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Phytochemical Profiling of *Helianthus annuus* L. seed and Investigation of Developmental Toxicity in Zebrafish Embryos with Anti-Osteoporotic Evaluation in Adult Models

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KEYWORDS

Osteoporosis, Antioxidant, Zebrafish, Osteoclasts, Antioxidant Activity, Helianthus annuus

ABSTRACT:

This study investigates the pharmacological potential of Helianthus annuus L. (sunflower) defatted seed cake, a discarded residue after oil extraction. Rich in phytochemicals like flavanones and polyphenols, this material is shown to exhibit various activities, including anti-inflammatory and antioxidant properties. The extract was evaluated for its phytochemical analysis, antioxidant and anti-inflammatory activities. The primary focus was evaluating its anti-osteoporotic activity, given the lack of scientific data on this specific property. Osteoporosis was induced in a zebrafish model using Ferric ammonium citrate (FAC), and promising results were observed with extract administration (100 and 200 µg/ml) in preventing osteoclast formation and enhancing osteoblast formation. Furthermore, the embryo toxicity of the extracts on developing zebrafish embryos was assessed at various concentrations (3.125-1000 µg/ml) over 24-96 hours, demonstrating its safety as per OECD guideline. The presence of bioactive constituents revealed phenolic and flavonoid content in the extract. LC-MS/MS profiling further confirmed the presence of several polyphenolic compounds. In vitro antioxidant assays and anti-inflammatory protein denaturation assays demonstrated promising effect. Developmental toxicity testing demonstrated the LD50 lies in between 12.5 and 3.125 µg/ml. In vivo, pretreatment with FAC (250 µg/ml) significantly attenuated a quite improvement in bone resorption and bone density The findings suggest that the anti-osteoporotic effects of Helianthus annuus L. may stem from its anti-inflammatory and antioxidant properties, highlighting its potential as a natural therapeutic agent for bone density improvement and supporting further investigation into its active constituents.

Introduction

Chronic Disease, characterized by long-lasting condition and slow progression, has become a health burden that is having a very significant impact on global health. This skeletal disorder is characterized by bone deformation, bone fragility, increase of osteoclast cells in bone etc. Global estimation of this chronic disease epidemiology in Asia, this statistical data is among 10-30% of women aged 40 and older, 26.6- 28.6 % of adults are combating with this disorder. Highlighting in India, an estimated of 60 million people have osteoporosis with women accounting 80% of cases. There are various synthetic drugs in modern era that are well known for their potent anti-osteoporotic activity like Bisphosphonates, SERMs, PTH analogues, Sclerostin Inhibitor etc. class of drugs with monoclonal antibodies. These compounds have a high degree of potent activity on osteoporosis but also have a large number of side effects i.e. Bisphosphonates class of drugs are having Osteonecrosis, femoral fracture, musculoskeletal pain. *Helianthus annuus* L. with a well-known common name sunflower, is an important global oil rich source, mainly in seed part. It has a root origin in North America, belonging to the family Asteraceae which is also known as the Compositae or daisy family. The extensive reservoir of bioactive chemicals found in *Helianthus annuus* L. seeds, leaves, and other plant components have attracted growing scientific attention in addition to its commercial significance as a source of edible oil, confectionary seeds, and animal feed.[1] The one of the most

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highlighted parts of the plant is seed which is recognized for unsaturated fatty acid, linoleic acid, oleic acid etc. Aside from fats, the seed provides a good source of protein (15–20%), dietary fiber, and important vitamins, particularly vitamin B (niacin, thiamine, folate). It is also a good source of vitamin E (Tocopherol), which is a strong antioxidant. It is also rich in various source of minerals like zinc, phosphorus, selenium, potassium etc. There are various animal models like rat, mouse in the section of rodents, Rabbit, monkey in the circle of non-rodents, and the successive model of Zebrafish in miscellaneous.[2] 70-80% genetic similarity to humans, easy breeding and hatching technique, availability make this zebrafish (*Danio rerio*) model as a successful one. Also, the clear microscopic view of embryos depicts this model as a popular one for embryo toxicity. This study concentrates on the extraction and appropriate solvent system evaluation for *Helianthus annuus* L. seeds, assessing developmental toxicity in zebrafish embryos and conducting an anti-osteoporotic investigation on *Danio rerio.*

Materials and methods

Collection, identification and Extraction of plant

In August 2024, the entire fresh *Helianthus annuus* L. plant was gathered from the village of Murshidabad in the West Bengal district. The air-dried seeds get supplied by UPL Limited, C/O Bharathi Bhramha Seeds that is situated in the district of Rangareddy, Telengana. The plant was identified and authenticated by the survey of Botanical Garden, Howrah with the authentication number JISU/2023/HA/001.

Sunflower seeds were dried, pulverized into a fine powder, and subjected to defatting using n-hexane to remove lipid components. The defatted seed powder was then processed for extraction. Maceration was selected as the extraction method due to its suitability for thermolabile phytoconstituents. Briefly, 100 g of defatted *Helianthus annuus* L**.** seed powder was macerated with 300 mL of distilled water. The aqueous extract was subsequently subjected to successive solvent partitioning using solvents of increasing polarity, namely ethyl alcohol, ethyl acetate, chloroform, and hydro-alcoholic solvent system, to obtain respective solvent fractions. The extracts were filtered, concentrated under reduced pressure, and stored for further phytochemical and pharmacological evaluation.

Phytochemical Screening

Using slight modifications, the qualitative phytochemical analysis of the *Helianthus annuus* L. seed extract was conducted in accordance with Kokate et al., 2005. The analysis for alkaloid, flavonoid, glycoside, cardiac glycoside, tannin, terpenoids, saponin glycoside, carbohydrate was performed. According to Ainsworth EA, Gillespie KM [3] with a slight modification in procedure, the prepared stock solution and Folin–Ciocâlteu reagent (FCR) were mixed. After incubation, 75% Na2CO3 solution is added. After that by incubating again in dark environment, it is centrifuged, and absorption is checked at 725 nm against a standard gallic acid. The total phenolic content in the extract was estimated by using the following formula: C = c (V/m) where C = total phenolic content mg GAE/g dry extract c = concentration of gallic acid obtained in mg/ml (for extract the concentration can be calculated from the equation obtained from gallic acid standard curve) V = volume of extract in ml. It was carried out by the procedure by Yee et. al. [4] with small modification, test sample was mixed with 5% sodium nitrite followed by 10% aluminium chloride and 1M NaOH is added. After incubation in dark the absorbance is checked at 510 nm against standard quercetin. The total flavonoid content (TFC) of the extract was calculated using the formula: C = c (V/m) Where C = total flavonoid content mg QE/g dry extract c = concentration of quercetin obtained in mg/ml (for extract the concentration can be calculated from the equation obtained from quercetin standard curve) V = volume of extract in ml m = mass of extract in gm

***In Vitro* Antioxidant and Anti-inflammatory Assays**

Reducing Power Assay

For this test described as per Benzie et. al. [5] with slight modification, samples were mixed with PBS buffer and potassium ferricyanide. TCA was added followed by treatment with 1% FeCl3. The absorbance is taken at 700 nm by UV-visible spectrophotometer. The percentage of antioxidant property is calculated by (%) = (absorbance of control – absorbance of sample)/ absorbance of sample *100.

