

Evaluating the Osteogenic potential of *S. officinale* against Glucocorticoid-induced Osteoporosis using in vivo model

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Abstract

Glucocorticoid-induced osteoporosis is characterized by impaired bone formation, increased bone resorption and disruption of bone mineralization. The present study investigated the osteoprotective potential of *Symphytum officinale* (*S. officinale*) aqueous root extract using a dexamethasone-induced osteoporosis model in adult zebrafish (*Danio rerio*). Phytochemical screening and LC–MS analysis were performed to characterize the extract, followed by developmental toxicity assessment. Adult zebrafish were divided into control, dexamethasone-treated and low- and high-dose *S. officinale*-treated groups. Bone regeneration and mineralization were evaluated using histopathology, Alizarin Red staining, scale morphometry and ICP-MS analysis of calcium and phosphorus. Hydroxyproline, alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) assays were performed to assess collagen content and bone remodelling. The extract showed the presence of alkaloids, terpenoids, phytosterols and other bioactive constituents, including quercetin derivatives and biochanin A, while no developmental toxicity was observed at the tested concentrations. Dexamethasone induced marked alterations in tissue architecture, reduced mineral deposition and scale size and disrupted calcium and phosphorus levels. *S. officinale* treatment improved skeletal morphology, mineralization, scale size and mineral content, with pronounced effects on several parameters at the higher dose. The extract also modulated hydroxyproline, ALP and TRAP activities, indicating concentration-dependent effects on bone remodelling. Hence, *S. officinale* demonstrates osteoprotective potential against dexamethasone-induced skeletal alterations in zebrafish.

Introduction

Osteoporosis is classified as bone disorder characterized by reduced bone mass, deterioration of bone microarchitecture, and increased risk to fractures [1]. It is regarded as a significant public health concern, especially among the elderly population [2]. Bone homeostasis is sustained by a complex equilibrium between bone formation, carried out by osteoblasts, and bone resorption, performed by osteoclasts. The imbalance in bone remodelling, characterized by excessive formation driven by osteoclasts and resorption mediated by osteoclasts, results in significant skeletal fragility. Osteoporosis currently impacts more than 200 million individuals globally, with projections indicating approximately 61 million cases in India by the year 2026 [3]. Secondary osteoporosis is common among people who take glucocorticoids, which are commonly used to treat and manage a variety of autoimmune, inflammatory, and immune-mediated conditions [4]. Dexamethasone, a synthetic glucocorticoid, is commonly used as an experimental agent to induce bone loss in both animal and cellular models of osteoporosis. Its pathological effects result from the suppression of osteoblast proliferation and differentiation, along with the stimulation of osteoclast mediated bone resorption. Increased systemic glucocorticoid levels, whether from endogenous overproduction or from prolonged pharmacological use, are closely linked to osteoporosis, decreased bone mineral density, and an elevated risk of fractures [4]. In clinical settings, Dexamethasone is provided via oral tablets, intravenous injections, or inhalation formulations to treat conditions including autoimmune disorders, inflammatory disorders and specific cancers. While it offers therapeutic benefits, prolonged or high dose use markedly elevates the risk of glucocorticoid induced

osteoporosis, establishing it as a recognized model compound for investigating skeletal fragility. Current pharmacological approaches for the prevention of osteoporosis such as bisphosphonates, hormone replacement therapy, and monoclonal antibodies, demonstrate efficacy in decreasing fracture risk and enhancing bone strength. Nevertheless, these treatments are not devoid of drawbacks. Prolonged use has been linked to negative effects, including atypical femoral fractures (AFF), osteonecrosis of the jaw (ONJ), and complications related to the gastrointestinal or cardiovascular systems, highlighting the necessity for safer and more sustainable therapeutic options [5]. Natural alternatives are gaining recognition for their ability to maintain bone mineral density and promote regeneration without negative effects linked to conventional method of treatments. Comfrey (*Symphytum officinale*), a well - known medicinal herb, has been extensively utilized in the management of fractures, inflammatory joint conditions, and wound healing. In addition to its ethnopharmacological applications, phytocompounds derived from plants, including flavonoids, polyphenols and terpenoids, exhibit considerable potential for osteoblast differentiation, inhibiting osteoclast mediated resorption, modulating essential signalling pathways and reducing oxidative stress [6]. While *S. officinale* has been traditionally employed for centuries in the healing of fractures and the repair of bones, the scientific evidence supporting its protective effects remains complex. Recently, the zebra fish has gained recognition as an exceptionally valuable vertebrate model, owing to its genetic resemblance to humans, its transparent skeletal development, and its rapid regenerative abilities, which render it particularly effective for studying metabolic bone disorders and regenerative processes [7]. The model will facilitate the investigation of glucocorticoid-induced osteoporosis and potential therapeutic interventions that influence bone mineralization and skeletal architecture [8]. The present study aims to elucidate the osteoprotective effects of *S. officinale* extract on a dexamethasone-induced osteoporotic model utilizing adult zebrafish. This investigation aspires to provide experimental evidence supporting *S. officinale* as an effective therapy for glucocorticoid-induced osteoporosis.

Materials & Methods

Materials

Dried roots of *S. officinale* was purchased from Sodium bicarbonate (NaHCO_3), Chloroform, Sulphuric acid, Copper acetate solution, Nitric acid, Methanol, Formic acid, Acetonitrile, Sodium hydroxide (NaOH), 4% Paraformaldehyde (PFA), Dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT reagent), Haematoxylin and Eosin stains are purchased from Sisco research laboratory (SRL).

Methods

Preparation of Aqueous Extract

The dried roots of *S. officinale* were purchased from Nishtha exports and used for the extraction of the aqueous extract [9]. Initially, the dried roots were ground finely and weighed to an amount of 10g. The weighed powder was added to 100ml of distilled water in a conical flask. This was done to make sure there is a ratio of 1:10 (w/v) in the material added to the solvent. Heat extraction method was used and the temperature maintained below 50°C. The process of heat extraction was performed for 30 minutes, after which the extract was cooled to room temperature. After the extraction, the resulting mixture was left to cool at room temperature. Then, the mixture was poured in falcon tubes for centrifuging for 20 minutes at 7200 rpm [10]. The resulting supernatant was collected and placed in Petri dishes and finally freeze-dried and lyophilized to make a crude extract of the sample [11].

Phytochemical analysis

The produced *S. officinale* root extract were analysed to find the presence of various phytochemicals through qualitative analysis [9]. The crude extract was subjected to various test to identify the presence and absence of phytochemical constituents.

Alkaloids

Mayer's Test

A volume of 2 to 3mL of the sample was introduced, subsequently followed by the addition to 1 to 2 drops of Mayer's reagent. The appearance of a white or yellow precipitate indicated the presence of alkaloids [12] .

Dragendroff's test

A sample volume of 2 to 3mL was taken, followed by the addition of 1 to 2mL of Dragendroff's reagent. The appearance of brown precipitate indicated the presence of alkaloids [12].

Reducing sugars

Benedict's test

0.5mL of the sample was mixed with 0.5mL of Benedict's reagent, and the resulting mixture was mixed thoroughly. The solution was subsequently heated in a water bath for a duration of 2 to 5 minutes. A colour change to green, yellow, or red signified the presence of reducing sugars [13].

Fehling's test

1mL of sample was mixed with 1mL each of Fehling's A and Fehling's B reagents. The resulting mixture was heated in a water bath for a duration of 2 to 5 minutes. The appearance of a red precipitate

indicated the presence of reducing sugar [13].

Saponins

Foam Test

1mL of the sample was mixed with 2mL of water and thoroughly shaken. The resulting mixture was kept in rest for 1 to 2 minutes. The appearance of the foam indicated the presence of saponins [14].

Sodium bicarbonate (NaHCO_3) Foam test:

1mL of the sample was mixed with 1mL of saturated sodium bicarbonate. the observation of bubbles/effervescence confirmed the presence of saponins [14].

Phytosterols

Salkowski's test (Hesse's variation)

3mL of chloroform was mixed with 3mL of the sample and shaken well. Following this, 3mL of sulphuric acid was added slowly, and the mixture was permitted to rest. The formation of a pink ring or red colour in the chloroform layer confirmed the existence of phytosterols [15].

