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Therapeutic Potential of Chrysin in Improving Bone Health

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Abstract: Vitamin D deficiency is endemic worldwide. Although several strategies have been established to enhance vitamin D_3 levels, studies specifically focusing on the inhibition of vitamin D metabolism, which may prolong the availability of active vitamin D in pathological conditions, have been less explored. Studies also suggest that higher doses of vitamin D_3 fail to achieve optimum vitamin D levels. In this context, we focused on the enzyme CYP3A4, which promotes the inactivation of active vitamin D. The current study aimed to decipher the impact of chrysin, a proven CYP3A4 inhibitor, as an intervention and its effects in combination with low-dose vitamin D_3 (40 IU) and bone health in vitamin D deficiency conditions. The in vivo activity of chrysin was evaluated in female Wistar albino rats fed a vitamin-D-deficient diet to attain vitamin D deficiency for 28 days. Chrysin was given alone and in combination with calcium carbonate ($CaCO_3$) and/or vitamin D_3. All therapeutic interventions were assessed for serum 25-hydroxyvitamin D3(25-OH-D_3) by LC-MS and biochemical, urinary, and bone parameters. Animals treated with chrysin alone and in combination with low-dose vitamin D_3 and/or $CaCO_3$ showed an eminent rise in serum 25-OH-D_3 levels along with increased serum biochemical parameters. In contrast, a significant decrease in the urinary parameters followed by beneficial effects on bone parameters was noticed in contrast with the vitamin-D-deficient diet group. Our findings revealed that although chrysin alone showed a notable effect on 25-OH-D_3 and osseous tissue, comparatively, it showed an intensified therapeutic effect in combination with vitamin D_3 and $CaCO_3$, which can be employed as a cost-effective option to improve bone health.

Keywords: bone; chrysin; CYP3A4; vitamin D deficiency; osteoporosis; 25-OH-D_3

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1. Introduction

Currently, the magnitude of musculoskeletal diseases is emerging systematically among all age groups, affecting the social life of people and leading to pain and disability [1]. Osteoporosis is the most discussed burden of approximately 1 in 3 women and 1 in 5 men [2]. The predominant risk factors projecting bone diseases such as osteoporosis are age, sex, genetic defects, sedentary lifestyle, vitamin D deficiency/insufficiency, minimal intake of calcium, and early menopause [3]. However, the possible aspects that can reduce the intensity of skeletal disorders can be the recognition of the disease in its early stage followed by an effective treatment that includes ingestion of appropriate vitamin D and calcium supplements to combat bone loss [4]. Vitamin D is broadly recognized as a "bone vitamin" for its magnificent impact on osseous tissue. Conversely, its insufficient distribution leads to vitamin D deficiency, which has a tremendous effect on skeletal health

and worsens pathological conditions such as osteoporosis [5,6]. It was formerly known that the imperative relationship between active vitamin D and transcellular calcium uptake is necessary for maintaining bone architecture [7]. The observational findings on calcium kinetics specified that systematic absorption of calcium is approximately 30–40% in the case of adequate vitamin D (>32 ng/mL) and elicits only 10–15% in the case of low vitamin D (<15 ng/mL) [8].

On the other hand, although hypovitaminosis D is a modifiable risk factor, the medical community is facing several challenges in terms of maintaining or achieving optimum serum vitamin D_3 levels and retaining bone mass [9]. Respective research strategies are implemented to conquer vitamin D insufficiency by targeting sunlight exposure and climatic conditions [10], gut absorption of vitamin D supplements [11], vitamin D receptor (VDR) genotyping [12], and metabolic expression of cytochrome P450 (CYP450) enzymes and their interplay with vitamin D [13,14]. CYP450 enzymes are the major class of catabolic enzymes that play a decisive role in the pharmacokinetics of drugs as well as endogenous substances. Since CYP enzymes metabolize 90% of medicinal drugs, numerous investigations implying CYP450 have become crucial for revealing unveiled drug–drug interactions [15]. It is possible that the interactions caused by enzymatic inhibition/induction result in diminished/enhanced metabolic compounds, which may have a greater emphasis on the kinetic and dynamic profiles of therapeutic targets [16]. In this regard, we focused on the multifunctional class of enzymes CYP3A4, which catalyzes 25-hydroxylated-mediated conversion of the active form of vitamin D into its inactive form [14]. Interestingly, research studies revealed that therapeutic substrates (inducers/inhibitors) of CYP3A4 enzymes may contribute to altering the $1,25(OH)_2D_3$ levels in the serum [15]. Various systematic studies have indicated that higher doses of vitamin D_3 fail to achieve optimum serum 25-OH-D_3 levels in humans [17]. From this perspective, subsequent inhibition of vitamin D metabolism by CYP3A4 substrates may show an ameliorative effect on hypovitaminosis D. We hypothesized to enhance 25-OH-D_3 levels by incorporating the well-established CYP3A4 inhibitor chrysin [18], as shown in Figure 1. Chrysin is a natural flavonoid with beneficial pharmacological effects, such as anticancer, neuroprotective, hepatoprotective, cardioprotective, and antiarthritic effects [19,20]. It has also shown a promising outcome in osteoporotic rats [21]. Our research aimed to study the impact of chrysin on low-dose vitamin D_3 supplementation and its further implications on vitamin D deficiency conditions and bone health.