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DPPH Free Radical Scavenging Assay

The antioxidant property of the extract in various solvent system is assessed by studying DPPH free radical assay by Baliyan et. al [6] with little modification. In a 96-microtiter plate, 100 µl of DPPH solution and the same amount of extract solution was taken. Ascorbic acid was used as the standard. After that it was incubated in the dark and the absorbance is checked at 517 nm by ELISA microplate reader. The values were taken in a triplicate format. The percentage inhibition was calculated using the following formula: Percentage inhibition (%) = [{absorbance of control – (absorbance of sample – colour factor)}/ (absorbance of control)] *100

Egg albumin denaturation assay

The egg albumin denaturation assay is executed for in vitro anti-inflammatory activity. According to Dharmadeva et. al. [7] with some modification, egg albumin is collected from an egg and mixed with PBS buffer of pH 6.4. Then 2 ml of test sample is added, and it is incubated at 37°C for 15 min. After that the samples are rapidly heated and cooled down. The absorbance is checked at 660 nm. Blank absorbance is also taken. Percentage inhibition (%) = [(absorbance of control –absorbance of test sample)/ absorbance of control] ×100.

RBC membrane stabilization

To estimate the anti-inflammatory activity, RBC membrane stabilizing assay is one of the most important in vitro studies. For this study, described by Boniface et. al. [8] with some changes, freshly collected blood is stored in 4°C for 24 hours. Then the supernatant is removed by centrifugation, and the rest of the portion is washed thrice with an isotonic solution to make a 40% RBC suspension with PBS buffer (pH= 7.4). By mixing with extract, heating the absorbance was checked at 560 nm. % inhibition of hemolysis = [(absorption of the control – absorption of test sample mixture)/ absorption of the control] x 100.

LC-MS/MS Analysis

Liquid Chromatography–Mass Spectrometry (LC-MS) was employed to identify and characterize the bioactive compounds present in the water extract of *Helianthus annuus* L. seed. The analysis was conducted at the Sophisticated Analytical Instrument Facility, Panjab University, using a Waters SYNAPT-XS HDMS system (Model: DBA064) operated with MassLynx Version 4.2 software. Before analysis, the extract was centrifuged at 12,000 rpm for 10 minutes to remove any particulates. Chromatographic separation was achieved using a UPLC ACQUITY H CLASS Series system equipped with a Waters Acquity BEH C18 column (2.1 × 100 mm, 1.7 µm particle size). The flow rate was maintained at 0.2 mL/min, and the injection volume was 5 µL. The total run time for each analysis was 45 minutes. The system operated in data-dependent mode, allowing automatic switching between MS and MS/MS acquisition for comprehensive profiling of metabolites.

***In Vivo* Study**

Zebrafish Collection and Maintenance

In accordance to the approval given by Institutional animal Ethics committee by TAAB Biostudy Service with registration number 1938/PO/Rc/S/17/CPCSEA, zebrafish (Danio rerio) of AB strain and of both sex were collected from Centre for Laboratory Animal Research and Training, Buddha Park, Kalyani, Dist.-Nadia, West Bengal, Pin- 741235, Registration No. -2109/GO/ReRcBiBt/S/20/CCSEA. The average weight of each fish is 0.5 ± 0.2 gm and age 3-4 months. Total number of 48 adult zebrafish (>6 months old) were collected. The animals were initially housed for 30 days for acclimatization before the actual experiment. By using a circulatory air system in aquarium tank with maximum density 1g fish/L water, the zebrafish were adapted to regular testing, a maintained pH of 7.0-8.0 and temperature 26-30°C. Changing of water was performed thrice a week with dechlorinated water. The fishes were fed dry food twice daily. Proper environment and 14/10-hour light/dark cycle was maintained.

Zebrafish Embryo Hatching and collection

Zebrafish embryo hatching process was generally done between 48-72 hours post fertilization (hpf). The process was maintained at 28.5°C. The Breeding tank had been filled with 300 ml of water (pH 7.0-8.0). As per standard procedure described in Kimmel et. al. [9] the fishes are selected based on health and sex, The ratio of male to female fishes were 2:1 in pairs. Initially male and female fishes were separated by a separator for

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differentiating and fed with highly proteinaceous diet and later they were allowed to breed, and the tank was tilted a little. Generally, this process takes 30 minutes to 1 hour and the preferred time is dawn. Water quality and aquarium cleanliness were regularly checked during the spawning period, and excessive water filtration and feeding were avoided. Between 3 hpf the embryos are collected and kept in E3 medium containing specific salts and a balanced pH. These were incubated at 28°C. The compositions provided for 1X E3 medium are as follows. To make 1 liter of E3 50X stock solution,14.6 g NaCl, 0.63 g KCl, 2.43 g CaCl2·2H2O, 4.07 g MgSO4·7H2O and methylene blue [10].

Zebrafish Embryo Acute Toxicity

As per OECD Test Guideline 236, fish embryo Acute Toxicity (FET) Test, 2013 the acute toxicity study on embryo of fish-like zebrafish [11], are done over a short period of time usually 96 hpf for determining LD50. The different concentrations of water extract which were taken for this study are – 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, 800, 1000 µg/ml. Each concentration about 10ml of test solution and for each plate, 3 embryos were taken to additionally determine mortality rate. Temperature was maintained at 26 ± 1 °C and in a semi-static way the test was performed. Test solutions were prepared by dilution with double distilled water. The healthy embryos are transferred into the plate by wide-bore pipette after washing and examined under microscope. All the plates were sealed with parafilm to prevent evaporation, though some tiny, small spores were made on the parafilm. The observations were done on 3, 24, 48, 72, 96 hpf.