Salkowski's test (simple)

A single drop of concentrated sulphuric acid (H_2SO_4) was introduced to 1mL of the sample and permitted to rest. The appearance of a red colour in the acid layer verified the presence of phytosterol [15].

Fats and Oils

A volume of 1mL of the sample was placed on a filter paper and subsequently pressed with another filter paper. The appearance of oily or greasy marks on the filter paper indicated the presence of fats and oils [9].

Triterpenoids

Salkowski's test

6 to 7 drops of concentrated H_2SO_4 were added to 1mL of the sample in a test tube. The mixture was well shaken and left to rest for 2 to 3 minutes. The development of a golden colour in the lower acid layer confirmed the presence of terpenoids [15].

Diterpenoids

Copper acetate test

7 to 8 drops of copper acetate solution was added to 1 mL of the sample within a test tube. The resulting solution was allowed to rest for 3 to 5 minutes. A colour change to emerald green indicated the presence of diterpenoids [16].

Anthocyanins

Ammonia shift test

Few drops of ammonia (NH₃) solution were added to 1 ml of the sample. The change in colour from pinkish red to blue-violet confirmed the presence of anthocyanins [17].

Carboxylic Acids

Sodium bicarbonate (NaHCO₃) effervescence test

A volume of 1 mL was mixed with 3 mL of sodium bicarbonate (NaHCO₃) solution. The emergence of bubbles or effervescence, resulting from the liberation of carbon dioxide (CO₂) gas, validated the existence of carboxylic acids within the samples [9].

Proteins & Amino Acids

Xanthoproteic test

5 to 7 drops of concentrated nitric acid (HNO₃) were added to 1 mL of the sample and heated gently until a colour change is observed. The appearance of a yellow colour, (intensifies to orange on excess acid) confirmed the presence of amino acids [18].

Liquid Chromatography–Mass Spectrometry

The crude extract of *S. officinale* was used for LC-MS analysis. The crude extract was dissolved in methanol (LC-MS grade), sonicated, and centrifuged to precipitate the insoluble fractions in the sample. The filtrate was further diluted to the required concentration for analysis after being passed through a 0.22 µm filter [19]. UHPLC (ultra-high-performance liquid chromatography) and MS systems were used to profile the extracts. The compounds were separated using a reverse phase C18 column using two solvents including; solvent A consisting of water and 0.1% formic acid & solvent B having acetonitrile and 0.1% formic acid. A linear gradient elution was conducted with flow rate set at 0.3 ml/min. Mass spectrometric data acquisition was done in positive and negative ESI modes ranging between m/z of 50 and 1200 [20]

Animal housing maintenance

The studies were performed using standard IAEC (Institutional Animal Ethical Committee) protocol. Adult male zebrafish were selected to exclude any biasing variables arising due to sex-dependent

differences in hormone regulation [21]. The fish were acclimatized to the experimental environment for three days prior to the experiments [22]. Zebrafish were kept in an aquarium equipped with a recirculatory water system at temperatures ranging from $28 \pm 1^\circ\text{C}$ and pH between 7.0 and 7.5, with adequate concentrations of dissolved oxygen [23]. Aquariums were cleaned after every 48 hours by changing part of the water and eliminating debris. A constant amount of food was administered once daily, and excess food was discarded within 10–15 minutes [24].

Developmental Toxicity Assessment

Healthy embryos and larvae of zebrafish were obtained and incubated in laboratory settings [24]. Embryos were maintained under optimum temperature and lighting conditions throughout the experiment [23]. Aqueous extract concentrations of *S. officinale* were prepared using different concentrations of 1, 3, 6, 12, 25, 50, and 100 $\mu\text{g/mL}$, as well as a control treatment. Embryos were then randomly allocated into different experimental groups, where they were exposed to specific concentrations of extract. The duration of exposure was four continuous days (Days 1–4) [25]. Developmental toxicity testing was conducted by evaluating several factors of the embryo on a daily basis. These include mortality rate, hatching rate, heartbeats, length of body size, and general development progress [26]. All data acquired through observations were documented and analysed each day in comparison to the control group to examine any toxic effects at various concentrations of extract. The study found that there was no apparent developmental toxicity of the extract; 100% hatching rate and survival rates were obtained from all concentrations tested respectively [27].

Experimental treatment grouping

The adult male Zebrafish species were assigned to four different groups, and there were five fish in each group ($n = 5$) [24][23]. This was done using random allocation in order to avoid any biases when grouping the animals according to their body size, age, and health conditions [22]. All groups were housed in the same conditions [7].

Group I – Control (CT group)

Animals of this group were not subjected to any pharmacological treatment but remained in-system water under routine husbandry practices [24]. The control group provided the baseline physiological values of bone mineralization, skeletal anatomy, and tissue histomorphology for normal adult male zebrafish that are free from osteoporosis [28].

Group II – Disease Control (DC group)

Animals in this group was subjected to dexamethasone in a dose of $3.92 \mu\text{g/mL}$ in systemic water through the method of immersion to develop a model for glucocorticoid-induced osteoporosis [29][30]. This group was used as disease control to verify the osteoporotic impacts caused by the prolonged use

of glucocorticoids[31], which include poor bone mineralization, skeletal architecture disruption, and calcium-phosphate balance disturbance[32].

Group III – Low Dose Treatment (S01 group): Animals in this group was subjected to treatment with dexamethasone at a concentration of 3.92 µg/mL together with aqueous extract of *S. officinale* at a very low concentration of 6.25 µg/ml[33]. The purpose of this group was to find out whether osteo-protection by the plant extracts was feasible even at a low dose[34].

Group IV – High Dose Treatment (S02 group): Animals in this group received concurrent administration of dexamethasone at 3.92 µg/ml alongside the aqueous extract of *S. officinale* at a high concentration of 12.5 µg/ml[35]. This group was designed to evaluate the full osteoprotective potential of the extract at an increased therapeutic dose and to assess dose-dependent restoration of skeletal integrity relative to both the disease control and low-dose treatment groups[7] [36]

Treatment Protocol

The experiment was held for around three weeks, including the acclimatization stage before euthanasia and sample collection were done[37]. Cultures of adult zebrafish were reared under laboratory settings prior to conducting the experiment. The experiment included four groups that have been mentioned earlier. The treatments in this experiment followed a cycle where on the first day, the zebrafishes from all groups apart from the control were treated with dexamethasone. In the second day, both S01 and S02 groups were treated with the aqueous extract of *S. officinale*, and the same procedure was repeated every three days. After completing the study period, the fish were euthanized by rapid chilling procedures[38][39].

Histopathology

The specimens were fixed in 10% neutral buffered formalin for 24 hours and then underwent dehydration by serial passage through grades of ethanol and clearing in xylene followed by embedding in paraffin wax[40]. Sections measuring 5µm thick were prepared using a rotary microtome and were mounted on glass slides where deparaffinization and staining of Hematoxylin and Eosin were done[41].

Alizarin Red Staining

Scales from zebrafish were fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature, then rinsed thrice in PBS before staining with 2% Alizarin Red S solution (pH 4.2) for 10 minutes to detect calcium deposits[42]. Staining excess was washed out using PBS, and the scales were then prepared on slides for microscopic examination under bright-field microscopy[42].

Collection of scales for Biochemical assays

The Zebrafish scales were removed from the mid-body area of the individual fish after euthanization. The removal was performed using fine tips of forceps under a stereo microscope to avoid causing any damage to the scales themselves[42]. The scales were placed in Eppendorf tubes with ice-cold phosphate buffered saline (PBS) and washed thrice to remove the remaining mucous cells as well as any other contaminant from the scales. After washing, the scales were dried on sterile filter papers, either used immediately in downstream experiments or stored frozen at -80°C [43].

Measurement of size of scales

Scale measurements were taken from all fish groups using a digital vernier caliper. Differences in size and shape were observed across groups. Mean values were calculated for each group and compared graphically[44].

Inductively Coupled Plasma Mass Spectrometry – ICP-MS

In analysing the elemental composition of calcium and phosphorus, samples were digested with 100 μL of 61% supra-pure nitric acid (HNO_3) solution at 100°C for 8 hours[29] to ensure total digestion of the calcium-phosphate mineral matrix, as well as the collagen and other organic matrixes[45]. After this, the digested sample was diluted with 1% HNO_3 to reach its optimal working strength, then analysed via ICP-MS for calcium and phosphorus content quantitation[46].