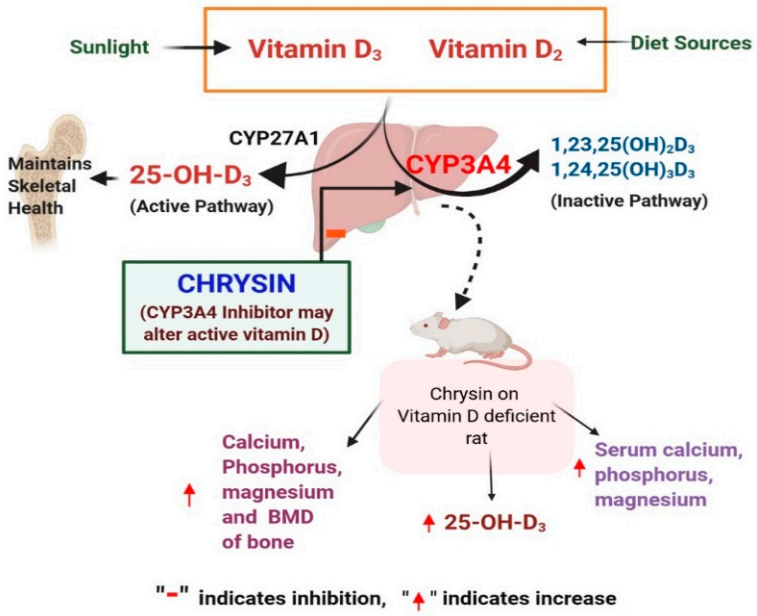

Figure 1. Chrysin CYP3A4 inhibition pathway: impact of chrysin on vitamin D and bone health.

2. Materials and Methods

2.1. Materials

Chrysin, also known as 5,7-dihydroxyflavone, was acquired from Sigma Aldrich, Bengaluru, India. Vitamin D_3 capsules of 2000 IU were used and obtained from UAV Private Limited, Mumbai marketed as D-RISE 2000 soft gelatin capsules. Calcium carbonate and all other chemicals used for the preparation of a vitamin-D-deficient diet were of analytical grade and purchased from SD-fine chemicals and Merk Pvt Ltd., Hyderabad, India. The kits used for the estimation of various biochemical parameters were from Erba Manheim, Pvt Ltd., Mumbai, India.

2.2. Preparation of Vitamin-D-Deficient Diet

The required ingredients for the preparation of the diet were weighed accordingly and blended for approximately 45 min followed by the addition of 10% water to make a dough. Then, it was granulated by using an 8 mm sieve followed by making round-shaped balls, dehydrated, and dried for approximately 3 days at room temperature at 37 °C. The diet prepared was standardized to ensure uniform nutrient values similar to those of the normal diet. The prepared diets were stored at 2–8 °C to minimize oxidation [22]. The composition of the normocalcemic vitamin D deficiency was followed as per the earlier reference [23] (Supplementary Table S1).

2.3. Dose Selection for the Study

Based on earlier research studies, the doses of chrysin, vitamin D_3, and $CaCO_3$ were fixed at 100 mg/kg, 40 IU, and 50 mg/kg, respectively [24,25].

2.4. Experimental Animals

Adult healthy female Wistar albino rats weighing 180–200 g were obtained from a certified breeder: Sri Venkateshwara Enterprises, Bangalore, India and housed under standard environmental conditions such as ambient temperature (22 ± 2 °C), relative humidity (30–70%), and a 12/12 h light/dark cycle in individual polypropylene cages and maintained hygienic conditions throughout the study according to the committee for control and supervision of experiments on animals (CPCSEA) guidelines. All experimental protocols were approved by the Institutional Animal Ethics Committee (Approval. No. CPCSEA/1677/PO/Re/S/2012/IAEC/34).

2.5. In Vivo Experimental Design

Vitamin D deficiency was induced by the diet prepared. According to the research of interest, the animals were divided into six groups (n = 6), and all the treatment groups (groups II, III, IV, V, and VI) were fed a vitamin-D-deficient diet except for the normal diet (group I) followed by treatment for 28 days. The treatment in the various groups were administered by mouth i.e., per oral (*po*). The schedule was as follows:

1. Group I: Normal (standard laboratory diet and drinking water ad libitum).
2. Group II: Disease control (fed a vitamin-D-deficient diet).
3. Group III: Therapeutic intervention 1 (chrysin (100 mg/kg, *po*).
4. Group IV: Therapeutic intervention 2 (chrysin (100 mg/kg, *po*) + $CaCO_3$ (50 mg/kg, *po*).
5. Group V: Therapeutic intervention 3 (chrysin (100 mg/kg, *po*) + vitamin D_3 (40 IU/kg, *po*).
6. Group VI: Therapeutic intervention 4 (chrysin (100 mg/kg, *po*) + $CaCO_3$ (50 mg/kg, *po*) + vitamin D_3 (40 IU/kg, *po*).

The required quantities of test drug solution (chrysin) and calcium carbonate ($CaCO_3$) were prepared by suspension in 1% w/v carboxy methyl cellulose (CMC) and vitamin D_3 in distilled water every day before administration to the animals. The prepared solution was administered with the help of an oral feeding needle (18 G).

2.6. Body Weights

The body weights of all the animals were taken on the 7th day (short term effects) and 28th day (long term effects) of the study, and the differences in the weights of the animals were compared among the different groups.

2.7. Biochemical Evaluation

Blood samples were collected from the retro-orbital plexuses on the 7th and 28th days of the study. The serum was separated and analyzed for calcium, phosphorus, magnesium, and alkaline phosphatases (ALP) with the help of kits by using an autoanalyzer (Erba Mannehim). The collected serum samples were also used for the estimation of vitamin D metabolites for which the samples were processed and stored in amber-colored Eppendorf tubes at 4 °C to avoid degradation of vitamin D metabolites [26].