Anti-Osteoporotic study

The anti-osteoporotic activity was performed on the adult model of Danio rerio. As per Yan et. al. [12] Adult zebrafish were subjected to high iron stress to form osteoporosis. The test drug was utilized to see its anti-osteoporosis effect in such conditions. There was total 6 groups of fishes for the study and each group contained 3 fishes. The fish water (either with or without FAC) was changed once every day. Adult zebrafish were randomly divided into two groups. Group 1 zebrafish were in the holtfreter water and Group 2 Zebrafish were in Ferric Ammonium Citrate (FAC) solution. After 15 days, FAC group was sub-divided into Group 3,4,5 and 6. The grouping method was used as: Group 1: Control group (Only cultured in Holtfreter H2O); [Holtfreter water (3.5 g/L NaCl, 0.5 g/L KCl, 0.1 g/L CaCl2 and 0.025 g/L NaHCO3)]. Group 2: Ferric Ammonium Citrate (FAC)- Treated group (kept in 250 µg/ml-1 FAC for 25d). Group 3: FAC+ H2O. group (Exposed to 15d FAC and an additional 10 d in H2O). Group 4: FAC+AL (Alendronate) (Standard group – 30 µM) (Treated with FAC for 15 d, following exposure to alendronate for 10d). Group 5: FAC + Test group-1 (100 µg/ml) (Treated with FAC for 15 d, following exposure to test extract for 10d). Group 6: FAC + Test group-2 (200 µg/ml) (Treated with FAC for 15 d, following exposure to test extract for 10d). The alizarin red staining area and cumulative optical density (IOD) were selected as measures of bone mineralization. Adult zebrafish bone was stained using alizarin red. After the 10 days of treatment the Zebrafish was subjected to following treatment. Zebrafish were anesthetized and fixed in 4% paraformaldehyde at 4°C for 2d. Samples were dehydrated in 75% ethanol for 2d. The abdomen was cut open to increase stain permeability. After that Samples were then subjected to 0.5% alizarin red staining on a shaker for 24h. After staining, samples were briefly washed with water and then soaked in trypsin solution for 3d. After trypsin soaking, samples were submerged in 1% KOH solution for 5d before being gently scraped and scaled using tweezers. There after Samples were finally put in glycerine by soaking in 1% KOH solution for 2 days followed by 100% glycerine. After the successful staining the zebrafish were seen under the stereomicroscope and the images are observed in the ImageJ software for its bone density.

Statistical analysis

The data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed with one-way ANOVA followed by a post-hoc Tukey test.

Result

The post extraction yield percentages for water, hydro alcohol, ethanol, chloroform, and ethyl acetate are 6.9%, 7.9%, 10.48%, 9.55%, and 6.46%, respectively.

Phytochemical Analysis

In the current study, the qualitative phytochemical tests on Chloroform, Ethyl acetate, Ethanol, Hydroalcoholic and Water extracts of *Helianthus annuus* L. seed were

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carried out. Table 1 contains the overall phytochemical test results of different solvent extracts of *Helianthus annuus* L. seed. The tests gave positive results for flavonoids, glycosides, terpenoids and saponins which were suspected to be the potential reasons behind the therapeutic efficacy shown by the extract. Terpenoids were especially found in each extract except the water extract. Terpenoids can show multiple pharmacological activities including promising effects against inflammation and pain.

Table 1: Various phytochemical tests performed on different solvent extracts along with their corresponding results.

Sl. No.	Phytoconstituents	Phytochemical test	Result	
1	Alkaloid	Mayer's test	Methanolic Extract	-
			Ethyl acetate extract	-
			Chloroform extract	-
			Hydroalcoholic	-
			Water extract	+
2	Cardiac glycoside	Bromine water test	Methanolic Extract	-
			Ethyl acetate extract	-
			Chloroform extract	-
			Hydroalcoholic	-
			Water extract	+
3	Flavonoid	Alkaline reagent test	Methanolic Extract	+
			Ethyl acetate extract	-
			Chloroform extract	-
			Hydroalcoholic	+
			Water extract	+
4	Terpenoid	Test for Terpenoid	Methanolic Extract	+
			Ethyl acetate extract	+
			Chloroform extract	+
			Hydroalcoholic	+
			Water extract	-
5	Glycoside	Test for Glycoside	Methanolic Extract	+
			Ethyl acetate extract	-
			Chloroform extract	-
			Hydroalcoholic	

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			Water extract	-
6	Saponin glycoside	Test for Saponin glycoside	Methanolic Extract	-
			Ethyl acetate extract	-
			Chloroform extract	-
			Hydroalcoholic	+
			Water extract	+
7	Carbohydrate	Seliwanoff test	Methanolic Extract	+
			Ethyl acetate extract	-
			Chloroform extract	-
			Hydroalcoholic	
			Water extract	-
8.	Tannin	Gelatin test	Methanolic Extract	
			Ethyl acetate extract	
			Chloroform extract	+
			Hydroalcoholic	-
			Water extract	+

Total Phenolic Content and Total Flavonoid Content

The results are shown in Figure 1, from the values alone, it can be said that all the extracts except the hydroalcoholic contained ample amount of phenols. The chloroform extract contained 872.89 µg of Gallic acid equivalent (GAE)/ mg of extract. This extract had the highest amount of phenols present making it the most effective in extracting phenolic contents. The aqueous extract also had high amount of phenols present as it was calculated to

e 751.66 µg of GAE/mg of extract. Both ethanolic and ethyl acetate extracts showed moderate to high results. The former contained 649.76 µg and the later had 659.74 µg of GAE present in 1 mg of extract. Finally, the hydroalcoholic extract, which had the lowest phenolic content, showed a concentration of 263.44 µg of GAE in 1 mg of extract. For TFC from the results are shown in Fig. 4 the values it can be interpreted that the water and ethyl

acetate extracts yielded the highest amount of Flavonoid content. The aqueous extract had 421.44 µg of Quercetin equivalent (QE)/mg of extract and quite similarly, the ethyl acetate extract also had 415.97 µg of QE/mg of extract. This may indicate that water and ethyl acetate have a much better capacity as a solvent for the flavonoids present in the extract. The ethanolic and chloroform extract also performed well as it had a flavonoid content of 352 µg

of QE/mg of extract and 319.66 µg of QE/mg of extract respectively. The only solvent that had the lowest concentration of flavonoids present in them was the Hydroalcoholic extract. Total Flavonoid Content present in this extract was drastically lesser than the other solvents. It had 168.71 µg of QE/mg of extract, marking it the lowest.

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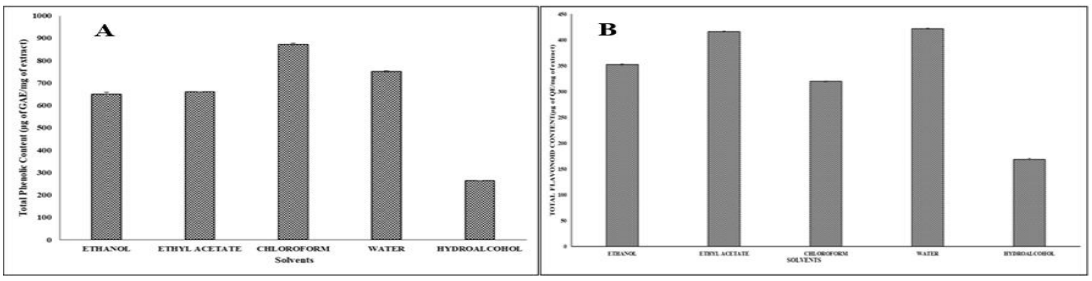

Fig 1: Estimation of Total phenolic content (A) and Total Flavonoid content (B) of various extracts using various solvents. Data are expressed as Mean ± Standard deviation (n=3). The significance from the control group is represented by * when p<0.05.