Results were expressed as fold change relative to the normal control group using the formula

$$\text{Ratio} = \frac{\text{Sample value}}{\text{Control value}}$$

Hydroxyproline assay

Hydroxyproline content in liquid samples was estimated using a colorimetric method[47]. The materials required included liquid sample (serum, tissue extract, culture supernatant, or other biological fluid), hydroxyproline standard, 6N hydrochloric acid for hydrolysis (if required), sodium hydroxide for neutralization, chloramine-T reagent, perchloric acid solution, Ehrlich's reagent (p-dimethylaminobenzaldehyde reagent), distilled water, test tubes, centrifuge tubes, micropipettes, centrifuge, water bath, vortex mixer, and spectrophotometer[48]. Briefly, 1 mL of the liquid sample was taken in a test tube and, if protein-bound hydroxyproline was to be measured, the sample was hydrolysed with 6N HCl at 110°C for 18–24 hours, followed by cooling, neutralization with sodium hydroxide, and filtration. Then, 1 mL of chloramine-T reagent was added and incubated at room temperature for 20 minutes for oxidation[49]. After incubation, 1 mL of perchloric acid solution was added and mixed thoroughly, followed by the addition of 1 mL of Ehrlich's reagent. The mixture was incubated in water bath

at 60°C for 20 minutes for colour development, cooled to reach room temperature, and the absorbance was measured at 557–560 nm against a reagent blank using a spectrophotometer[50]. A standard curve was prepared using known concentrations of hydroxyproline, and the hydroxyproline content of the sample was calculated accordingly.

Alkaline phosphatase (ALP) assay

ALP activity in liquid samples was estimated using a colorimetric assay based on the hydrolysis of p-nitrophenyl phosphate (pNPP)[51]. The materials required included liquid sample (serum, plasma, cell culture supernatant, or tissue extract), p-nitrophenyl phosphate substrate, alkaline buffer (glycine buffer, pH 10.4, containing magnesium chloride), sodium hydroxide solution (if stopping reagent is required), distilled water, test tubes or microplates, micropipettes, incubator/water bath, and spectrophotometer or microplate reader[52]. Briefly, an appropriate volume of liquid sample (typically 50–100 µL) was added to test tubes containing 1 mL of freshly prepared substrate solution and incubated at 37°C for 15–30 minutes. During incubation, ALP hydrolyses p-nitrophenyl phosphate to produce p-nitrophenol, resulting in a yellow colour. The reaction was stopped by adding 0.1 N sodium hydroxide, if required[53]. The absorbance was measured at 405 nm against a reagent blank using a spectrophotometer. A standard curve using p-nitrophenol or enzyme activity calculation formula was used to determine ALP activity, and the results were expressed as U/L or IU/L depending on the sample type[54].

TRAP Assay

Tartrate-resistant acid phosphatase (TRAP) activity in liquid samples was estimated using a colorimetric assay based on the hydrolysis of pNPP in the presence of tartrate[55]. The materials required included liquid sample (serum, plasma, cell culture supernatant, or tissue extract), p-nitrophenyl phosphate substrate, sodium tartrate solution, acetate buffer (pH 5.5), sodium hydroxide solution (stopping reagent), distilled water, test tubes or microplates, micropipettes, incubator/water bath, and spectrophotometer or microplate reader[56]. Briefly, an appropriate volume of liquid sample (50–100 µL) was mixed with acetate buffer containing sodium tartrate and incubated with p-nitrophenyl phosphate substrate at 37°C for 30–60 minutes[57]. TRAP present in the sample hydrolyses the substrate to release p-nitrophenol[58]. After incubation, the reaction was terminated by adding sodium hydroxide solution, resulting in the development of a yellow colour[59]. The absorbance was measured at 405 nm against a reagent blank using a spectrophotometer[60]. A standard curve using p-nitrophenol or an appropriate calculation formula was used to determine TRAP activity, and the results were expressed as U/L or IU/L[55].

Results and discussion

Preparation of Aqueous extract of *S. officinale*

The aqueous extract of *S. officinale* is illustrated through pictorial representation to depict its preparation (Fig. 1).

Phytochemical analysis

Phytochemical analysis results show that extract confirms the presence of bioactive molecules like alkaloids, terpenoids (triterpenoids and diterpenes), and phytosterols (**table 1**) [6]. Strongly positive results concerning alkaloids indicate considerable content of these substances, which play an important role in modulation of inflammatory processes and prevention of osteoclasts actions during bone resorption[61]. Terpenoids are highly represented, may have protective effects on osteoblasts by decreasing oxidative stress that promotes development of osteoporosis. Phytosterols present demonstrate possible estrogenic activity, contributing to the preservation of bone mineral density and regulation of bone remodelling process[62]. The presence of reducing sugars in small amounts may promote metabolic and healing processes in tissues, but their specific role in osteogenesis might be not too significant. Lack of saponins, fats, and anthocyanins is beneficial for enhanced bioavailability and biological activity. In general, alkaloids, terpenoids, and phytosterols appear to provide synergic mechanisms for the improvement of antioxidative, anti-inflammatory, and bone-protecting effects[63].

Table 1

Phytochemical screening of the aqueous extract showing the tests performed, observations recorded, and corresponding results for each identified compound.

PHYTOCHEMICALS	TEST PERFORMED	OBSERVATION	RESULT
Alkaloids	Mayer's reagent test	Creamy white ppt	++
Alkaloids	Dragendroff's test	Reddish brown ppt	++
Reducing sugars	Benedict's test	Mild yellow	+
Reducing sugars	Fehling's test	Red precipitate	+++
Saponins	NaHCO ₃ Foam test	No observation	-
Phytosterols	Salkowski's test (Hesse's variation)	Red colour ppt	+
Phytosterols	Salkowski's test (simple)	Red colour in lower layer	+
Fats & oils	Filter paper test	No oils stains	-
Triterpenoids	Salkowski's test	Golden yellow	+++
Diterpenes	Copper acetate test	Emerald green	+++
Anthocyanins	Ammonia shift test	No observation	-
Carboxylic acid	NaHCO ₃ effervescence	No observation	-
Proteins & amino acids	Xanthoproteic test	Mild yellow colour	+

LC-MS

LC-MS profiling of the aqueous root extract of *S. officinale* revealed the presence of several putative bioactive constituents. The analysis identified signals corresponding to quercetin derivatives, biochanin A, and various glycoside-containing compounds, indicating the chemical complexity of the extract (**table 2**). These findings are consistent with the preliminary phytochemical screening, which demonstrated the presence of alkaloids, terpenoids, phytosterols, reducing sugars, and proteins/amino acids. Among the compounds detected by LC-MS, quercetin derivatives and biochanin A are of particular interest because of their reported biological activities relevant to bone metabolism (**Fig. 2**) [64]. Quercetin-related compounds have been associated with the regulation of osteoblast differentiation and osteoclast-mediated bone resorption, whereas biochanin A, an isoflavonoid, has been reported to have potential bone-preserving effects. The detection of these constituents therefore provides a possible phytochemical basis for the osteoprotective effects observed in the subsequent zebrafish experiments. However, the present LC-MS analysis was primarily used for compound profiling, and further targeted

analysis and structural confirmation are required to establish the identity, abundance, and individual contribution of these compounds to the observed osteoprotective activity [65][66].

Table 2

Identification of phytochemical compounds detected in *S. officinale* using LC-MS analysis, showing retention time, compound name, exact molecular weight, and molecular formula.