2.8. Estimation of Urinary Parameters

The animals were hydrated with 5 mL of water and were individually housed in metabolic cages for 6 h, and the collected urine was then centrifuged at 2000 rpm for 5 min. The supernatant was collected and stored in airtight containers at 4–8 °C for further evaluation of urinary excretion of calcium, phosphorus, and magnesium.

2.9. Bone Parameters

On the 28th day of the study, the animals were sacrificed by cervical dislocation. The bones, such as the femur, tibia–fibula, 4th lumbar, and 8th thoracic vertebrae, were collected, weighed, and stored. The stored bone samples were further used to determine various bone parameters as follows:

2.9.1. Bone Weight

Freshly isolated femurs and tibia fibulas were taken, adhering tissues were removed and weighed by using an electronic digital balance [27].

2.9.2. Bone Length

The isolated femurs and tibia–fibula were cleaned by removing adhering tissues, and length was measured from the proximal tip of the head to the distal tip of the medial condyle by using the vernier calipers scale [28].

2.9.3. Bone Hardness

The isolated femurs and tibia–fibula were cleaned, placed in a Pfizer hardness tester, and pressure was applied until the bone broke. The breaking strength is considered the bone hardness, and the readings were obtained in Newtons (N) [29].

2.9.4. Vertebral Hardness

The fourth lumbar vertebra and eighth lumbar vertebra were spotted, isolated, and placed in a Pfizer hardness compression tester, and the readings were obtained in Newtons [29].

2.9.5. Bone Mineral Density

The bones were cleaned and freed of soft tissue and then frozen for further analysis of bone mineral density. Before examination, the bones were defrosted for approximately 30 min followed by analysis using dual-energy X-ray absorptiometry (Elite ACCLAM series) for a scan time of approximately 3 min, and the results obtained were evaluated accordingly [30,31].

2.9.6. Bone Ash Content

The bones were cleaned, and adhering soft tissue was removed for the estimation of bone mineral content. They were kept in a muffle furnace at 600 °C for 24 h. The collected

bone ash was weighed with the help of a digital balance, further diluted with 6 N HCl, and assayed for calcium, magnesium, and phosphorus content by calorimetric estimation using the respective diagnostic kits [30].

2.10. Estimation of 25-OH-D_3 by LC-MS/MS

The concentration of 25-OH-D_3 in the serum was quantified using the most reliable LC-MS/MS method. The extraction of 25-OH-D_3 from the serum samples was obtained by simple liquid-liquid extraction. Initially, 200 µL of serum sample was mixed with 25 µL of internal standard in 200 µL of methanol and equilibrated for 5 min. Then, 200 µL of methanol was added, vortexed for 10 s, and allowed to stand for precipitation. Then, 2 mL of n-heptane was added and vortexed for 10 min. The samples were centrifuged at 10,000 rpm for 10 min to separate the organic layer. The organic layer was transferred into clean tubes and evaporated to dryness under nitrogen at room temperature. The prepared samples were reconstituted in 100 µL of 2 mM ammonium acetate and 0.1% formic acid in 80:20 methanol/water [27,32].

The 20 µL of the sample was initially analyzed using an Agilent 1200 series HPLC instrument (Agilent Technologies, Santa Clara, CA, USA) with C18 (250 mm × 4.6 mm i.d) column maintained at ambient temperature (30 °C) coupled to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Q-TOF-LC/MS 6510 classic G6150A, Agilent Technologies, Santa Clara, CA, USA) with a dual ESI as an ion source. The data acquisition was performed under the control of Mass Hunter workstation software. Mobile phase A: 2 mM ammonium acetate, 0.1% formic acid in water, B: 2 mM ammonium acetate, and 0.1% formic acid in methanol was filtered with a 0.45 µm nylon membrane and pumped with a flow rate of approximately 10 mL/min. The standard calibration curve of 25-OH-D_3 was plotted by selected serial dilutions of 5, 10, 15, 20, and 25 ng/mL. The standard 25-OH-D_3 peak was identified and quantified by comparing the selected fragment ion (m/z 383.5 → 401.3).

2.11. Histopathology

Bone specimens collected from various treatment groups were fixed in 10% formalin followed by further decalcification and dehydration with ascending grade alcohol, washed with distilled water, and processed for paraffin sectioning. Finally, the sections were stained with hematoxylin and eosin for evaluation. The prepared sections were focused under a fluorescence microscope (Olympus Private Limited, Miami, FL, USA) for histopathological changes [33].

2.12. Statistical Analysis

Data were expressed as the mean ± standard deviation (SD) of six observations and evaluated by using one-way and/or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test by using GraphPad Prism software version 8 (GraphPad software, Inc., La Jola, CA, USA). The confidence level of $p < 0.05$ was statistically significant.

3. Results

3.1. Effect of Therapeutic Interventions on Body Weight (gm)

A significant ($p < 0.05$) decrease in the body weight of animals treated with a vitamin-D-deficient diet (group II) was noticed at regular intervals compared with normal animals (group I). In the case of animals treated with chrysin and in combination with $CaCO_3$ and/or vitamin D_3 (groups III, IV, V, and VII), a significant ($p < 0.05$) increase in body weight was shown when compared with the diet control group, indicating a positive impact on improving vitamin D deficiency. The overall effect of drug treatments on body weight is summarized in Table 1.

Table 1. Effect of therapeutic interventions on body weight, serum calcium, phosphorus, magnesium, and alkaline phosphatases (ALP) levels.