***In vitro* Antioxidant and Anti-inflammatory Study**

The DPPH free radical scavenging activity of different solvent extracts in the concentration of 100µg/ml are listed in Fig. 5 It is seen that the aqueous extract shows the highest level of

percentage antioxidant activity of 64.6%. Following that, the hydroalcoholic and chloroform extract shows the second highest activity with a negligible contrast being 61.89% and 61.43%. Lastly, The Ethyl acetate extract and Ethanolic extract had 60.13% and 54.28% of antioxidant activity respectively. As shown in Fig. 6, the Chloroform extract shows the highest antioxidant potential with a reducing power of 80.3%. Ethyl acetate and Ethanolic extract also showed very good antioxidant activity, almost equal to chloroform. On the other hand, the water extract had moderate antioxidant activity. It worked but was not as strong as the previous ones. Finally, the hydroalcoholic extract had the lowest antioxidant activity and was the least effective among all. Fig. 7 showcases the anti-inflammatory or protective activity of the extract in different solvent systems (100µg/ml) against protein denaturation. Here, the hydroalcoholic extract has the maximum protective activity against inflammation or stress as it shows a solid 91.14% of inhibition of degradation. Water and ethyl acetate extracts also show quite effective activity as they have 89.32 and 86.86% of inhibitory activity respectively. But the chloroform and ethanolic extracts, being drastically less than previous ones, fail to show any satisfactory effective activity. Their percentage of inhibition is 31.67 and 32.07%. The values are listed in Fig. 8 Each one of the solvent extract systems was able to produce promising results in HRBC Membrane stabilization. Among them, the hydroalcoholic extract produced the highest resistance against haemolysis. The percentage of inhibition against haemolysis was 79.74%. Ethanolic and ethyl acetate extract produced quite similar activity as their inhibitory percentage was 74.42 and 74.78% respectively. Water showed a 75.31% inhibition against haemolysis which was satisfactory. The values were quite close to each other and all of them produced quite good activity, the chloroform extract was the lowest in effectiveness with an inhibitory percentage of 73.70%.

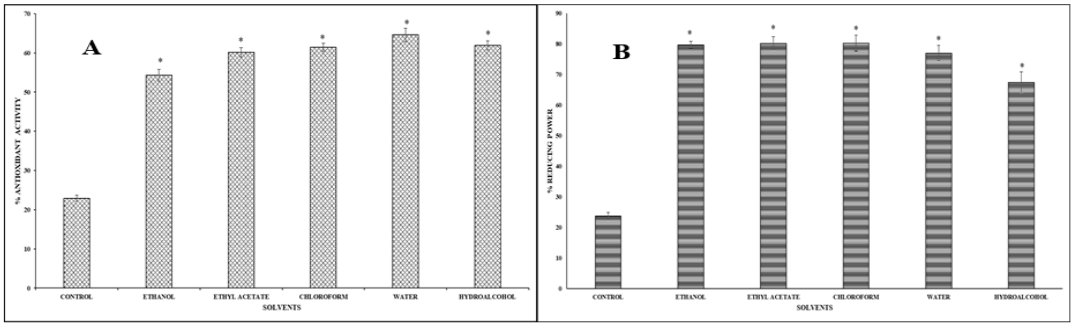

Fig 2: Evaluation of the percentage antioxidant (DPPH free radical scavenging) activity (A) and percentage reducing power (B) of *Helianthus annuus* L. seed extract in different solvents. Data are expressed as mean ± SD (n=3). The significance from the control group is represented by * when p<0.05

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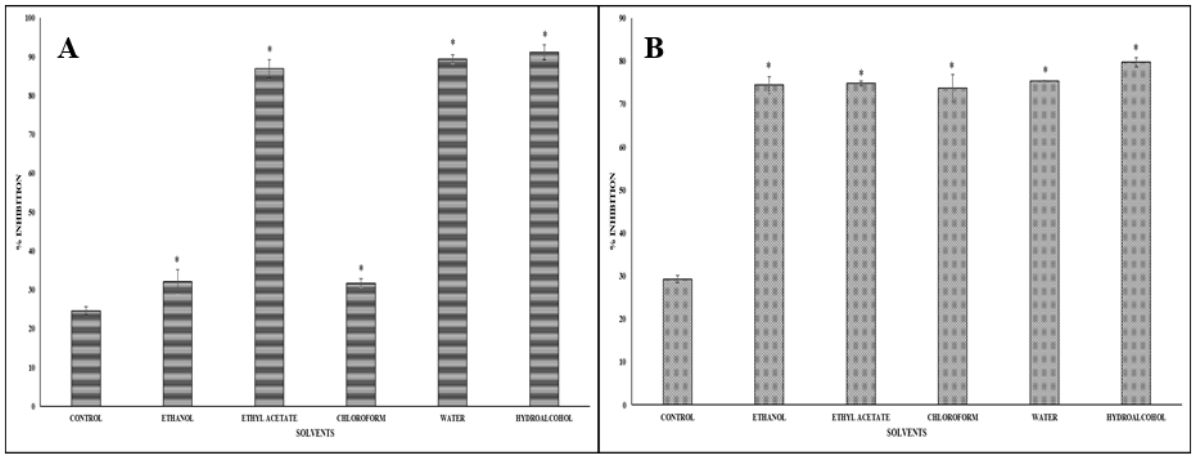

Fig 3: Estimation of the percentage inhibition of protein denaturation by Egg albumin denaturation assay (A) and hemolysis produced by the water extract of *Helianthus annuus* L. seed through HRBC membrane stabilization assay (B) in different solvent systems. Data are expressed as mean ± SD (n=3). Significance from the control group is indicated by * ($p<0.05$).

Finalization of Solvent system:

The initial qualitative phytochemical analysis hinted a suitability of water system as the solvent of choice as the presence of desired phytochemical were plenty in that. Afterwards that initial impression was manifested to be right through multiple qualitative tests where a yield of abundant amount of phytocompounds can be seen. For the reason of such outcome, it can be speculated that the present photoactive components responsible for the desired effects of the compound are polar in nature hence their good solubility in water. Also, as a solvent water is capable of almost no harm on top of being extremely easily available. Therefore water as a solvent system of choice was the most justifiable.

