the normal levels in all the groups.

TABLE 10: URINE AND SERUM LEVELS OF CALCIUM, CREATININE AND PHOSPHOROUS OF NORMAL, CPSP TREATED, LLLT TREATED, CPSP+LLLT TREATED, OSTEOPOROTIC RATS AT 8th WEEK

Group	Serum			Urine		
	Ca^{2+} (mg/dl)	Po_4^- (mmol/dl)	Creatinine (mg/dl)	Ca^{2+} (mg/dl)	Po_4^- (mmol/dl)	Creatinine (mg/dl)
Normal control	16.58 ± 1.33	11.03 ± 0.13	0.98 ± 0.03	10.21 ± 0.43	4.44 ± 0.04	16.68 ± 1.24
Dexamethasone	$5.74 \pm 0.64^{***a}$	$5.65 \pm 0.08^{***a}$	$0.15 \pm 0.01^{***a}$	$23.03 \pm 1.63^{***a}$	$9.43 \pm 0.10^{***a}$	$24.76 \pm 1.16^{***a}$
Dexamethasone + LLLT	$12.20 \pm 0.54^{***b}$	$8.36 \pm 0.05^{***b}$	$0.43 \pm 0.03^{***b}$	18.04 ± 0.45	$6.11 \pm 0.21^{***b}$	$20.61 \pm 0.51^{***b}$
Dexamethasone + CPSP	$13.92 \pm 1.76^{***b}$	$10.36 \pm 0.21^{***b}$	$0.46 \pm 0.03^{***b}$	$13.34 \pm 0.87^{*b}$	$4.82 \pm 0.05^{***b}$	$13.37 \pm 0.45^{***b}$
Dexamethasone + Alendronate sodium	$16.38 \pm 0.55^{***b}$	$12.93 \pm 0.11^{***b}$	$0.69 \pm 0.07^{***b}$	$10.81 \pm 0.85^{***b}$	$3.77 \pm 0.09^{***b}$	$15.51 \pm 0.99^{***b}$
Dexamethasone + LLLT + CPSP	$14.72 \pm 1.12^{***b}$	$11.93 \pm 0.13^{***b}$	$0.95 \pm 0.01^{***b}$	$12.13 \pm 0.61^{***b}$	$4.38 \pm 0.09^{***b}$	$11.26 \pm 0.47^{***b}$

Effect of CPSP and LLLT on Serum ALP on 4th Week & 8th Week: There is a significant increase ($234.91 \pm 11.99^{***a}$) in serum levels of ALP in group II treated with dexamethasone when compared with the normal group of animals. Group III animals irradiated with LLLT showed a significant decrease ($177.20 \pm 3.86^{***b}$) in the serum ALP levels when compared to the group II animals. Group IV animals treated with CPSP demonstrated a significant decrease ($167.09 \pm 6.31^{***b}$) in the calcium levels in serum when compared to group II. A significant increase ($144.07 \pm 4.43^{***b}$ & $149.84 \pm 3.41^{***b}$) in serum creatinine levels was observed in group V & VI respectively. Similarly on 8th week there is a significant increase ($238.01 \pm 2.80^{***a}$) in serum levels of ALP in group II treated with dexamethasone when compared with the normal group of animals. Group III animals irradiated with LLLT showed a significant decrease ($163.02 \pm 6.43^{***b}$) in the serum ALP levels when compared to the group II animals. Group IV animals treated with CPSP demonstrated a significant decrease ($166.87 \pm 3.48^{***b}$) in the calcium levels in serum when compared to group II. A significant increase ($104.70 \pm 5.07^{***b}$ & $128.82 \pm 2.91^{***b}$) in serum creatinine levels was observed in group V & VI respectively. It was observed that when compared with 4th week, there was a further increase in serum calcium levels on 8th week which almost returned to the normal levels in all the groups.