Parameters	Body Weight (gm)		Calcium (mg/dL)		Phosphorus (mg/dL)		Magnesium (mg/dL)		ALP (mg/dL)	
Days	**7th**	**28th**	**7th**	**28th**	**7th**	**28th**	**7th**	**28th**	**7th**	**28th**
Group I	167.9 ± 0.91	166.6 ± 2.22	7.24 ± 0.38	9.83 ± 0.55	11.19 ± 1.00	11.53 ± 0.58	1.87 ± 0.28	1.76 ± 0.28	17.04 ± 0.91	17.52 ± 1.88
Group II	162.4 ± 1.24 #	155 ± 1.92 #	7.24 ± 0.39	9.60 ± 0.68	9.024 ± 0.98 #	9.12 ± 0.61 #	1.34 ± 0.41	1.16 ± 0.27 #	19.26 ± 0.63 #	29.63 ± 1.74 #
Group III	156.66 ± 2.1 *	162.5 ± 0.96 *	7.29 ± 0.47 *	11.12 ± 0.34 *	9.98 ± 0.66	10.2 ± 0.52 *	1.66 ± 0.32	1.98 ± 0.18 *	17.45 ± 0.58 *	15.31 ± 1.45 *
Group IV	161.6 ± 1.04	156.66 ± 1.24	7.50 ± 0.85 *	11.63 ± 1.14 *	10.08 ± 0.96 *	11.81 ± 0.78 *	2.06 ± 0.25 *	2.18 ± 0.35 *	17.83 ± 0.84	16.51 ± 1.53 *
Group V	173.3 ± 0.96 *	165 ± 2.35 *	7.69 ± 0.84 *	11.56 ± 0.51 *	9.84 ± 0.72 *	12.08 ± 0.85 *	1.67 ± 0.35 *	2.01 ± 0.24 *	17.92 ± 0.12	15.15 ± 1.30 *
Group VI	162.85 ± 2.51	171.6 ± 2.63 *	7.55 ± 0.32 *	12.53 ± 0.43 *	10.95 ± 0.83 *	12.04 ± 0.83 *	1.95 ± 0.41 *	2.08 ± 0.28 *	17.63 ± 0.49	13.50 ± 1.37 *

Note: Data are expressed as the mean ± SD (n = 6), analyzed by two-way ANOVA followed by Tukey's multiple comparison test. # = $p < 0.05$ is considered statistically significant when comparing group, I (normal) vs. group II (diet control group). * = $p < 0.05$ is considered statistically significant when compared with the other treatment groups (group III, IV, V, and VI) vs. diet control group.

3.2. Effect of Therapeutic Interventions on Serum Calcium, Phosphorus, Magnesium, and ALP Levels

The research study revealed that there was no significant effect on serum calcium levels of disease control animals (group II) in normal animals, which resembles the diet supplied, which does not modulate calcium levels. There was a significant decrease in the serum phosphorus and magnesium levels among DC animals compared with normal animals, which particularly demonstrates the significance of vitamin D_3 in mineral homeostasis. Other treatment groups, such as III, IV, V, and VI, showed a significant ($p < 0.05$) increase in serum calcium, phosphorus, and magnesium at regular intervals compared with the vitamin-D-deficient diet control group. This finding suggests a definite effect of chrysin in vitamin-D-deficient conditions and is illustrated in Table 1. Moreover, a significant ($p < 0.05$) increase in the serum ALP levels was noticed among animals in the group that received a vitamin-D-deficient diet compared with group I, which indicates bone deterioration. However, a significant ($p < 0.05$) decrease in the serum ALP levels was noticed in groups III, IV, V, and VI when compared with group II animals fed a vitamin-D-deficient diet, which revealed decreased bone damage in contrast with the disease control (DC) animals (Table 1).

3.3. Effect of Therapeutic Interventions on Urinary Calcium, Magnesium, and Phosphorus Levels

The study showed a significant ($p < 0.05$) surge in the excretion of urinary calcium, magnesium, and phosphorus levels in vitamin-D-deficient, diet-treated animals when compared with normal animals. This hinted toward the increased excretion of calcium, magnesium, and phosphorus, which coincides with the decreased availability in the serum. A significant ($p < 0.05$) decline in the excretion of urinary parameters was found in the groups treated with chrysin along with other treatment combinations, i.e., groups III, IV, V, and VI, when compared with the vitamin-D-deficient diet groups (Table 2).

Table 2. Effect of therapeutic interventions on urinary calcium, magnesium, and phosphorus levels.

Parameters	Calcium (mg/dL)		Phosphorus (mg/dL)		Magnesium (mg/dL)	
Days	7th	28th	7th	28th	7th	28th
Group I	6.68 ± 0.49	6.98 ± 0.40	1.21 ± 0.02	2.91 ± 0.22	1.4 ± 0.06	2.45 ± 0.022
Group II	7.73 ± 0.59	10.40 ± 0.38 #	1.03 ± 0.021	4.61 ± 0.19 #	1.98 ± 0.098 #	4.88 ± 0.98 #
Group III	6.88 ± 0.30 *	6.05 ± 0.22 *	0.88 ± 0.01	2.31 ± 0.12 *	0.92 ± 0.021 *	2.94 ± 1.20 *
Group IV	6.76 ± 0.73 *	5.11 ± 0.42 *	1.63 ± 0.09	1.97 ± 0.12 *	1.02 ± 0.065 *	1.99 ± 0.06 *
Group V	6.165 ± 0.67 *	5.00 ± 0.41 *	1.92 ± 0.06	2.01 ± 0.11 *	1.24 ± 0.08	2.42 ± 0.071 *
Group VI	6.66 ± 0.39 *	4.6 ± 0.23 *	1.52 ± 0.087	2.55 ± 0.24 *	1.11 ± 0.056 *	3.01 ± 0.06 *

Note: Data are expressed as the mean ± SD (n = 6), analyzed by two-way ANOVA followed by Tukey's multiple comparison test. # = $p < 0.05$ is considered statistically significant when comparing group, I vs. group II. * = $p < 0.05$ is considered statistically significant when compared with the other treatment groups (group III, IV, V, and VI) vs. diet control group.