LC-MS Analysis

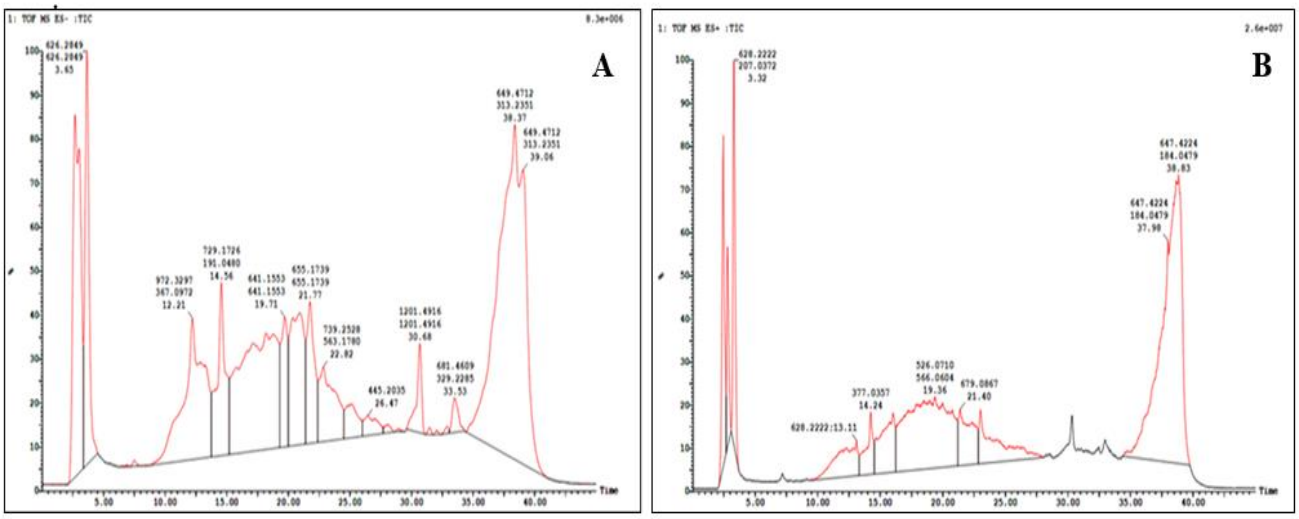

Figure 4: LC-MS chromatograms of the water extract of *Helianthus annuus* L. seed recorded in (A) positive and (B) negative ionization modes

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Table 2: Identified phytoconstituents from the water extract of *Helianthus annuus* L. seed as determined by LC-MS analysis (The resource libraries are Natural Products Atlas, HMDB, ChemSpider, PubChem, PubChem, LipidMaps)

Sl. No	m/z Value	RT (min)	Ionization Mode	Compound Name	Molecular Formula	Area %
1	262.0954	2.79	Positive	Dietziamide A	$C_{13}H_{14}N_2O_4$	2.79%
2	262.0954	2.79	Negative	L-cis-Cyclo(aspartylphenylalanyl)	$C_{13}H_{14}N_2O_4$	5.11%
3	207.0372	3.32	Positive	3-Hydroxybenzoic acid	$C_7H_6O_3$	5.92%
4	377.0357	14.24	Positive	Quercetin-3-O-glucoside (Isoquercitrin)	$C_{21}H_{20}O_{12}$	2.83%
5	191.048	16.55	Negative	Kaempferol-3-O-rutinoside	$C_{27}H_{30}O_{15}$	5.11%
6	526.071	19.36	Positive	Digoxin	$C_{41}H_{64}O_{14}$	21.87%
7	503.1612	2.7	Negative	Dihydromyricetin	$C_{15}H_{12}O_8$	9.85%
8	729.1726	14.56	Negative	Rutin	$C_{27}H_{30}O_{16}$	4.95%
9	649.4712	38.37	Negative	Triacylglycerol (C36:2)	$C_{39}H_{72}O_6$	31.28%

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10	972.3297, 367.0972, 641.2088	12.21	Polyphenolic compound	Proanthocyanidin dimer	$C_{30}H_{26}O_{12}$	9.98%

In vivo study: Zebrafish Embryo Acute Toxicity

Table 3 shows the morphological Observations in a total of 10 groups navigating the different concentrations of test extract ranging from 1000 to 3.125 µg/ml in context of different hpf. Each group contained 3 embryos on which the experiment was executed. In 1000 – 100 µg/ml a complete embryogenic mortality was observed by 96 hpf in this concentration range. Although embryos survived up to 24 hpf, the survival rate rapidly declined afterwards. This may indicate severe toxicity. In 50 µg/ml all the embryos died by 96 hpf. This may suggest witnessing a sub-lethal toxicity at this concentration. Table 3 shows the survival rate of the emryos which showcases in 25 µg/ml by 96 hpf, developmental arrest engulfed the vitality of the remaining embryo. This may also contain a capacity to produce sub-lethal toxicity. The final survival rate was found to be 66.67%. On 12.5 µg/ml the embryos in this group showed an even lower survival rate of 33.3% as by 96 hpf, only 1 of the 3 embryos remained alive. The extract may have been hypersensitive regarding the development of the embryos. Ultimately in 6.25 – 3.125 µg/ml the lowest two doses were the only optimum concentrations where no developmental deformities were observed in the embryos. All the embryos survived up to 96 hpf giving a perfect survival rate of 100%. These findings may suggest a therapeutic window which exists below or at 6.25 µg/ml.

