

Nano-Encapsulated Volatile Oil of *Ruta Graveolens* Leaves Inhibited Glucocorticoid-Induced Osteoporosis in Zebrafish Larval Model

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Abstract

Background: The present research aims to screen anti-resorptive activity to alleviate osteoporosis and other bone and joint disease of the Nano encapsulated essential oil isolated from the *Ruta graveolens* (NELVORG), Family: Rutaceae using a novel, fast, economical and genetically tractable method to evaluate developmental aspects of bone formation in a high throughput fashion through the visualization of embryonic and larval skeleton of zebrafish (*Danio rerio*) model. **Method:** The volatile oil (VO) was isolated from the leaves of *R. graveolens* and extracted VO was subjected to GC-MS Analysis. Nano-encapsulated volatile oil of *R. graveolens* leaves (NELVORG) was prepared by solvent displacement method. The Nanoparticle size was standardized by scanning electron microscope. We implemented 3Rs (Reduction, Replacement, Refinement) ethical principle to minimize harm to the vertebrate animals. Preliminary toxicological studies were evaluated on whole embryo and larvae, showed no mortality up to 1µl/ml. At 3 days post fertilization (3 d.p.f) zebrafish larvae (n=6 / group) were taken in 96 well plates containing embryo medium. Osteoporosis (OP) was induced by immersing zebrafish larvae from 3dpf to 6dpf in embryo medium containing 10µg/ml prednisolone. At 6 d.p.f, the E3 medium containing prednisolone was removed and replaced with the combination of 10µg/ml prednisolone and NELVORG in triplicate till 9 d.p.f was taken as a test group. Alendronate (10µg/ml) and (0.1%) DMSO was taken as a standard and control group respectively. At 9 d.p.f zebrafish larvae were collected and subjected to alizarin red staining for the labelling of the skeleton and the area of stained portion were analyzed using image pro plus analysis to calculate the density of staining. **Results:** The result showed NELVORG significantly prevented Glucocorticoid-induced bone loss and density and comparable to the standard drug Alendronate in zebrafish larval (ZF) model in vivo. So, Nano encapsulated volatile oil of the leaf of *R. graveolens* may be developed as a novel nontoxic potential candidate for the prevention or treatment of osteoporosis.

Keywords: Glucocorticoid, leaf volatile oil, NELVORG, Osteoporosis, *Ruta graveolens*, *Ruta graveolens*, Zebrafish

INTRODUCTION

Traditional systems of medicine have been practiced continuously worldwide on many accounts. Increase in population rise, insufficiency distribution of drugs, expensive treatments, side effects of several synthetic drugs and development of resistance to currently prescribed drugs for infectious diseases have led to increased emphasis on the use of plant

materials as a source of medicines for a wide range of human ailments. Recently, the WHO (World Health Organization) estimated that 80 percent of people worldwide rely on herbal medicines for some aspect of their primary health care needs. According to WHO, around 21,000 plant species have the potential for being used as medicinal plants. Disease of bone loss like osteoporosis contributes to the

major worldwide health problem with an estimated of 100 million people at the risk of developing the disease [27, 28]. A gradual reduction in bone loss in osteoporosis to a point where the skeleton is compromised ends up with bone fragility and susceptibility to the fractures [13]. It was reported that patients taking glucocorticoids (GC) for 6 months or longer, 50% develop osteoporosis with a 30% increase of incidence of fracture. Thus osteoporosis is a major limiting factor in the usage of steroids [1, 36]. Only fewer therapies are prone to be effective in increasing bone mass and improving the defects in bone microarchitecture to establish advanced osteoporosis or for fracture healing improvement.

It has been reported that, the very first tiny fish on earth was susceptible to arthritis. This discovery may fast track the therapeutic research in preventing bone related disability. According to Pixar's Dory, zebra fish and other ray finned fish have synovial fluid that are creaky and susceptible to arthritis. "Developing zebra fish as a first arthritis model – an emerging regenerative model for research to cure arthritis" said Gage Crump. It was also been reported that, zebra fish jaw and fins have certain features that resemble the synovial joints found in mammals.

In our study, the 3Rs ethical principle (Replacement Refinement Reduction) was implemented to minimize harms to vertebrate animals used in science. Recently zebrafish are becoming an established model of several human disease states including for investigating developmental aspects of bone formation [15, 11]. Further the zebra fish genome has been sequenced and shown to contain similarities of genes compared to the human genome with high homology across key protein binding domain in many cases [15].

Volatile oil is valuable natural product found applications in many areas including

pharmaceuticals, cosmetics, perfumes, phytotherapy, spices etc. Attention of many scientists was attracted towards the screening of plants to study the biological activities of oils from phytochemical and pharmacological to therapeutic aspects. This may be hopefully led to new direction on plant applications and new perspective on the potential therapeutic use of the natural products. Volatile oil is a complex mixture comprising of many single components.

The family Rutaceae consists of wide range of aromatic plants and is the largest plant family with approximately 150 genera and 1500 species known for its citrus fruits and also called as citrus family [19, 37]. A variety of plants of this family used in traditional system of medicine worldwide. The most common medicinal plants of this family *R. graveolens* L. commonly known as garden rue. Rue is an ornamental evergreen shrub and has considerable medicinal importance. More than 120 natural compounds including acridone alkaloids, coumarins, essential oil, flavonoids, and furocoumarins were reported in this plant.