3.4. Effect of Therapeutic Interventions on Bone Ash Parameters Such as Ash Weight, Calcium, Phosphorus, and Magnesium

Among all the bone ash parameters, the disease control (group II) showed a significant ($p < 0.05$) decrease in ash weight and calcium levels, which indicates a decrease in bone calcification and bone formation. No significant ($p < 0.05$) difference in phosphorus and magnesium levels in bone ash samples of the vitamin-D-deficient group was noticed when compared with normal groups. However, the animals treated with different therapeutic interventions, such as groups III, IV, V, and VI, showed a significant ($p < 0.05$) increase in bone ash weight and calcium. This indicates increased bone calcification and strong bone formation. A significant increase in the phosphorus levels of bone ash was noticed only in group 4 (Chrysin + $CaCO_3$) and group 6 (Chrysin + $CaCO_3$ + Vitamin D_3) when compared with the vitamin-D-deficient group. The levels of magnesium were not significantly altered between any of the groups (Table 3).

Table 3. Effect of drug treatment groups on bone ash content (ash weight, calcium, phosphorus, and magnesium levels) and femur bone mineral density (BMD).

Parameters	Bone Ash Content				BMD
	Ash Weight (g)	Calcium (mg/dL)	Phosphorus (mg/dL)	Magnesium (mg/dL)	Femoral Bone Mineral Density (g/cm^2)
Group I	0.79 ± 0.64	17.40 ± 1.01	9.29 ± 0.48	5.05 ± 0.36	0.158 ± 0.007
Group II	0.66 ± 0.80 #	15.16 ± 0.80 #	9.23 ± 0.44	4.98 ± 0.17	0.140 ± 0.0015 #
Group III	0.82 ± 0.69 *	18.20 ± 0.96 *	9.85 ± 0.75	5.21 ± 0.18	0.154 ± 0.011 *
Group IV	0.95 ± 1.43 *	19.38 ± 0.66 *	11.34 ± 1.09 *	5.21 ± 0.18	0.161 ± 0.008 *
Group V	0.86 ± 1.50 *	17.45 ± 0.72 *	10.46 ± 1.11 *	5.10 ± 0.32	0.169 ± 0.0095 *
Group VI	0.98 ± 0.99 *	18.59 ± 0.86 *	11.34 ± 1.06 *	5.13 ± 0.41	0.186 ± 0.003 *

Note: Data were expressed as the mean ± SD (n = 6), analyzed by one-way ANOVA followed by Tukey's multiple comparison test. # = $p < 0.05$ is considered statistically significant when comparing group, I vs. group II. * = $p < 0.05$ is considered statistically significant when compared with the other treatment groups (group III, IV, V, and VI) vs. diet control group.

3.5. Effect of Therapeutic Interventions on The Femur and Tibia–Fibula Weights, Lengths, and Hardness

The study showed that there was a significant ($p < 0.05$) decrease in the femur and tibia–fibula weights and hardness in DC animals when compared with normal animals, representing decreased bone strength, which may increase the risk of osteoporotic-related fractures. In contrast, animals treated with chrysin and in combination with $CaCO_3$ and/or vitamin D_3 showed a significant ($p < 0.05$) increase in the femur and tibia–fibula weights and hardness, demonstrating increased bone strength. However, no significant ($p < 0.05$) difference was found in the lengths of the femur and tibia–fibula in any of the groups studied (Table 4).

Table 4. Effect of therapeutic interventions on femur and tibia–fibula weights, lengths, and hardness.

Parameters	Femur			Tibia-Fibula		
	Length (cm)	Weight (gm)	Hardness (N)	Length (cm)	Weight (gm)	Hardness (N)
Group I	2.93 ± 0.10	0.74 ± 0.06	9.8 ± 0.21	3.27 ± 0.23	0.72 ± 0.036	14.17 ± 2.44
Group II	2.93 ± 0.19	0.61 ± 0.08 #	8.94 ± 0.12 #	3.23 ± 0.27	0.55 ± 0.07 #	6.98 ± 1.91 #
Group III	3.02 ± 0.10	0.74 ± 0.03 *	9.93 ± 0.24 *	3.55 ± 0.27	0.66 ± 0.035 *	11.91 ± 2.10 *
Group IV	3.08 ± 0.12	0.74 ± 0.06 *	10.43 ± 0.83 *	3.60 ± 0.21	0.74 ± 0.066 *	13.86 ± 1.52 *
Group V	3.03 ± 0.15	0.79 ± 0.05 *	10.02 ± 0.41 *	3.57 ± 0.38	0.75 ± 0.096 *	14.39 ± 2.213 *
Group VI	3.13 ± 0.12	0.88 ± 0.06 *	11.76 ± 0.67 *	3.65 ± 0.33	0.84 ± 0.016 *	15.60 ± 2.70 *

Note: Data were expressed as the mean ± SD ($n = 6$), analyzed by one-way ANOVA followed by Tukey's multiple comparison test. # = $p < 0.05$ is considered statistically significant when comparing group, I vs. group II. * = $p < 0.05$ is considered statistically significant when compared with the other treatment groups (group III, IV, V, and VI) vs. diet control group.

3.6. Effect of Therapeutic Interventions on 4th Lumbar Hardness and 8th Thoracic Hardness

The study showed that there was a significant decrease ($p < 0.05$) in the 4th lumbar hardness and 8th thoracic hardness among group II animals compared with group I, indicating decreased bone mass or strength due to vitamin D insufficiency. However, the animals treated with other therapeutic interventions showed a significant ($p < 0.05$) increase in the 4th lumbar hardness and 8th thoracic hardness compared with animals fed a deficient diet (Table 5).