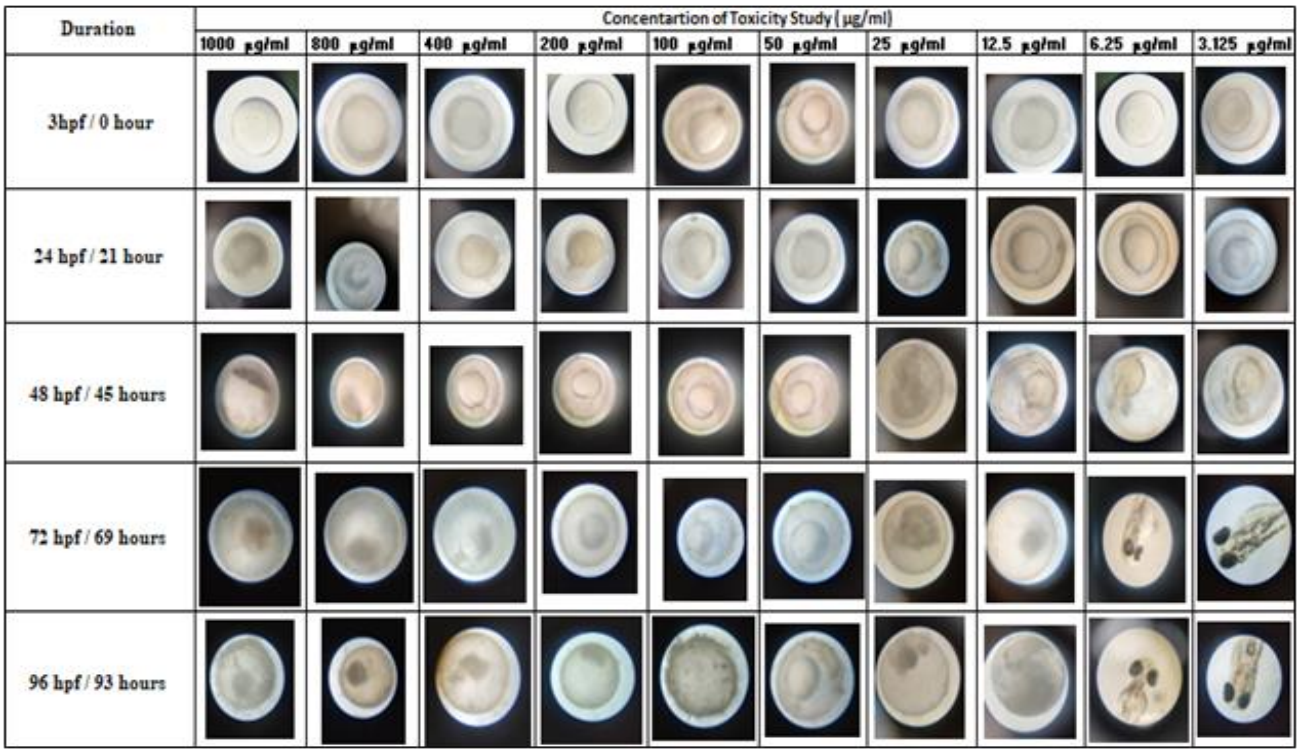

Fig 5: Acute Toxicity Study in different concentrations of *Helianthus annus* L. extract in context of different hpf on zebrafish embryos

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Table 3: Toxicity Study based on Morphological Deviation

Concentration (µg/ml)	No. of Embryo	Duration of Exposure					
		3hpf / 0 hour	24 hpf / 21 hour	48 hpf / 45 hours	72 hpf / 69 hours	96 hpf / 93 hours	Percentage survival
1000 µg/ml	3	All have survived	2 survived	1 survived	All dead	All dead	0
800 µg/ml	3	All have survived	2 survived	1 survived	All dead	All dead	0
400 µg/ml	3	All have survived	2 survived	1 survived	All dead	All dead	0
200 µg/ml	3	All have survived	3 survived	1 survived	All dead	All dead	0
100 µg/ml	3	All have survived	3 survived	2 survived	All dead	All dead	0
50 µg/ml	3	All have survived	3 survived	2 survived	1 survived	Developmental arrest	0
25 µg/ml	3	All have survived	3 survived	3 survived	2 survived	2 survived	66.70%
12.5 µg/ml	3	All have survived	All have survived	2 survived	1 survived	1 survived	33.30%
6.25 µg/ml	3	All have survived	All have survived	All have survived	All have survived	All have survived	100%
3.125 µg/ml	3	All have survived	All have survived	All have survived	All have survived	All have survived	100%

Table 4: Developmental observations of embryos at different life span in various concentrations

Concentration(µg/ml)	Life span(hour)			
	24 hpf / 21 hour	48 hpf / 45 hours	72 hpf / 69 hours	96 hpf / 93 hours
	Developmental Observation			
1000 µg/ml	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No

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800 µg/ml	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No
400 µg/ml	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Slightly present Fin presence- Not Frome Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No
200 µg/ml	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Moderate Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No
100 µg/ml	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No
50 µg/ml	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No
25 µg/ml	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No
12.5 µg/ml	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Normal Fin presence- Visible Hatching rate-
6.25 µg/ml	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Normal Fin presence- Visible Hatching rate- High

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3.125 µg/ml	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Absent Fin presence- Not formed Hatching rate- No	Spinal curvature- Normal Fin presence- Visible Hatching rate- High

Development of osteoporosis in adult zebrafish model

Osteoporosis in adult Zebrafish models are studied because of their high similarity with humans. It is also believed that their bone structure is quite indifferent to human's. In vehicle treated group (no disease induction), the zebrafishes in this group remained in normal Holtfreter's water for the whole process. No Osteoporosis was induced in them. A normal bone structure and density can be seen. Only FAC zebrafishes were placed in the FAC media from the get-go. The induction of Osteoporosis was seen first in them. Their bone condition was much more severe than other groups. Microscopic image depicts the bone condition of the Zebrafishes from this group. In Vehicle + FAC treated group the bone fractures are visible in the zebrafishes, indicating the success of Osteoporosis induction. In standard drug treated group the results were as the Osteoporotic symptoms are clearly reduced or less for the Zebrafish that are clearly visible in microscopic image. In Test 1 (100µg/ml), the results were quite optimum as the bone degeneration for the Zebrafishes seems to be less than has been shown in microscopic image, the results were not satisfactory. In Test 2(200µg/ml), the results which are seen in the Figure 6 are most acceptable. The bone breakdown is stopped to the point that it seems like Osteoporosis was never induced in these Zebrafishes. This specific concentration of the water extract was able to produce a protective effect against Osteoporosis or bone breakdown and able to somehow increase the bone density. This was a remarkable result in its own way.

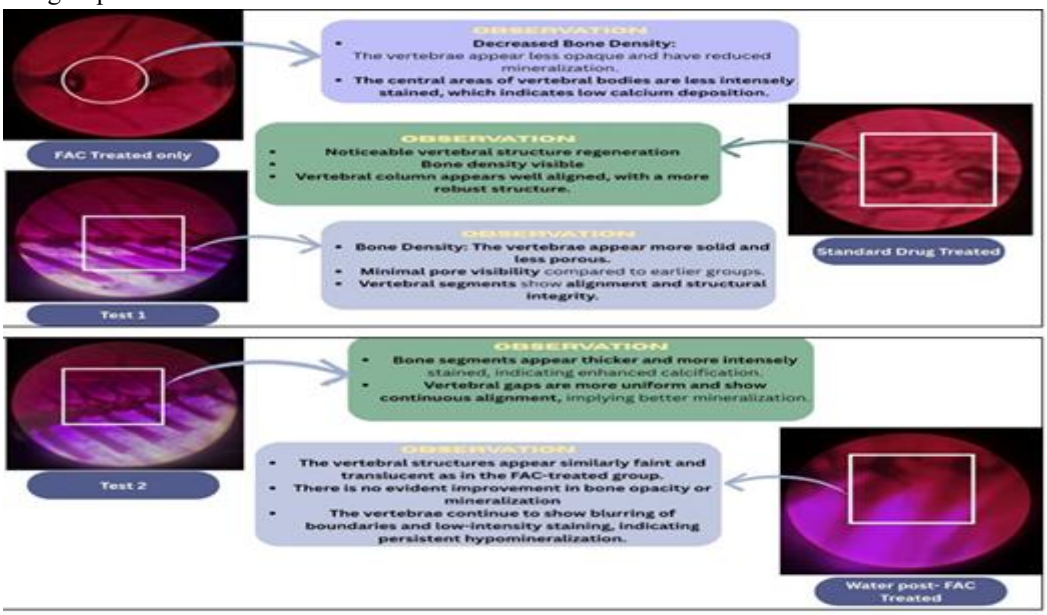

Figure 6: Representative alizarin red–stained images of zebrafish vertebral columns showing changes in bone mineralization following FAC induction and treatments. FAC-treated fish show reduced vertebral opacity and faint staining, indicating hypomineralization. Standard drug and Test treatments improve vertebral alignment and bone density, with Test 2 showing more intense staining and thicker vertebral segments. Water post-FAC fish continue to exhibit faint staining. White boxes indicate regions of interest