S.no	Retention Time	Compound name	Molecular weight (exact mass)	Molecular formula
1	12.264	Yohimbic acid hydrate	358.1893	C20H26N2O4
2	4.840	6-[5,7-dihydroxy-2-(4-hydroxyphenyl)-4-oxochromen-6-yl]-5,7-dihydroxy-2-(4hydroxyphenyl) chromen-4-one	538.09	C30H18O10
3	1.103	1,7-Dimethyluric acid	196.0596	C7H8N4O3
4	14.840	Syrosingopine	666.2789	C35H42N2O11
5	14.436	Thalrugosaminine	652.3149	C39H44N2O7
6	14.284	Picroside II	512.153	C23H28O13
7	13.577	3-[(Z)-5-[6-hydroxy-5,5,8a-trimethyl-2-methylidene-3-[3,4,5-trihydroxy-6(hydroxymethyl)oxan-2-yl] oxy-3,4,4a,6,7,8-hexahydro-1H-naphthalen-1-yl]-3-methylpent-2enoxyl]-3-oxopropanoic acid	570.304	C29H46O11
8	13.123	5-(4-hydroxyphenyl)-1-[2-(4-hydroxyphenyl) ethyl] pentyl 6-O-(6-deoxy-alpha-Lmannopyranosyl)-Beta-D-Glucopyranoside	608.2833	C31H44O12
9	12.416	Aleuretic Acid	304.225	C16H32O5
10	11.860	Liensinine	610.3043	C37H42N2O6
11	10.648	Biochanin A 7-O-beta-d-glucoside-6"-O-malonate	532.1217	C25H24O13
12	9.486	Decanoylcarnitine	316.2482	C17H34NO4+
13	8.173	[(1S,3aR,5R,5aR,8aR,9S,9aR)-1,5,8a-trimethyl-2,8-dioxo-3a,4,5,5a,9,9a-hexahydro1H-azuleno[6,5-b] furan-9-yl] 2-methylpropanoate	334.178	C19H26O5
14	7.567	5-(4-hydroxyphenethylamino)-4-acetamido-5-oxopentanoic acid	308.1372	C15H20N2O5
15	7.264	Dihydrokavain	232.1099	C14H16O3
16	6.608	5,14,15-O-triacetoxy-7-O-benzoyloxy-3-O-propanoyloxymyrsinol	652.2884	C36H44O11

S.no	Retention Time	Compound name	Molecular weight (exact mass)	Molecular formula
17	5.446	(3R,7R,8R,8aS)-3,4'-dihydroxy-7'-(2-hydroxyethyl)-4,4,7,8a-tetramethylspiro[2,3,4a,5,6,7-hexahydro-1H-naphthalene-8,2'-3,8-dihydrofuro[2,3-e] isoindole]-6'-one	429.2515	C ₂₅ H ₃₅ N ₅ O ₅
18	5.042	Rutarin	424.1369	C ₂₀ H ₂₄ O ₁₀
19	4.789	bis(2-(pyridin-3-yl) piperidin-1-yl) methane	336.2314	C ₂₁ H ₂₈ N ₄
20	4.587	Nylidrin	299.1885	C ₁₉ H ₂₅ N ₂ O ₂
21	4.284	Quercetin-3,7-O- α -L-dirhamnoside	594.1585	C ₂₇ H ₃₀ O ₁₅
22	3.527	N, N-Dimethyldodecylamine	213.2457	C ₁₄ H ₃₁ N
23	3.072	2-(4-(2,3-dihydrobenzo[b] [1, 4] dioxin-6-yl)-3-methyl-1H-pyrazol-5-yl)-4-ethyl-5methoxyphenol	366.158	C ₂₁ H ₂₂ N ₂ O ₄
24	2.113	N-Acetylneuraminic acid	309.106	C ₁₁ H ₁₉ N ₂ O ₉
25	1.507	Anabasamine	253.1579	C ₁₆ H ₁₉ N ₃
26	6.103	(1r,5R,7S)-2-(1H-indol-4-yl)-5,7-dimethyl-1,3-diazaadamantan-6-one	295.1685	C ₁₈ H ₂₁ N ₃ O

Developmental toxicity

Zebrafish embryos were exposed to varying concentrations of the *S. officinale* extract (1, 3, 6, 12, 25, 50, and 100 μ g/mL) to evaluate its effect on early development (**Fig. 3**) Developmental progression and hatching were monitored over four days across all concentration groups[22]. The mortality rate, hatching rate, heartbeats, length of body size, and general development progress were observed. Besides that, embryos were continuously observed to detect any morphological deformities or abnormalities[26]. Based on the results obtained from zebrafish embryo toxicity evaluation, the extract did not show any toxicity even within the wide concentration range used (1-100 μ g/mL). This fact is proven by a 100% hatching and survival rate within 48 hpf [67]. Hatching and survival rates were recorded. Both parameters were evaluated across concentrations ranging from 1 to 100 μ g/mL to determine any concentration dependent effects. The hatching & survival rate was kept at 100% during the time course analysis (24–96 hpf) (**Fig. 4**). Such results demonstrate the toxicity profile of the plant extract.

Zebrafish grouping and maintenance

The fishes were grouped as mentioned in the protocol and proper care was taken. All experiments were done in accordance with the IAEC protocol. The schematic representation of the grouping was shown as **Fig. 5**.

Histopathology and Alizarin red staining

Histopathology confirmed that CT group had normal and well-organized muscle fiber structures, but DC group had severe disorganized tissues and muscle breakdown which confirms osteoporosis (**Fig. 6a-d**). The SO1 group had partial recovery with slight abnormality, but the SO2 group exhibited nearly normal tissue structure similar to the control group. Alizarin Red stain showed dense and even calcium accumulation in the CT group (**Fig. 6e-h**). But the DC group had less and unevenly stained tissues with faint staining, suggesting a mineralization defect. The SO1 group had fair recovery compared with DC group where the SO2 group had strong and even calcium accumulation, demonstrating significant mineralization recovery. These results are clear evidence that *S. officinale* administration resulted in a dose dependent response to mineralization defect under osteoporotic condition.

Measurement of size of the scales

Scale size was measured from all groups using a digital vernier calliper, and mean values were calculated and compared across groups (**Fig. 7a**). The size of the scales on zebrafish showed significant differences in the experimental groups due to mineral deposition and dynamics of bone growth[68]. The control group had normal sizes of scales, proving healthy mineralization processes. The DC group showed decreased size of scales, which indicates impairment of mineral deposition and proves the known osteoporotic effects of the drug. Treatment of the fish with the extract led to dose-dependent effect. The group SO1 (6 µg/mL) had relatively bigger size of scales in comparison with the group that received dexamethasone, demonstrating partial increase in mineral content. Particularly, the group SO2 (12 µg/mL) had the biggest size of scales, approaching the control group, which proves effective mineralization and regeneration of bone-like structures[29][7]. The increase in scale sizes is explained by bioactive substances in the extract like flavonoids, terpenoids, phytosterols, which stimulate osteoblastic activity while preventing osteoclast-mediated resorption[69].

ICP-MS Analysis

The calculation of calcium and phosphorus fold changes in experimental groups led to understanding of particular pattern of disruption and recovery of mineralization processes (**Fig. 7b**) [29]. The CT group, indicates regular mineralization of scales. In DC group, presence of dexamethasone led to reduction in mineralization rates. As a result, we can assume that lack of minerals occurred due to its well-known catabolic effect that interfere with calcium regulation, increasing resorption rate and decreasing osteogenesis. In treatment with SO1 (6 µg/mL), we observe partial recovery of the mineral concentration compared to CT group, and SO2 (12 µg/mL) causes even greater recovery and is dose-dependent. It

should be noted that the recovery process was maximal in group treated with SO₂, and their fold change of calcium reached the maximum point than the control[35].

Hydroxyproline, Alkaline phosphatase (ALP), TRAP Assay

From the obtained results for hydroxyproline content, it is clear that the level of hydroxyproline was decreased in DC group (15 µg/mL) as compared with CT group (19 µg/mL)[29][37]. The decrease level of hydroxyproline suggests glucocorticoid-dependent reduction of collagen synthesis and destruction of the extracellular matrix (**Fig. 8a**). Administration of SO₁ produced some improvement in the content of this biomarker but not enough significant since it showed a low increase in its levels (15.5 µg/mL). However, the introduction of SO₂ demonstrated the highest increase of the levels of hydroxyproline (21.5 µg/mL), which were even higher than those in the CT group. As for TRAP activity which indicates osteoclastic activity, it became clear that, the levels of this marker were notably increased in DC group (10 U/L) compared with CT group (9.2 U/L) (**Fig. 8b**). Administration of SO₁ showed a positive effect on this activity level, as the level became lower (7.5 U/L). Yet, SO₂ showed no positive effect and had similar activity levels indicating reduced osteoblast function at higher concentration. ALP activity, which is an osteogenic biomarker indicates osteoblastic activity and ability to mineralize, was observed to decrease in the DC group (6.9 U/L) when compared to control group (7.15 U/L), supporting the idea that dexamethasone inhibited the activity of osteoblasts (**Fig. 8c**). On the other hand, SO₁ treatment resulted in an increase in ALP activity (7.3 U/L), thus demonstrating recovery and enhancement of osteoblastic activity. In contrast, SO₂ showed the lowest level of ALP activity (6.9 U/L) among all three groups under study, which points out reduced activity of osteoblasts. Analysis of differences between TRAP and ALP activities gives the understanding that the effect of *S. officinale* on bone remodelling is biphasic and concentration-dependent[37]. At a lower concentration of *S. officinale* (SO₁), this compound is observed to stimulate osteoblast activity (increase in ALP activity) but inhibit osteoclast activity (decrease in TRAP activity). In higher concentration of *S. officinale* (SO₂), the balance shifts towards increase in osteoclast activity (increased TRAP activity) and reduction osteoblastic activity (decrease in ALP activity). Hydroxyproline content was found to be significantly higher in SO₂ group, which could point out to enhanced collagen synthesis independent from osteoblastic ALP activity[29].