Table 5. Effect of therapeutic interventions on 4th lumbar vertebrae hardness and 8th thoracic vertebrae hardness.

Groups	4th Lumbar Hardness (N)	8th Thoracic Hardness (N)
Group I	14.64 ± 0.96	15.66 ± 1.64
Group II	10.66 ± 0.71 #	10.21 ± 1.45 #
Group III	12.96 ± 0.85 *	14.26 ± 1.21 *
Group IV	15.20 ± 1.01 *	14.94 ± 1.99 *
Group V	14.96 ± 1.86 *	15.21 ± 0.98 *
Group VI	15.62 ± 1.59 *	16.04 ± 1.29 *

Note: Data were expressed as the mean ± SD ($n = 6$), analyzed by one-way ANOVA followed by Tukey's multiple comparison test. # = $p < 0.05$ is considered statistically significant when comparing group, I vs. group II. * = $p < 0.05$ is considered statistically significant when compared with the other treatment groups (group III, IV, V, and VI) vs. diet control group.

3.7. Effect of Therapeutic Interventions on Femur Bone Mineral Density

There was a significant ($p < 0.05$) decline in the BMD of animals fed DC compared with normal animals. However, the animals treated with other therapeutic interventions (groups III, IV, V, and VI) showed a significant ($p < 0.05$) increase in bone mineral density compared with animals fed a vitamin-D-deficient diet (Table 3). Among all the studied interventions, group VI (chrysin + $CaCO_3$ + vitamin D_3) showed better improvement in all parameters (serum, urinary, and bone).

3.8. Effect of Therapeutic Interventions on 25-OH-D_3 by LC-MS/MS

A significant ($p < 0.05$) decrease in serum 25-OH-D_3 levels of animals treated with a vitamin-D-deficient diet was noticed compared with normal animals, which correlated with the altered biochemical and bone parameters. However, comparatively, there was a significant ($p < 0.05$) increase in 25-OH-D_3 levels in serum samples of animals treated with other therapeutic interventions, i.e., III, IV, V, and VI, in contrast with group II animals (Figure 2).

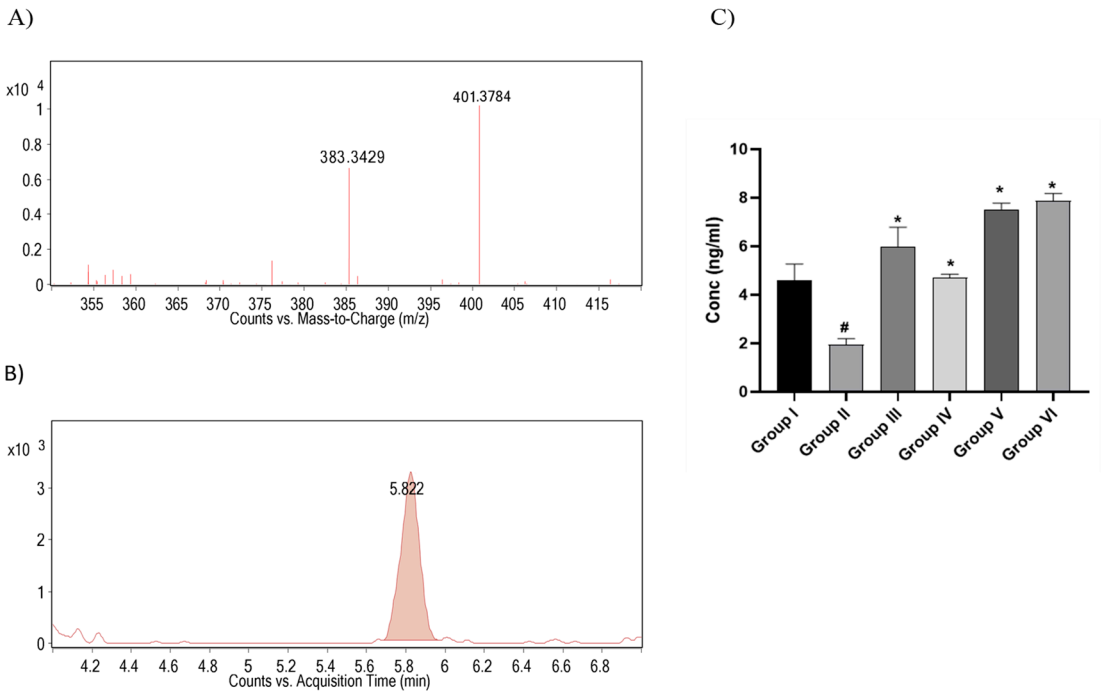

Figure 2. Quantification of 25-OH-D_3 levels by LC-MS/MS. (**A**) Mass fragmentation of 25-OH-D_3. (**B**) Ion chromatogram of the selected fragment ion (m/z 383.5 → 401.3). (**C**) Quantification of serum 25-OH-D_3 from the area under the curve (data are expressed as the mean ± SD (n = 4), analyzed by one-way ANOVA followed by Tukey's multiple comparison test. # = $p < 0.05$ is considered statistically significant when comparing group, I vs. group II. * = $p < 0.05$ is considered statistically significant when compared with the other treatment groups (group III, IV, V, and VI) vs. diet control group).