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Discussion and Conclusion

The phytochemical tests suggest various rich phytochemical's presence, which can also be justified by the polarity of the solvents. Flavonoid's presence is observed in polar extract like methanol, hydroalcoholic and water extract. Alkaloids and cardiac glycosides are mainly identified in only water, that has potent medical application or physiological effect. Only in non-aqueous extracts, terpenoids are present which are expected due to their nonpolar nature. Terpenoid's presence suggest the possibility of antimicrobial, anti-inflammatory activities [13]. Saponin glycosides are also an indicative compound that are present in hydroalcoholic, and water extract. This suggests the potential of cholesterol lowering effect, antiviral activity [14]. The varying results across different extracts highlight the importance of solvent selection in phytochemical extraction. Total Phenolic content and Total Flavonoid content both aimed to estimate two important phytoconstituents: Phenolic compound and flavonoids. Chloroform extract showed the highest amount of presence of phenolic compound where water is the second highest. Because of the presence of OH group in phenols, they show a potent antioxidant activity, while also having good antimicrobial and cardioprotective activity [4][15]. In flavonoid estimation, water extract proves the highest amount of presence of flavonoid which can cause a promising activity of anti-inflammatory, anti-cancer and neuroprotective action [6][16-17]. The investigation into antioxidant activity is crucial for understanding the potential health benefits of various compounds. The antioxidant property of the sample is checked for the test sample by DPPH free radical scavenging assay and reducing power assay. 1-diphenyl-2-picrylhydrazyl or DPPH is a free radical, that always tends to accept electron from the compound which have a potent antioxidant and transforms into DPPH-H (with an extra hydrogen) by scavenging reduction [18]. This reaction produces a colour change from yellow or colourless to violet. The result showed from DPPH assay, Water and hydroalcoholic extract demonstrated the highest antioxidant activity. While ethanol had the lowest antioxidant property. High antioxidant property of hydroalcoholic and water supports the promising potential as an antioxidant. Selected solvent by screening, water extract, shows increasing antioxidant activity on increasing concentration. On the other hand, one study shows that reducing power assay also helps to justify the antioxidant properties. The main key component of this assay is Fe3+ ion, from potassium ferricyanide which is converted into Fe2+ ion in presence of buffer and heat. This is a prove that the test compound has antioxidant property and it can neutralize the free radicals and act as an antioxidant [19]. Correspondingly in the previous test, water extract is showing a potent activity like DPPH assay, but chloroform, ethyl acetate is also showing a good potency. The anti-inflammatory assay called Heat induced RBC membrane haemolysis assay is very important to elucidate the potential of *Helianthus annuus* L. seed extracts in modulating inflammatory responses. Various endogenous factors like pH change, cytokines and heat can initiate inflammation. For this test the albumin protein of egg (white part of egg) is denatured, and the 3-dimensional structure of the protein is destroyed, and the test compounds potential anti-inflammatory activity is measured against it. Water and hydroalcoholic extract both showed a good potency in having the capacity to resist the protein structure degradation from inflammation characterized by heat. But ethanol and chloroform had the least inhibition capability indicating poor antioxidant activity. Additionally, the human RBC membrane stabilizing assay is also performed as an anti-inflammatory assay. Normally due to heat induction or any mediator-based inflammation, RBC membrane proteins and lipids rupture and the erythrocyte structure uniformity is disrupted. As a result, hemoglobin and other material comes outside and RBC gets hemolyzed. If the test compound has any property of anti-inflammatory, it will protect the RBC membrane from getting hemolyzed by membrane stabilization while also limiting the release of pro-inflammatory cytokines [20]. Here similar to the Egg albumin assay, hydroalcoholic extract showed the best efficacy and water also exhibited a good percentage of anti-inflammatory activity followed by ethyl acetate. The water and hydroalcoholic extract were the best.

LC-MS analysis of the water extract of *Helianthus annuus* L. seed was performed in both positive and negative ionization modes, revealing a complex phytochemical profile composed of several bioactive constituents. A total of ten major compounds were tentatively identified based on accurate m/z values,