TABLE 11: SERUM ALP OF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 4th AND 8th WEEK

Group (n=6)	Treatment	4th week	8th week
		ALP (IU/L)	ALP (IU/L)
I	Normal control	124.31 ± 5.06	128.53 ± 2.90
II	Dexamethasone	$234.91 \pm 11.99^{***a}$	$238.01 \pm 2.80^{***a}$
III	Dexamethasone + LLLT	$177.20 \pm 3.86^{***b}$	$163.02 \pm 6.43^{***b}$
IV	Dexamethasone + CPSP	$167.09 \pm 6.31^{***b}$	$166.87 \pm 3.48^{***b}$
V	Dexamethasone + Alendronate sodium	$144.07 \pm 4.43^{***b}$	$104.70 \pm 5.07^{***b}$
VI	Dexamethasone + LLLT + CPSP	$149.84 \pm 3.41^{***b}$	$128.82 \pm 2.91^{***b}$

Effect of CPSP and LLLT on Femur Weight, Length, Thickness on 8th Week: The femur weight ($0.22 \pm 0.01^{***a}$), thickness ($2.66 \pm 0.02^{***a}$) is significantly decreased in group II animals supplemented with dexamethasone when compared to the normal group. Group III animals irradiated with LLLT showed a significant increase in femur weight ($0.30 \pm 0.00^{**b}$), insignificant increase in thickness (2.64 ± 0.05) when compared to the group II animals. A significant increase in femur weight ($0.41 \pm 0.00^{***b}$), insignificant increase in thickness (2.71 ± 0.01) was observed in group IV animals. A significant increase in the femur weight, thickness ($0.5 \pm 0.01^{***b}$, $2.89 \pm 0.01^{***b}$) was

observed in group V animals when compared to that of the group II animals. A significant increase in the femur weight, (0.47 ± 0.01^{***b}, 2.89 ± 0.01^{***b}) was observed group V animals and in group VI there is a significant increase in femur weight (0.47 ± 0.01^{***b}), insignificant increase in thickness (2.71 ± 0.02) when compared to that of the group II animals. An insignificant decrease in the femur length (30.5 ± 0.78) was observed in group II animals which were back to near normal in the groups treated with CPSP, LLLT, sodium alendronate as well as CPSP + LLLT.

TABLE 12: FEMUR WEIGHT, LENGTH, THICKNESSOF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 8th WEEK

Group (n = 6)	Treatment	Weight (per 100 g b.w.)	Length (mm)	Thickness (mm)
I	Normal control	0.63 ± 0.02	32.3 ± 1.15	2.82 ± 0.02
II	Dexamethasone	0.22 ± 0.01^{***a}	30.5 ± 0.78	2.66 ± 0.02^{***a}
III	Dexamethasone + LLLT	0.30 ± 0.00^{**b}	30.83 ± 0.43	2.64 ± 0.05
IV	Dexamethasone + CPSP	0.41 ± 0.00^{***b}	31.6 ± 0.83	2.71 ± 0.01
V	Dexamethasone + Alendronate sodium	0.5 ± 0.01^{***b}	33.0 ± 0.40	2.89 ± 0.01^{***b}
VI	Dexamethasone + LLLT + CPSP	0.47 ± 0.01^{***b}	32.0 ± 1.74	2.71 ± 0.02

Effect of CPSP and LLLT on Mechanical Strength of Femoral Bone on 8th Week: The results of bone mechanical strength revealed that the group II animals administered with dexamethasone reduced the force required to break (10.68 ± 0.60^{***a}) significantly when compared to the normal group of animals.

TABLE 13: BONE MECHANICAL STRENGTHOF NORMAL, CPSP TREATED, LLLT TREATED, CPSP + LLLT TREATED, OSTEOPOROTIC RATS AT 8th WEEK

Group (n = 6)	Treatment	Force at break (kg/cm^2)
I	Normal control	18.08 ± 0.38
II	Dexamethasone	10.68 ± 0.60^{***a}
III	Dexamethasone + LLLT	13.18 ± 0.60^{**b}
IV	Dexamethasone + CPSP	15.80 ± 0.26^{***b}
V	Dexamethasone + Alendronate sodium	17.75 ± 0.38^{***b}
VI	Dexamethasone + LLLT + CPSP	17.95 ± 0.91^{***b}

Group III animals when irradiated with LLLT showed a significant improvement in the mechanical strength of the femur (13.18 ± 0.60^{**b}). Treatment with CPSP significantly improved the mechanical strength of the femur (15.80 ± 0.26^{***b}). Group V & VI animals when treated with alendronate sodium and CPSP in combination with LLLT also showed a significant improvement in the mechanical strength (17.75 ± 0.38^{***b} & 17.95 ± 0.91^{***b}) of the femur and increased the strength required to break the femur bone.