Discussion

Phytochemical analysis shows that *S. officinale* extract is rich in bioactive compounds such as alkaloids, terpenoids, and phytosterols.[6], which collectively contribute to osteoprotective effects by reducing oxidative stress, inhibiting osteoclast-mediated bone resorption.[61][62]. The presence of reducing sugars in small amounts may promote metabolic and healing processes in tissues. [63]. The LC–MS analysis of *S. officinale* extract revealed presence of several bioactive constituents, including quercetin derivatives, biochanin A, and glycosides. Quercetin has been reported for its anti-osteoporotic activity, promoting osteoblast differentiation and suppressing osteoclast-mediated bone resorption, favouring bone formation[65]. Biochanin A, a phytoestrogen, may contribute to bone conservation[70]. While

looking towards the developmental the embryo's development proves that the tested extract does not have toxic and lethal properties[71]. The tissue architecture evaluated through histopathological examination revealed marked architectural variations among groups. They had well organized muscle fibers without any signs of degeneration, proving that the control group had well-developed architecture. The group treated with dexamethasone showed severe architecture alterations like weak organization of muscle fibers, low density, and presence of degraded and shrunken tissues because of the mineralization defect caused[72]. The treatment of a lower concentration (S01) yielded better results compared to the dexamethasone group, showed partial recovery as they retained some architectural features and some muscle fibers were slightly misaligned. In comparison, treatment with a higher dose (S02) caused marked improvement in the tissue architecture which explains the potential of the extract. Muscle fibers became better organized, dense, and more aligned, resembling those of the control group. Such results reveal a considerable therapeutic effect provided by higher concentration of the extract[35]. It should be noted that same results could also be obtained while studying mineralization process with use of Alizarin Red stain. The control group exhibited strong and homogeneous staining, indicating healthy calcium deposition[37]. But, the group treated with dexamethasone showed much weaker staining and heterogeneous distribution[35][37]. While S01 group displayed fair progress in deposition of calcium salts, evidenced by the intensity of staining, which was greater than that of the dexamethasone group, there was still an uneven distribution pattern, implying incomplete recovery. It is also important to note that S02 group displayed significant improvement in both intensity and uniformity of staining, similar to the control group. The results obtained from zebrafish scale sizes analysis confirmed clear differences between the experimental groups, showing alterations in the process of mineralization and bone formation. In the case of the control group, normal scale sizes were recorded, showing adequate mineralization. On the other hand, in the group treated with dexamethasone, a drastic decrease was observed, confirming the negative effect of this substance on mineralization and bone density loss. *S. officinale* treatment resulted in dose-dependent scale size improvement, where S01 showed a moderate effect, and S02 had a pronounced increase in the scale size, which was close to the control group scale sizes[69]. Continuing with ICP-MS analysis with the scales measured the calcium and phosphorus fold change analysis revealed disrupted mineralization in the dexamethasone-treated group due to impaired calcium regulation and increased resorption, while *S. officinale* treatment showed dose-dependent recovery[29], with the higher concentration restoring mineral levels close to normal, indicating effective reversal of bone loss. Thus the result of ICP-MS clearly states that the extract can be effectively used for osteoporosis treatment[35]. With ICP-MS findings indicating altered mineral levels, changes observed in hydroxyproline, TRAP, and ALP activities show clearly how there exists a link between osteoclast and osteoblast activities depending on the various treatment regimens employed. Increase in hydroxyproline results where S02 is higher than control shows that increase in collagen deposition which helps in increase in bone regeneration. The administration of dexamethasone lead to increased TRAP activity which confirms osteoporosis where the S01 is reduced tremendously and S02 comes in balance with control group showing development of bone regeneration. This shows that there is a stimulation of collagen production independent of ALP osteoblastic activity[29]. Collectively, this study highlights the bone-protective properties of *S. officinale* through various mechanisms of action, which

include mineral metabolism, collagen formation, and bone remodelling. However, it is important to regulate the doses because of the two effects that are dose-dependent. These results provide a promising foundation for future studies in osteoporosis management.

Conclusion

The present study demonstrates that *S. officinale* aqueous root extract possesses osteoprotective potential against dexamethasone-induced skeletal alterations in adult zebrafish. Dexamethasone exposure resulted in marked disruption of tissue architecture, reduced mineral deposition and scale size and alterations in calcium and phosphorus levels. Treatment with *S. officinale* improved skeletal morphology, mineralization, scale characteristics and mineral content, with several responses being more pronounced at the higher treatment concentration. The extract also influenced hydroxyproline, alkaline phosphatase and tartrate-resistant acid phosphatase activities, suggesting a modulatory effect on bone matrix formation and remodelling. Phytochemical and LC–MS analyses further indicated the presence of several potentially bioactive constituents that may contribute to these effects.

However, the precise molecular mechanisms underlying the osteoprotective activity of *S. officinale* remain to be established. Future studies should investigate the involvement of key osteogenic and osteoclastogenic pathways, particularly the RANKL/OPG and Wnt signalling pathways, through gene and protein expression analyses. Further dose-response studies, longer-term treatment studies, and assessment of additional bone turnover markers would help clarify the concentration-dependent effects observed in the present study. In addition, validation in appropriate mammalian osteoporosis models and subsequent pharmacological and safety investigations are required to determine the translational potential of *S. officinale* for glucocorticoid-induced bone loss.

Declarations

Data availability

The data generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Ethics declaration

The zebrafish experiments conducted in this study were performed in accordance with the relevant institutional and national guidelines for the care and use of laboratory animals. Ethical approval for the use of zebrafish was obtained from the Institutional Animal Ethics Committee (IAEC) of Sathyabama Institute of Science and Technology, prior to the commencement of the study (Approval No. SU/CLATR/IAEC/XXVII/04/2026). All experimental procedures were conducted in accordance with the approved protocol and applicable animal welfare regulations.

Competing interest

All authors declare no conflict of interest.