3.9. Effect of Therapeutic Interventions on Histopathology of the Femur Bone

The effect of therapeutic interventions on the histopathology of the femur bone was studied at 10× magnification, a detailed description was mentioned, and photomicrographs are given in Figure 3. The photomicrographs of group I (normal) showed the normal distribution of cells in the epiphyseal plate and the even distribution of osteocytes in the bone matrix. However, there was a decreased incidence of differentiating osteocytes and bone formation cells in the epiphyseal region among the animals fed group II (vitamin-D-deficient diet). Osteoclasts were also prominently noticed in the DC animals, which supports the process of bone resorption. However, the animals treated with group III (chrysin) showed a number of osteocytes at the epiphyseal plate, and bone formation cells, such as osteoblasts, were observed. In group III, few osteoclasts were also observed, but not as much as in the vitamin-D-deficient control group.

In group IV (chrysin + $CaCO_3$), increased proliferation of cells in the epiphyseal plate was observed compared with the diet control group, followed by subsequent calcification. In group V (chrysin + vitamin D_3), a considerable even distribution of osteocytes and matrix formation was observed, which indicated active bone growth. Moreover, epiphyseal plate architecture was observed to be better than the diet control group. In group VI (chrysin + $CaCO_3$+ vitamin D_3), increased bone formation due to increased osteoblast cells in the epiphyseal plate of the bone and comparatively fewer resorptive cells in contrast with the diet control group were noticed.

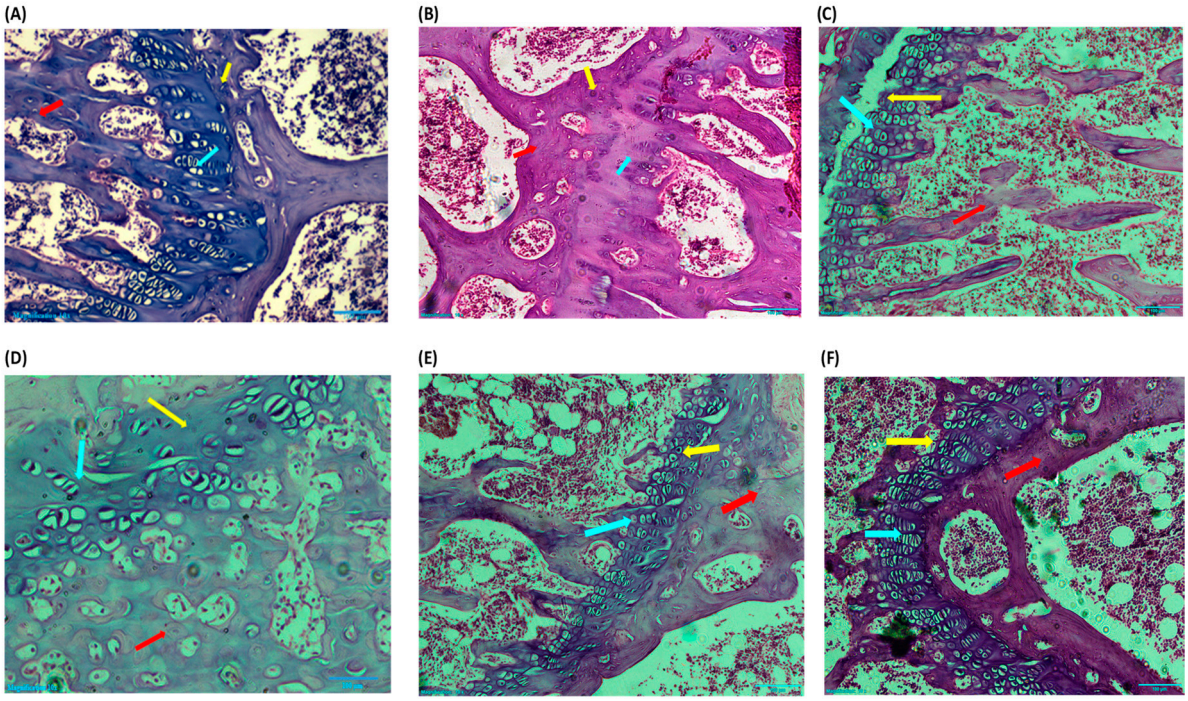

Figure 3. Impact of chrysin on histopathology of bone. (**A**) Group I (normal group); (**B**) Group II (disease control); (**C**) Group III (chrysin); (**D**) Group IV (chrysin + $CaCO_3$); (**E**) Group V (chrysin + vitamin D_3); and (**F**) Group VI (chrysin + vitamin D_3 + $CaCO_3$) showing alterations in normally growing cells (blue arrow) at the epiphyseal plate (yellow arrow) along with osteocytes (red arrow) in the bone matrix.

4. Discussion

Vitamin D has gained prominent value as a nutrient supplement from diverse sources and as an endogenous hormone biosynthesized from cholesterol by UV irradiation. Over the last few decades, the sunshine vitamin has gained optimistic importance in various pathological conditions in addition to former osseous effects [6]. The evidence stated that appropriate levels of serum 25-OH-D_3 have specified their essential role in the homeostasis of essential nutrients such as calcium, magnesium, and phosphorus, which accentuates bone mass and mineralization. Several studies reported that alterations in these mineral nutrients would result in elevated bone turnover, most significantly in vitamin-D-deficient conditions [34]. As females are more prone to osteoporotic fractures and bone loss, female rats were preferred for the current study [35].