Histopathology of Femur Bone: Normal rats showed typical osteoblasts, normal and compact trabeculae, bone matrix formed with mineral salts and osteon (Haversian system) reveal the active vasculature.

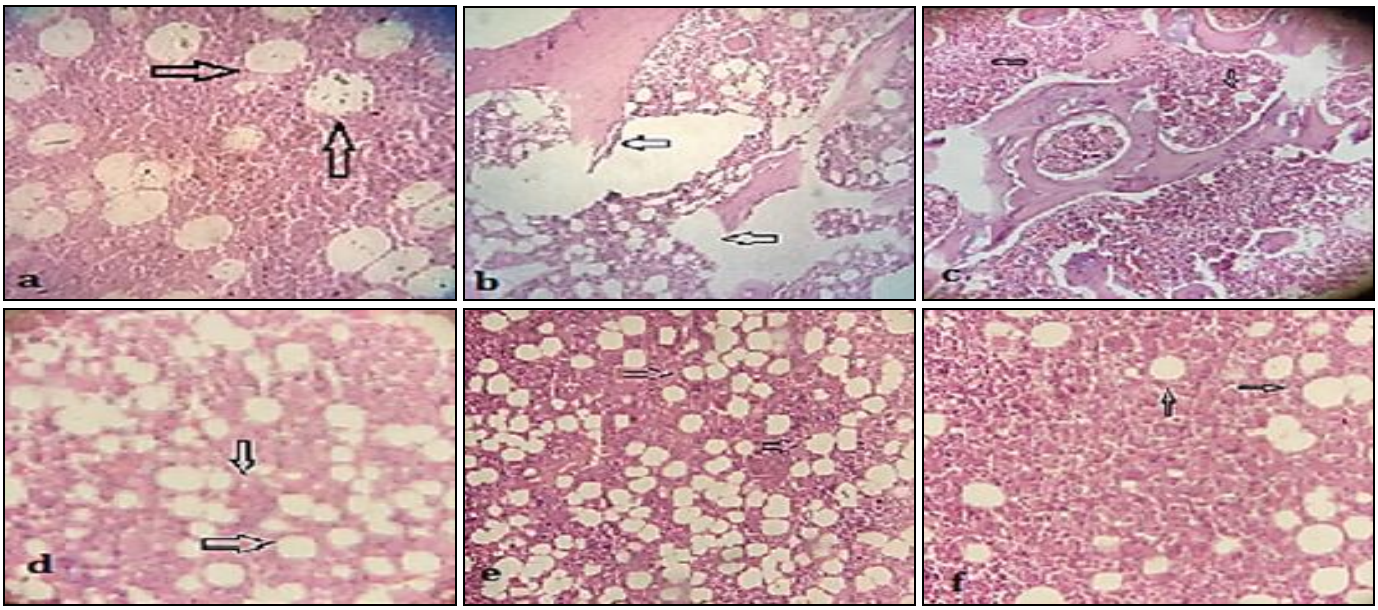

FIG. 1: a) NORMAL CONTROL b) DEXAMETHASONE c) DEXAMETHASONE + LLLT d) DEXAMETHASONE + CPSP e) DEXAMETHASONE + SODIUM ALENDRONATE f) DEXAMETHASONE + CPSP + LLLT

Poor osteoblastic lining, emptying of bone mineral salts from the matrix with thinning of trabeculae and loss of connectivity are observed in dexamethasone induced osteoporotic rats. Partial restoration of trabeculae with slightly active bone matrix. But bone undergoing resorption is seen with poor osteoblastic activity are observed in LLLT treated rats. Partial restoration of trabeculae with active bone matrix. But bone undergoing resorption is still seen in rats treated with CPSP treated rats. Restoration with thick elongated trabeculae with narrowed inter trabecular space. The appearances of mineral salts in the matrix is also evident in rats treated with alendronate sodium (standard). Maximum restoration of trabeculae into normal with improved activity of osteoblasts. The occurrence of mineral salts in bone matrix along with increasing bone cells is observed in the rats treated with CPSP + LLLT.