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Figures

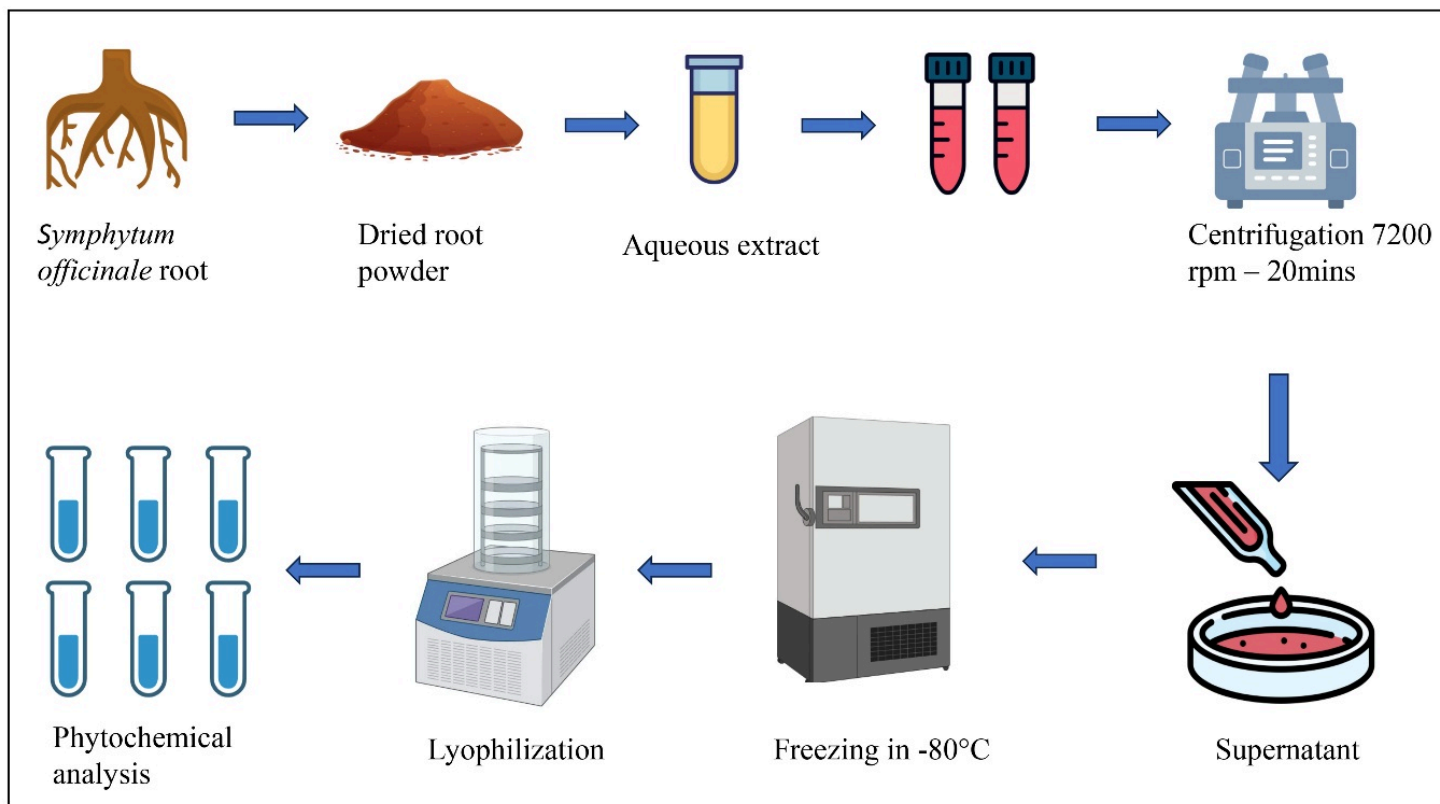


Figure 1

Schematic representation of aqueous extract preparation from *S. officinale* root, involving drying, extraction, centrifugation, supernatant collection, freezing, lyophilization, and phytochemical analysis to obtain crude extract from aqueous extract.

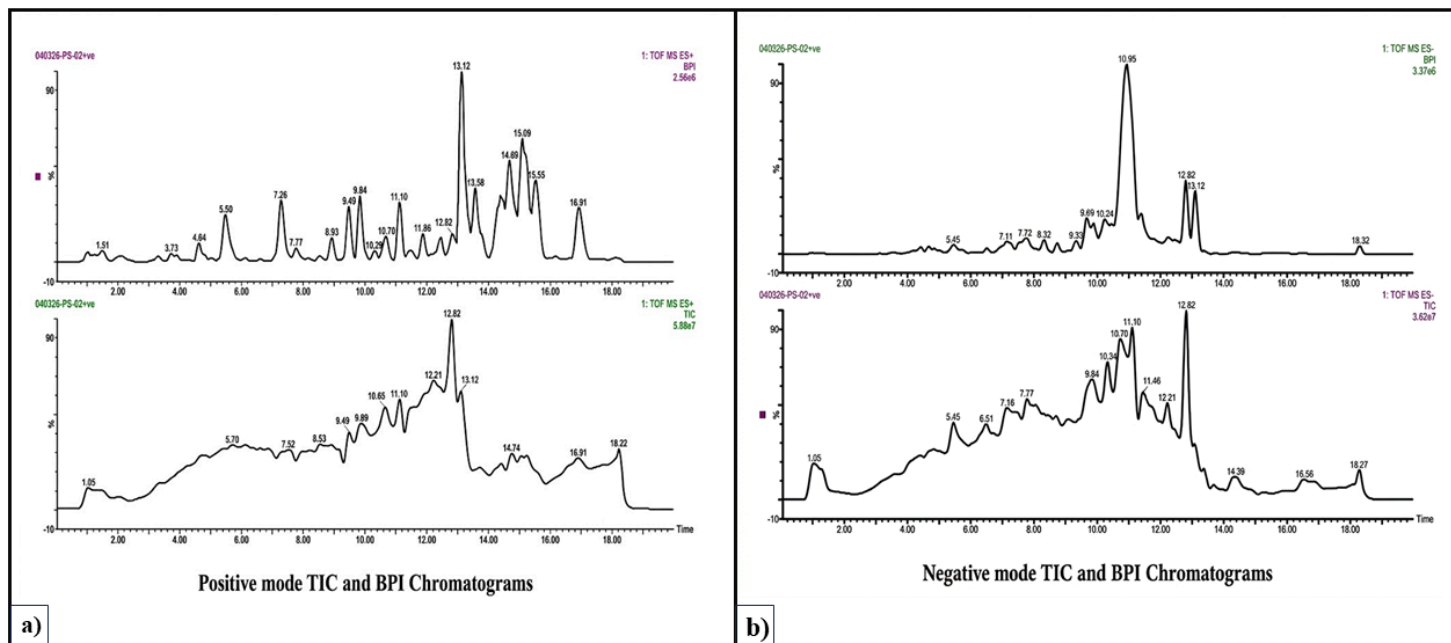


Figure 2

Total Ion Chromatogram (TIC) and Base Peak Ion (BPI) chromatograms of sample extract *S. officinale* acquired using TOF-MS in ESI mode. Positive ion mode (ESI+) chromatograms. Negative ion mode (ESI-) chromatograms were recorded to detect compounds with different ionization values. In each panel, the upper trace (coloured) represents the Base Peak Ion (BPI) chromatogram, while the lower trace (black) shows the Total Ion Chromatogram (TIC). Major peaks are labelled with their retention times (minutes), indicating the presence of diverse phytoconstituents with varying ionization properties.



Figure 3

shows the developmental progression of zebrafish embryos and hatched embryo (larvae) under different concentrations (1, 3, 6, 12, 25, 50, and 100 µg/mL) of the test post fertilization.

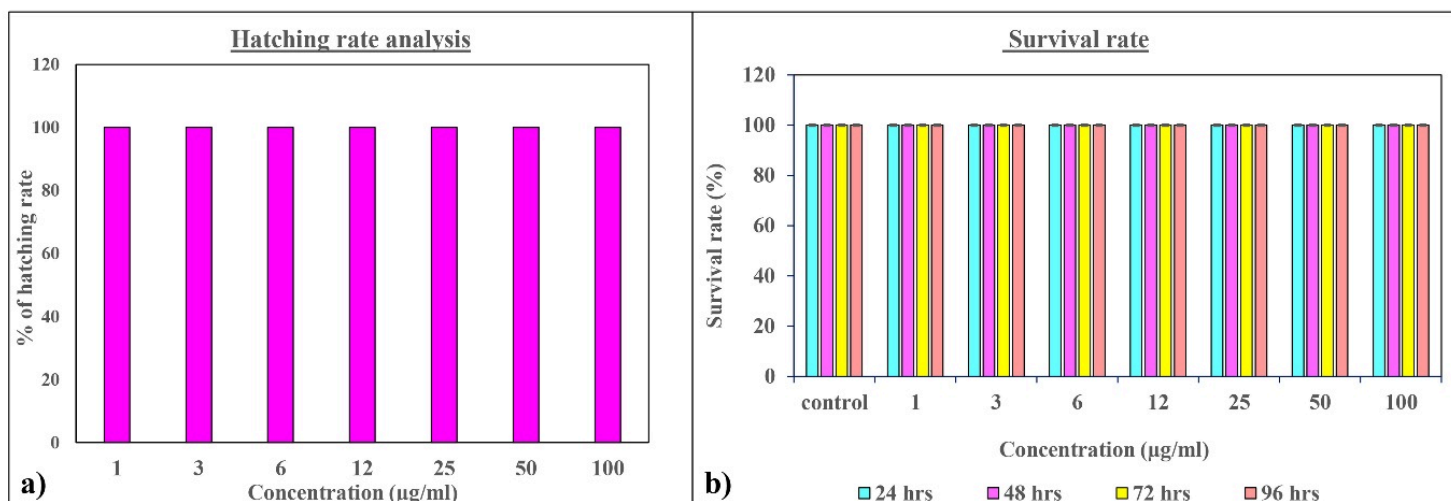


Figure 4

shows the results of zebra fish larvae 48 hours post fertilization **a)** depicts the hatching rate of zebrafish embryos across concentrations (1–100 µg/mL) of the extract 48 hours post fertilization which is recorded to be 100% **b)** Survival rate of zebrafish embryos at 24, 48, 72, and 96 hours across concentrations (1–100 µg/mL) of the extract is also 100% with 0% mortality