In our study, an eminent rise in the serum calcium, magnesium, and phosphorus levels was obtained among animals treated with different therapeutic interventions, suggesting their increased availability accompanied by the decline in urinary excretion, by which it was noticed that examined combinations of chrysin with $CaCO_3$ and/or vitamin D_3 showed potential interactions, which may also have a further impact on bone mineralization. Similar studies are available that support alterations in the serum and urinary levels of calcium with a vitamin-D-deficient diet and therapeutic interventions [36]. However, many more studies are warranted to conclude that this progress is dependent on decreased urinary excretion or may be due to increased absorption or decreased metabolism. In contrast, the animals fed a vitamin-D-deficient diet (group II) did not show any variance in calcium levels compared with normal animals, which suggests that the diet provided does not have any modulatory effect on calcium levels among DC animals. Nevertheless, diminished serum magnesium and phosphorus levels among vitamin-D-deficient animals assure the importance of vitamin D in maintaining appropriate mineral homeostasis. In the same vein, DC animals showed enhanced excretion of urinary calcium, phosphorus, and magnesium,

which further supports the importance of vitamin D in the renal excretion of essential minerals [37,38].

In this context, intensified serum ALP levels were noticed among group II animals, suggesting the induction of poor bone health, which also indicates the dynamic imbalance of bone tissue promoting bone resorption, which is consistent with previous reports [39]. Furthermore, a consistent decline in the serum ALP levels was obtained in animals treated with other therapeutic groups (groups III, IV, V, and VI), indicating considerable recovery of the disease condition.

Other supportive data obtained from the study presented a positive correlation between body weights and bone parameters such as weight, hardness of tibia–fibula, and the femur of the animals with the course of chrysin treatment retrieving bone mass and the importance of vitamin D in bone mineral absorption. In contrast, group II animals mitigated this effect, which resembles the results obtained from earlier studies indicating that increased body weight is proportional to bone weight [40,41]. No changes in the lengths of the femur bone and tibia–fibula bone were observed.

In addition, the diminished availability of serum biochemical estimations of essential minerals such as calcium, magnesium, and phosphorus witnessed in animals with insufficient vitamin D (group II) can also be analogous to the decreased hardness in the 4th lumbar and 8th thoracic vertebrae, suggesting a deterioration in bone mass that corroborates previous studies. Comparatively, other therapeutic groups showed amplified bone strength concerning increased 4th lumbar and 8th thoracic vertebral hardness. A comparable effect in bone strength was observed with increased serum calcium, magnesium, and phosphorus in earlier reports [42].

Previous research revealed the imperative link between bone mineralization and bone ash content in bone-related diseases. In this regard, the vitamin-D-deficient diet control group showed decreased bone ash content, i.e., calcium, phosphorus, and magnesium, which confirms decreased bone mineral deposits [43]. Nevertheless, all the treatment groups showed enhanced calcium content in bone ash, which can be attributed to the increased serum calcium levels. None of the therapeutic interventions caused any increase in magnesium deposits in the bones. However, chrysin, when given in combination with vitamin D_3 and $CaCO_3$, caused a prominent increase in the phosphorus content in bone ash. The role of $CaCO_3$ and vitamin D in increasing bone mineralization has been established in earlier studies [8].

Femur bone mineral density (BMD) is one of the gold standard tests for the determination of the mineral strength of bone and was assessed by performing DEXA [30]. The animals treated with a vitamin-D-deficient diet showed decreased bone mineral density compared with the normal group, which indicates decreased bone calcification, mineralization, and bone mass. Significantly, animals in different treatment groups showed intense bone mineral density, which supports the results obtained from biochemical and bone ash estimations [30]. To estimate the inhibitory effect of chrysin on vitamin D metabolism and its effect on low-dose vitamin D_3 (40 IU) supplementation, the estimation of serum 25-OH-D_3 levels was considered a reliable marker for vitamin D status [44]. From our experimental analysis, the diet control group showed decreased serum 25-OH-D_3. In contrast, increased serum 25-OH-D_3 levels were obtained in the animals treated with different therapeutic interventions, reinforcing its physiological effect on bone associated with the serum biochemical parameters discussed above. Chrysin, when given alone, effectively elevated 25-OH-D_3 levels, but the combination of chrysin with $CaCO_3$ and vitamin D_3 has shown a major impact in increasing the serum 25-OH-D_3 levels, which partly confirms the metabolic interactions of chrysin on vitamin D catabolism, even at a low doses of vitamin D_3 (40 IU).

The histopathological data also revealed a greater number of bone-forming cells in the epiphyseal plate of the animals treated with different therapeutic interventions, which also favors the data obtained from other biochemical parameters. However, an uneven distribution of osteocytes in the bone matrix was spotted in animals along with a

smaller number of bone-forming cells in the epiphyseal plate among the diet control group (group II). Bone specimens resemble decreased bone formation and can be attributed to hypovitaminosis D, as mentioned earlier [45].

5. Conclusions

Our preclinical research results reveal that the combination of chrysin, vitamin D_3, and $CaCO_3$ showed a greater effect on bone health than all other combinations studied. The result also clearly indicates that chrysin was able to increase the availability of active serum vitamin D, even with minimal vitamin D_3 supplementation (40 IU), but to confirm interactions at the site of metabolism, further studies are warranted. The study has opened a new option to treat vitamin D deficiency, which can be used as a cost-effective option to improve bone health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12178728/s1, Table S1: The composition of the vitamin D deficient diet as per the reference [23].

Author Contributions: Conceptualization, S.D. (Sujatha Dodoala); data curation, N.K.T.; formal analysis, S.S.K.; investigation, S.S.K., S.S. and N.K.T.; methodology, S.S.; project administration, S.D. (Sujatha Dodoala); supervision, S.D. (Sujatha Dodoala); visualization, V.J. and S.D. (Sathish Dyawanapelly); writing—original draft, S.S.K.; writing—review and editing, S.D. (Sujatha Dodoala), V.J. and S.D. (Sathish Dyawanapelly). All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data available within in the article. Additional data is available from S.D. (Sujatha Dodoala) upon reasonable request.

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