DISCUSSION: Normal rats of group I exhibited the body weight within the range, whereas the animals treated with dexamethasone alone showed a significant increase in the body weight of the animals by the end of the 4th week and continued to show the similar effects till the 8th week since there occurs a redistribution of fat cells in the body (called lipodystrophy), affects the metabolism, which can increase appetite leading to gain in the body weight. In the group III rats, after being treated with LLLT, showed a significant decrease in body which is due to the reduction in the thickness of subcutaneous fat [20]. Group IV rats showed a decrease in body weight after being treated with CPSP probably due to the presence of phytoestrogens and antioxidant vitamins. Group V ratstreated with sodium alendronate continued to decrease the increased body weight both at 4th week and 8th week respectively by increasing the bone mass by interacting with several adipokines in the bone remodeling by improving the effects on both formation and resorption. Group VI rats treated with the combination *i.e.,* CPSP + LLLT exhibited a decrease in increased body weight due the synergistic effect of CPCP & LLLT [21, 15].

When assessed on 4th week as well as 8th week, group II animals showed a significant decrease in the number of movements when compared to the normal group of animals which might be due to the depression and correlation between the osteoporosis and depression lies in the inflammatory cytokines which is a strong inducer of osteoclastogenesis and also in depression [22]. Group III animals have shown an insignificant increase in number of movements when compared with group II animals which is due to their ability to reduce the inflammatory cytokines [23]. A significant increase in the number of movements was observed in group IV animals treated with CPSP probably due to the reversal mechanism. Group V and group IV animals also showed a significant increase in the number of movement when compared with the group II animals which is due to the reversal of depression occurred. More increase in the number of movements as well as more decrease in the % reduction in locomotar activity was observed in 8th week when compared to the 4th week.

At 4th week, Group II rats showed less number of entries and also the time spent on open arm was significantly shorter when compared with that of the normal group of rats, whereas the number of entries and time spent in closed arm was significantly longer when compared with normal group and this was due to anxiety levels with the interleukin-2 levels in the brain, as osteoporotic individuals have higher risk of developing depression and also osteoporotic rats face difficulty to move [22]. The LLLT irradiated rats insignificantly increased the number of entries and time spent open arm and there was no change in the number of entries in the closed arm but the time spent in the closed arm was found to be decreased insignificantly when compared to group II animals.

Group IV rats treated with CPSP significantly increased the number of entries and time spent in the open arm and insignificantly decreased the number of entries and significantly decreased the time spent in the closed arm. Group V & VI animals showed significant increase in the number of entries and time spent in the open arm and a significant decrease in the number of entries and time spent in the closed arm. All these were probably probably due to their indirect mechanism on interleukin 2 levels owing to the reduction in the anxiety levels. Similar effects were observed even during the 8th week but when compared to the 4th week the overall activity was improved.

There was a significant increase in excretion of calcium, phosphorous and creatinine in group II treated with dexamethasone when compared with the normal group of animals. Calcium excretion increase was mainly due to increased bone loss by decreasing the rate of bone formation due to reduced intestinal calcium absorption, increase in phosphorous excretion was mainly due to decreased serum calcium concentrations, which increases serum PTH concentration thus increasing the number of osteoclast activity ultimately resulting in elevated bone resorption and increased creatinine excretion due to weaker interactions between the muscle and bone. Group III animals irradiated with LLLT showed an insignificant decrease in the excretion of calcium, phosphorous and creatinine when compared to the group II animals.

Group IV animals treated with CPSP demonstrated a significant decrease in the excretion of calcium, phosphorous and creatinine in urine when compared to group II and this may be attributed due to the phytoestrogens which is responsible for maintenance of proper bone density and due to the presence of minerals which acts as dietary source to increase the decreased serum calcium phosphorous and creatinine levels thus decreasing the levels of PTH. A significant decrease in urinary excretion of calcium was observed in group V & VI respectively owing the similar mechanism as stated above. It was observed that when compared to the 4^{th} week, there was more improvement in the levels of calcium, phosphorous and creatinine in the 8^{th} week.