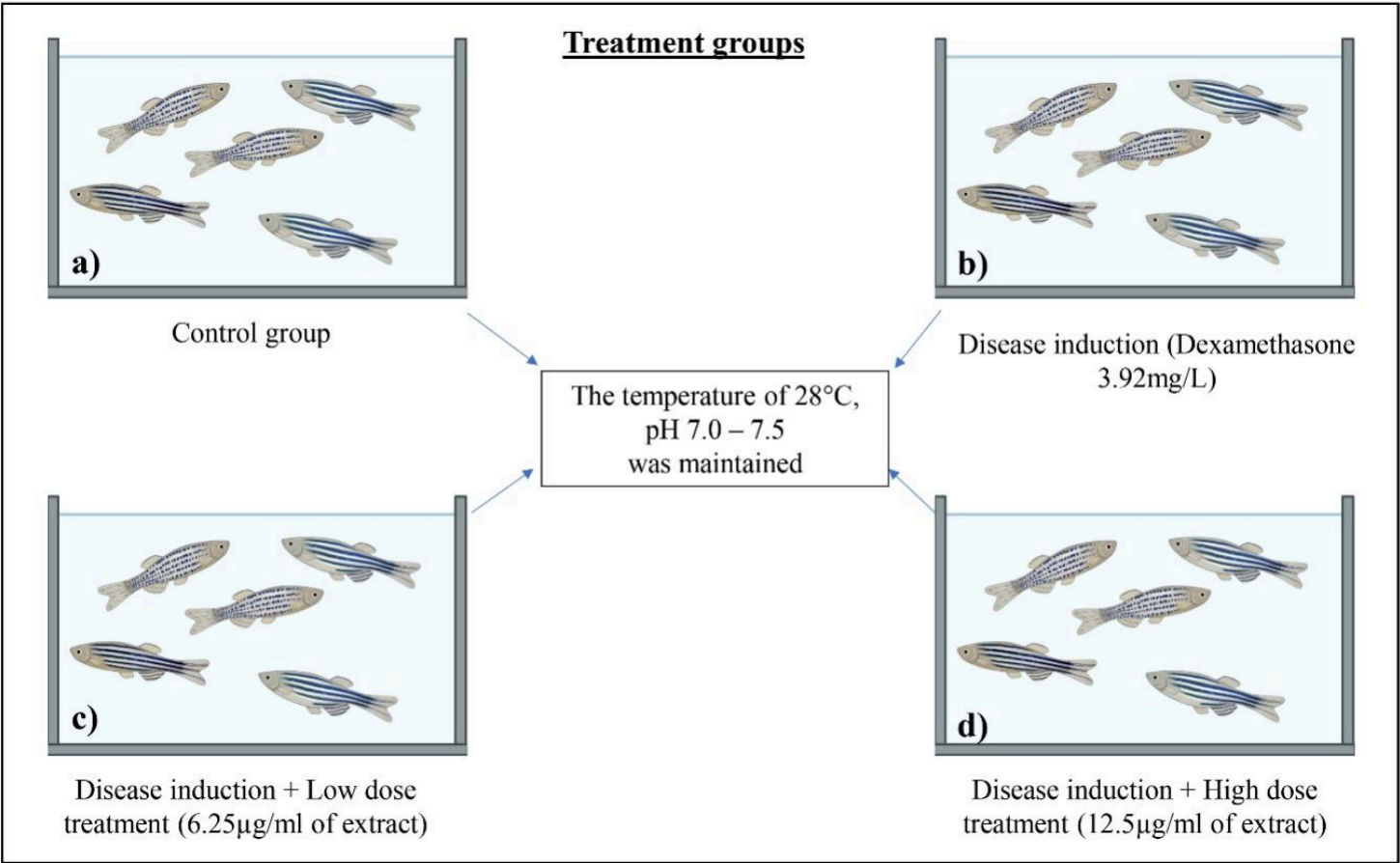


Figure 5

Schematic representation of grouping of fishes in tanks showing 4 different groups namely Control group, Osteoporosis developed group, Osteoporosis development with treatment of extract at lower does and osteoporosis development with treatment of extract at higher dose

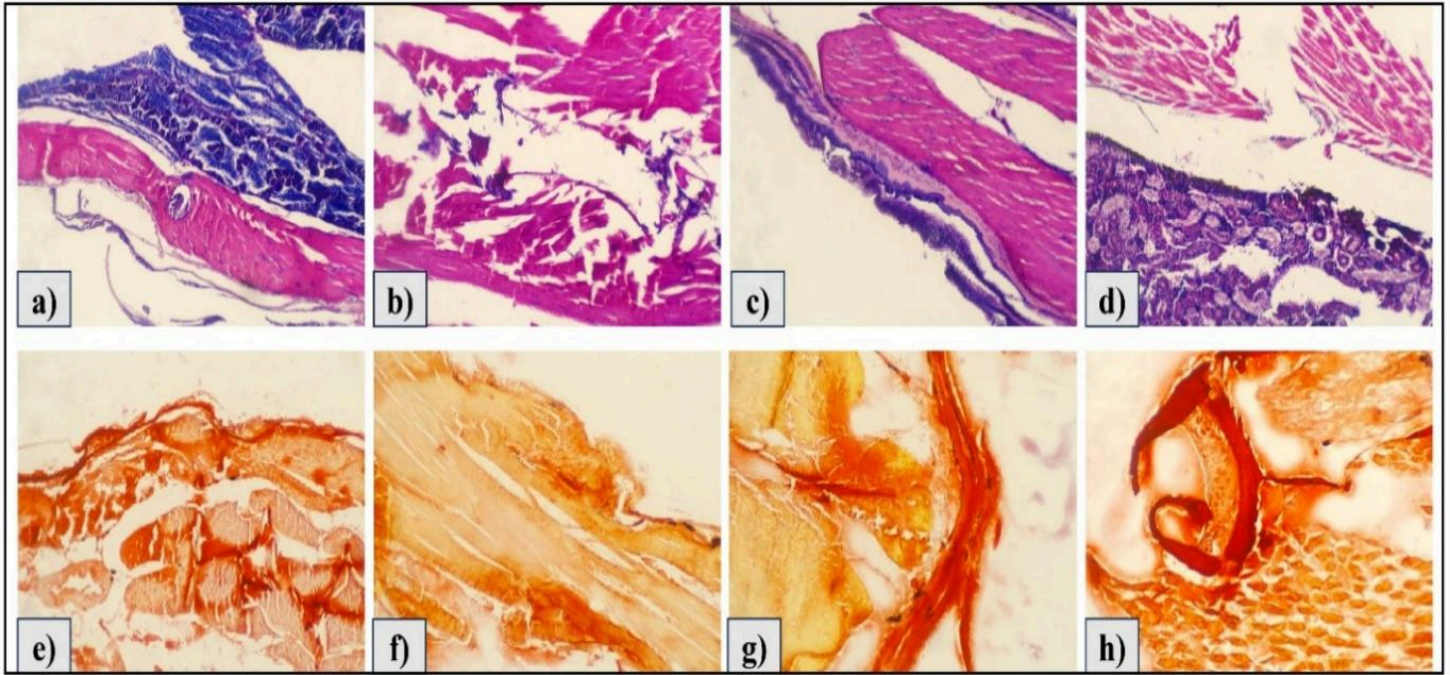


Figure 6

shows the images of results of histopathology and alizarin staining **a)** CT group showing normal, intact muscle fibers **b)** DC group exhibiting tissue disorganization, muscle fiber degradation **c)** SO1 group showing partial restoration with mild irregularities **d)** SO2 group demonstrating significant recovery resembling the control **e)** CT group (Alizarin Red staining) displaying dense and uniform calcium deposition **f)** DC group showing reduced, patchy, and faint staining **g)** SO1 group exhibiting moderate recovery of calcium deposition **h)** SO2 group showing strong and uniform staining.