retention times (RT), molecular formulas, and relative abundances, many of which have documented pharmacological activities. In the positive ionization mode, Digoxin (m/z 526.071, RT: 19.36 min) was the most prominent compound, comprising 21.87% of the total chromatographic area. Digoxin is a well-established cardiac glycoside with potent positive inotropic activity, acting via Na^+/K^+-ATPase inhibition, and is commonly used in the management of congestive heart failure and atrial fibrillation [21]. 3-Hydroxybenzoic acid (m/z 207.0372, RT: 3.32 min, 5.92%) was also identified and is known for its antioxidant and anti-inflammatory properties, having demonstrated radical scavenging and anti-proliferative effects in vitro [22]. Another phenolic compound, quercetin-3-O-glucoside (isoquercitrin) (m/z 377.0357, RT: 14.24 min, 2.83%), has been widely studied for its neuroprotective, anti-inflammatory, and anti-osteoporotic effects, including enhancement of bone formation and protection against oxidative stress in neuronal cells and osteoblasts [23]. Dietziamide A, a diketopiperazine detected at m/z 262.0954 (RT: 2.79 min, 2.79%), is reported to possess antimicrobial activity, although its pharmacological characterization remains limited and warrants further exploration [24]. In the negative mode, triacylglycerol (C36:2) (m/z 649.4712, RT: 38.37 min) was the most abundant compound with a relative area of 31.28%, representing a major class of neutral lipids that serve as metabolic energy reservoirs but may also reflect lipid metabolic activity in plant tissues [25]. Dihydromyricetin (m/z 503.1612, RT: 2.70 min, 9.85%) is a flavanonol with diverse bioactivities, including hepatoprotective, neuroprotective, antioxidant, and anti-alcohol intoxication effects, supported by evidence of modulation of GABAergic signaling and inhibition of lipid peroxidation [26]. Notably, recent studies have shown that dihydromyricetin also possesses anti-osteoporotic potential by suppressing osteoclast differentiation and activity and improving bone microarchitecture in ovariectomized models [27][28]. Proanthocyanidin dimers (m/z 972.3297, RT: 12.21 min, 9.98%) were also detected and are known for their strong antioxidant, anti-inflammatory, and cardioprotective properties, mediated through the modulation of oxidative stress and endothelial function [29]. The flavonol glycosides rutin (m/z 729.1726, RT: 14.56 min, 4.95%) and kaempferol-3-O-rutinoside (m/z 191.0480, RT: 16.55 min, 5.11%) were identified, both of which exhibit antioxidant, vasoprotective, and anti-inflammatory activities. Importantly, rutin has demonstrated anti-osteoporotic effects through its ability to enhance bone mineral density, inhibit osteoclastogenesis, and stimulate osteoblast differentiation in both in vitro and in vivo models [30-32]. Additionally, L-cis-Cyclo(aspartylphenylalanyl) (m/z 262.0954, RT: 2.79 min, 5.11%), a diketopiperazine derivative, has been reported to possess antitumor and antiviral activities by disrupting protein synthesis and interfering with cellular proliferation [33]. Together, these results highlight the presence of a diverse group of phytochemicals with significant therapeutic potential, including cardiovascular, neuroprotective, anti-inflammatory, antioxidant, and bone-protective (anti-osteoporotic) effects. The identification of these compounds supports the biological relevance of the sample and provides a strong foundation for further pharmacological evaluation. Though the mammalian models are best suitable for determining developmental toxicity, in recent years zebrafish as an alternative model has become quite popular. While fuelling many regulatory studies, zebrafish model with morphological observation developmental toxicity assessed through microscopy is at peak of interest. Defects affecting body curvature, hatching rate, fin development are key signals for toxicity study. By microscopic observation, at 24 hpf, 48 hpf, 72 hpf, 96 hpf the development was studied by microscopy. At the initial 3 hpf, the extract was not showing any effect on the embryos. But at higher concentration like 1000,800, 400 µg/ml, from 24 hpf, the extract was showing its toxicity. As 200 µg/ml and 100 µg/ml are comparatively lower concentration, these are showing effect from 48 hpf, but finally at 96 hpf, all five concentrations are showing their lethal effect. So as a result, the embryos of these concentrations had no development in spinal curvature, presence of fin, and with 0% hatching rate. At lower concentration for 50 µg at 96 hpf, the cell is in developmental arrest stage. But from 6.25 and 3.125 µg/ml the survival rate is 100% and the normal spinal curvature was observed. The fin was also present and with a high hatching rate. Changes in body curvature or absence of fin at 96 hpf, suggest the toxic effect on skeletal muscle might be the reason for malformation.

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This study aimed to evaluate the anti-osteoporotic efficacy of *Helianthus annuus* L. seed extract using an adult Zebrafish model. The experimental induction of osteoporosis in the Zebrafish was by the iron overloading method using FAC as a disease forming particle. It is a well-known and established method of inducing osteoporosis also known as Iron-overloaded osteoporotic model. In this model, the oxidative stress disrupts the balance between the osteoblasts and osteoclasts. The ultimate favour goes to osteoclast activity and as a result high amount of bone resorption occurs. Treatment with *Helianthus annuus* L. seed extract may have countered the deformities produced in the bones and restored the balance. This is supported by visually represented evidence and quantitative measurements. The only FAC treated group served as evidence of successful disease induction. Clear signs of osteoporotic changes can be seen. Furthermore, the reduced opacity of the vertebrae and the staining intensity being faint indicates a low deposition of calcium. As a result, the bone density has been diminished [31]. Observation in water post-FAC group, the vertebrae remained faint as the previous one. No calcification signs were either formed or seen. All of this suggests irreversible damage to the skeletal structure of the fish. An absence of therapeutic protection may have led to this situation. This group's result is a cross verification of the delayed or no treatment scenario after the disease induction as the observed signs were quite like the FAC only group. In the standard drug treated group the results were remarkable. The bone density and structure recovery were astonishing. The vertebral segments or neighboring portions showed enhanced mineralization and especially in calcium deposition. The consistent alignments and distinct features not only revalidated the efficacy of the standard drug but also served as a comparative benchmark for this study. Observations in Test 1 group (100 µg/ml) were quite moderate as the recovery was not that eye catching. On the bright side, the vertebrae appeared to be more solid, and the presence of fractures and pores were much less. This observational restoration suggests that the extract might initiate a bone-protective or barrier-like effect at this dose.

Observing Test 2 group (200 µg/ml) that exhibits the most promising results among all the experimental groups. The segments near the vertebrae were much thicker than before. The extract might have calcification capabilities at this dosage. The uniform vertebral gaps and thick bone density closely resembles the observations obtained from standard drug treated groups. The observed effects are likely to be attributed to the presence of various bioactive chemicals like flavonoids, polyphenols or unsaturated fatty acids. All of them possess strong antioxidants and anti-inflammatory activity. Osteoclastogenesis modulation may become easier in the presence of these compounds.

The study demonstrated that *Helianthus annuus* L. seed extracts possess significant antioxidant, anti-inflammatory, and anti-osteoporotic properties. Phytochemical screening revealed the presence of flavonoids, phenolics, glycosides, and terpenoids, with solvent polarity strongly influencing their extraction. Water and hydroalcoholic extracts showed the highest phenolic and flavonoid content, correlating with superior antioxidant and anti-inflammatory activity. LC–MS analysis confirmed the presence of bioactive compounds such as digoxin, quercetin-3-O-glucoside, dihydromyricetin, rutin, and kaempferol derivatives, which are known for their cardioprotective, neuroprotective, and bone-protective roles. Zebrafish embryotoxicity studies indicated safety at lower concentrations, while the adult iron-overload osteoporotic model revealed marked improvement in vertebral calcification and bone density, particularly at 200 µg/mL extract. These effects may be attributed to the synergistic action of polyphenols and flavonoids in restoring osteoblast–osteoclast balance under oxidative stress. Overall, H. annuus seed extract shows strong therapeutic potential as a natural antioxidant and bone-protective agent, warranting further mechanistic and clinical evaluation.

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Abbreviations:

HAE: *Helianthus annuus* L. extract; WHO: World Health Organization; CD: communicable diseases; OECD: Organization for Economic Co-operation and Development; DPPH: 2,2-diphenyl-1-picrylhydrazyl; UV: Ultraviolet; GAE: Galic Acid Equivalent; QE: Quercetin equivalent; %: Percentage; µg: Microgram; **µl: Microliter; PBS: Phosphate Buffer Solution; NaOH: Sodium Hydroxide; KOH: Potassium Hydroxide; oC: Degree Celsius; gm: Grams; w/v: Weight / Volume; FAC: Ferric Ammonium Citrate; TPC: Total Phenolic Content; TFC: Total Flavonoid Content; rpm: Revolution per minute; MIC: Minimum inhibitory concentration; SSE: Sunflower seed extract; GEC: Galic Acid Equivalents; CAE: Catechin equivalent

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