There was a significant decrease in serum levels of calcium, phosphorous and creatinine and increased level of ALP in group II treated with dexamethasone when compared with the normal group of animals. Calcium, phosphorous and creatinine levels were decreased due to increased excretion in urine (as already explained in urine parameters) and ALP values were found to be high since it is an important biochemical marker of bone turnover which promotes bone mineralization and in osteoporosis since the bone is porous, ALP levels will be released in the systemic circulation owing to increased ALP in serum. Group III animals irradiated with LLLT showed an insignificant increase in the serum calcium, phosphorous and creatinine and demonstrated a decrease in ALP levels when compared to the group II animals probably by enhancing bone formation and decreasing bone resorption.

Group IV animals treated with CPSP demonstrated a significant increase in the calcium, phosphorous and creatinine levels in serum and decrease in ALP values in serum when compared to group II owing to the presence of phytoestrogens and presence of minerals which serves as dietary source. A significant increase in serum calcium, phosphorous and creatinine levels was observed and showed a decrease in serum ALP levels in group V & VI respectively due to the reversal mechanism and synergistic effect. It was observed that when compared to the 4^{th} week, there was more improvement in the levels of calcium, phosphorous, ALP and creatinine in the 8^{th} week which almost returned to its normal levels.

Femur bone, length, thickness and mechanical strength were measured at the end of the 8th week. From the results it was found that the femur weight was significantly decreased and femur length, thickness was insignificantly decreased and reduced the mechanical strength in harness test in group II animals supplemented with dexa-methasone when compared to the normal group since dexamethasone increases the bone resorption and decrease the bone formation leading to diminishing bone mass and bone mineral density which affects the physical parameter of the bone (length, weight and thickness). Group III animals irradiated with LLLT showed a significant increase in femur weight and insignificant increase in femur length, thickness and increased the mechanical strength in hardness test when compared to the group II animals. A significant increase in femur weight and insignificant increase in femur length, thickness and increased the mechanical strength in hardness test was observed in group IV animals.

A significant increase in the femur weight and insignificant increase in femur length, thickness and increased the mechanical strength in hardness test was observed in group V & group VI animals when compared to that of the group II animals.

CONCLUSION: From this present study, it can be concluded that CPSP as well as LLLT has

antiosteoporotic activity it decreased the increased body weight due to the administration of dexamethasone. The combination of CPSP + LLLT also showed improved decrease in the increased body weight when compared to CPSP or LLLT group alone proving its synergistic activity. The combination group exhibited better improvement when compared to the singly treated groups. CPSP and LLLT treated rats showed improved number of movements in actophotometer, showed increase in the number of entries and time spent in the open arm and showed decrease in the number of entries and time spent in the closed arm of EPM than in disease control rats, reduced the excretion of calcium, phosphorous and creatinine in urine and increased the levels of calcium, phosphorous and creatinine levels in serum maintain the body homeostasis also reduced the increasing levels of ALP in serum compared to the osteoporotic rats.

Further the bone mechanical parameters supported our study, by showing increase in bone weight, length, thickness and increasing the force to break the bone in the rats treated with CPSP and LLLT group alone as well as in the combination group. The histopathological study also supported that CPSP and LLLT demonstrated protective activity for the maintenance of bone. From the results of the present study, it can be concluded that CPSP and LLLT has the potential to combat osteoporosis and contribute to the protective as well as curative activity against dexamethasone induced osteo-porosis in rats.

CPSP has proven its therapeutic efficacy in treating osteoporosis owing to the presence of minerals such as zinc, calcium, phosphorous, magnesium, iron, manganese, potassium, presence of vitamins and amino acid, also had rich source of phytosterols and phytoestrogens. The exact mechanism behind the activities shown by CPSP and LLLT on improving bone mass and qualities as well as its long term effect on maintenance of bone mass could not be elucidated through this invivo study hence further studies involving the recognition of the pathway and the mechanism of the drug is certainly warranted.

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