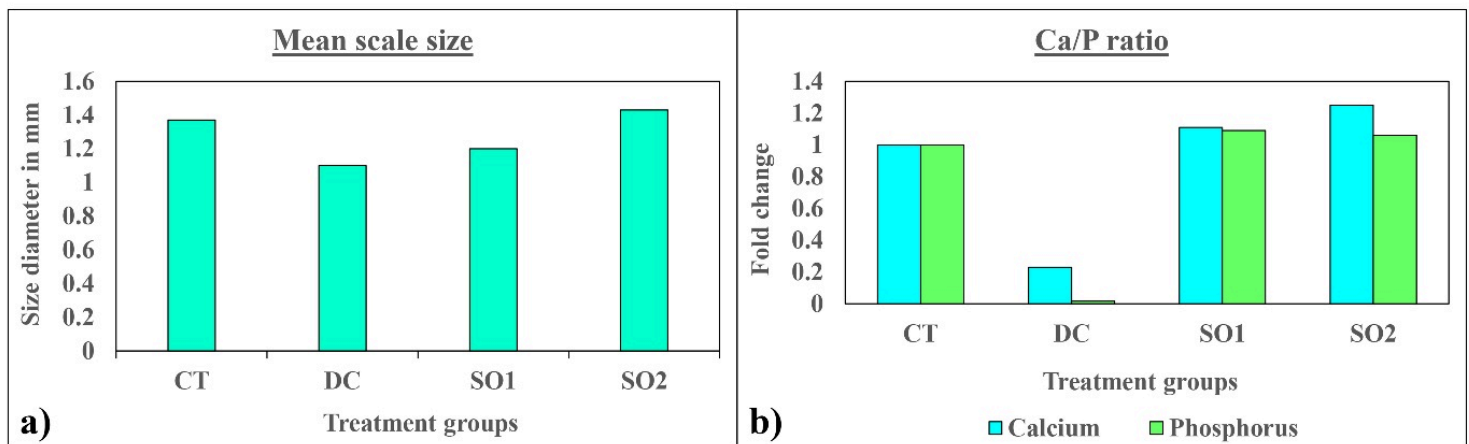


Figure 7

a) It shows the effect of treatment on the size of fish scales. The scale size in the group receiving Dexamethasone treatment was lower (1.10 mm) than that of the Control group (1.37 mm). In SO1 (6 µg/mL) treatment, the size of the scales was partially restored (1.20 mm), whereas in SO2 (12 µg/mL)

resulted in improved scale size (1.43 mm), which was close to the Control group **b)** Fold change analysis of calcium and phosphorus levels across experimental groups. Control shows normal mineral levels, dexamethasone-treated group exhibits significant depletion of these minerals, while SO1 and SO2 groups demonstrate dose-dependent recovery of mineral content. SO2 is recorded with highest Ca ratio which shows dose dependent effect in the scales

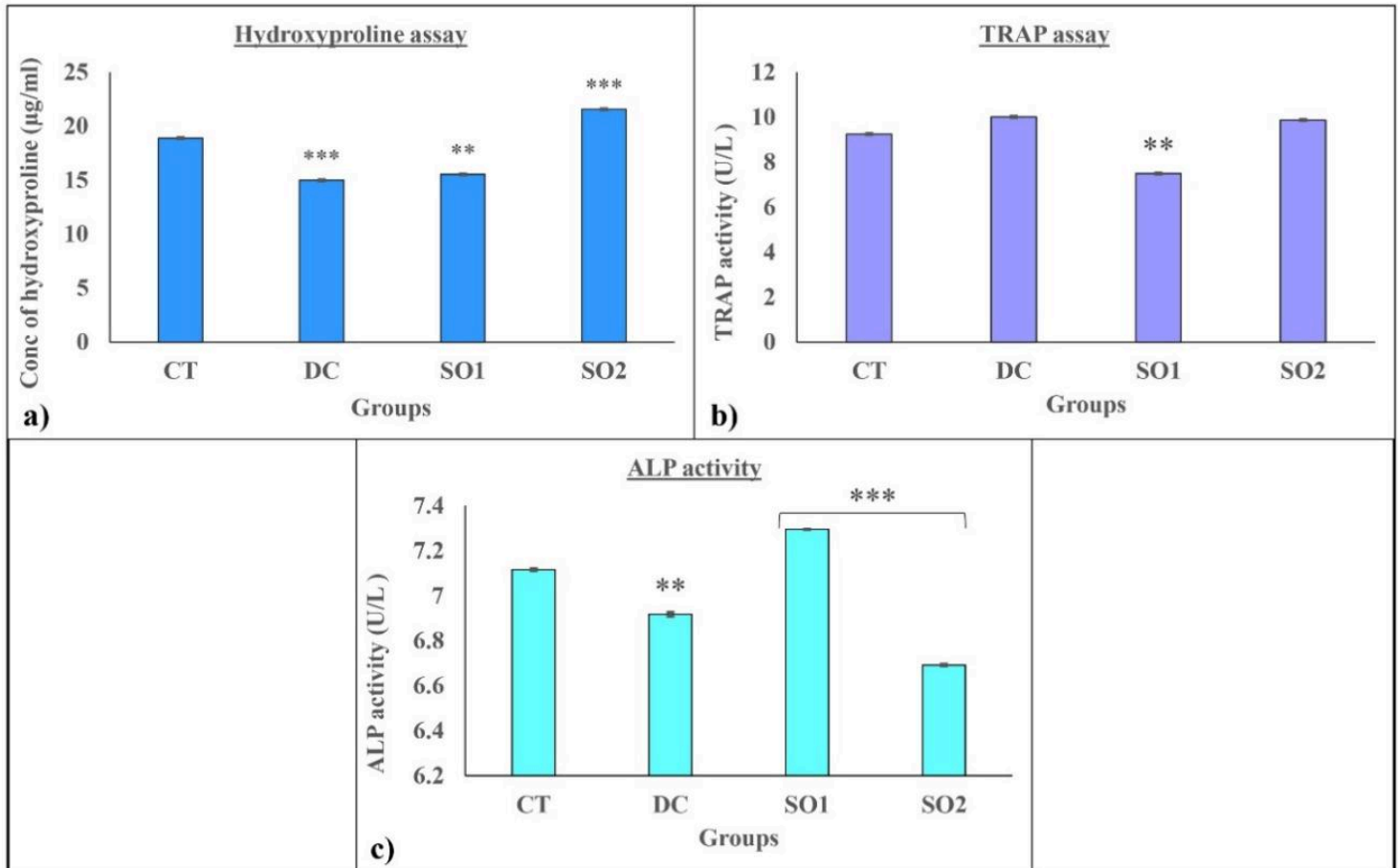


Figure 8

a) Hydroxyproline concentration was reduced in the DC group (15 µg/mL) compared to Control (19 µg/mL), indicating dexamethasone-induced collagen depletion. SO1 showed partial recovery (15.5 µg/mL), while SO2 restored levels beyond control (21.5 µg/mL), demonstrating a dose dependent increase in collagen content **b)** TRAP activity was elevated in the DC group (10 U/L) compared to control (9.2 U/L), reflecting increased osteoclast activity. SO1 showed a reduction in TRAP activity (7.5 U/L), while SO2 returned to levels comparable to DC (10 U/L), suggesting concentration-dependent variation in osteoclast modulation **c)** ALP activity declined in DC (6.9 U/L) relative to CT (7.15 U/L), indicating suppression of osteoblast function. SO1 showed an increase (7.3 U/L), which shows restoration of osteoblastic activity, while SO2 recorded the lowest activity (6.7 U/L), indicating a biphasic response across concentrations. On comparison with the ALP activity and TRAP assay it clearly shows that with increase in dose of SO2 in TRAP increased osteoclast activity while ALP activity SO2 is decreased a lot

due to osteoblast activity. *All experiments were done in triplicates (n=3). Mean $\pm$ SD was calculated, ** $p<0.01$ and *** $p<0.001$ were considered to be statistically significant*

Supplementary Files

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- [GraphicalabstractElizabeth.png](#)