In the traditional system of medicine, it is used as stimulant, emmenagogue, diuretic, abortifacient, and resolvent. The local use of this drug along with honey is good for the treatment of paralysis, tremors, joint pains, nerve disorder [35]. It has been used in homeopathy for injuries to the ligaments, tendons, and the periosteum or thinning of bones, overstrain, Carpal tunnel syndrome, eyestrain, hardened or thickened areas or nodules over the bones, periosteum or tendons caused by overuse or injury, back problem, sciatica, lameness after sprains especially ankles, wrists and knees, surgery, ganglion of the wrist, pain in the feet and dental and mouth problems [16].

It was reported that the leaves possess anti-nociceptive [24], anti-parasitic [14],

wound healing [38], antifungal [32], antiulcer [42], antiarrhythmic[21], anti H. pylori [34], The aerial parts showed relaxant activity [2], antioxidant [10], anti-Alzheimer's [3], antimicrobial [33], antibacterial and cytotoxicity[4], antiviral activity [30] and hepatoprotection activity [31], These initiated us to investigate the leaves especially volatile oil of the plant with strict scientific protocol so that the vast economic potential of the crop can be exploited properly. The study aims to scientifically explore the important medicinal use of the volatile oil of this plant on bones which have not been studied.

MATERIALS AND METHODS

Collection and authentication of *Ruta graveolens*:

Leaves of the plant *R. graveolens* were collected from Halieyberiya estate, Idukki district, Kerala, India during the month of July 2016. It was authenticated by Dr. Stephen, Dept. of Botany, The American College, Madurai and Dr. Sasikala, Director (Retd), Siddha Research Institute, Arumbakkam, Chennai.

Extraction of volatile oil from *R.graveolens*:

The fresh leaves of *R.graveolens* hydro distilled in Clevenger apparatus under cold water circulation for 4h. The pale yellow volatile oil was collected in brown vials and stored at 4°C until use.

Identification of compounds present in the volatile oil of leaves of *R. graveolens* by GC-MS analysis:

The extracted volatile oil of *R. graveolens* leaves was subjected to GC-MS analysis for the identification of important constituents and was compared with the standard instrument library.

Nano encapsulation of volatile oil of the *R.graveolens* leaves (NELVORG):

The following steps were involved in the production of Nano encapsulated *R. graveolens* leaves.

- 100mg of ethyl cellulose dissolved in 8ml of DMSO using magnetic stirrer
- 0.5ml of VO of *R. graveolens* was dissolved in 1ml of DMSO using magnetic stirrer.
- Then both the ethyl cellulose solution and VO solution mixed together.
- Then this solution was added drop wise to 30ml of distilled water by a syringe with constant stirring with the help of magnetic stirrer.
- Formation of nanoparticle was observed using microscope [18, 7, 41, 39].

MEASUREMENT OF NANOPARTICLES

Scanning Electron Microscope

SEM is a method for high-resolution imaging of surface. The sample was placed on a glass slide (1×1cm), after rinsing the slide with ethanol. A drop of nanoparticles was evenly distributed over the glass slide and allowed to dry in air. The NPs were subjected to SEM analysis under ambient conditions [18].

PHARMACOLOGICAL STUDY

Preliminary toxicological studies of Volatile oil of *R.graveolens* on zebrafish embryo and larvae. Toxicological studies rely on the utility of vertebrate animals which is expensive in both time and cost with debatable predictive power in safety aspects for a human. To streamline the drug development timeline prioritize drug candidates for animal testing and reduce an unnecessary cost for mammalian studies, drug screening assay using zebrafish is becoming increasingly popular [9, 26].

Whole Embryo Toxicity Study

At around 2-4 h post fertilization (blastula stage) were collected and rinsed several times with water. 200-300 embryos were maintained at 28°C in embryo medium. The development of blastula eggs was monitored at a specified time points (12,

36, 60, &80 hours) under a microscope. Endpoints used for assessing the effect of the drug during the major organ is visible included edema, eye malformation, bent tail, undulated notochord, twisted notochord, and death. Podophyllotoxin used as a standard at 0.01µg/ml concentration.

Larval Toxicity Study

5 d.p.f healthy zebrafish larvae (n=6) released in embryonic medium (10ml) in a petri dish in triplicate. Various concentrations of rue oil 0.5-2µl/ml dissolved in DMSO were treated and two controls (DMSO and embryonic medium). Podophyllotoxin (0.1µg/ml) was used as a standard toxin.

EFFECT ON GC-INDUCED OSTEOPOROSIS

Assay on bone formation and bone density in normal and prednisolone induced inhibition of osteogenesis in larval ZF model. At 3 days post fertilization (3 d.p.f) zebrafish larvae (n=6 / group) were taken in a 96 well plates containing embryo medium (5mM NaCl, 0.17mM KCl, 0.33mM CaCl₂, 0.33mM MgSO₄, methylene blue). OP was induced by immersing zebrafish larvae from 3dpf to 6dpf in E3 medium containing 10µg/ml prednisolone. At 6 d.p.f the E3 medium containing prednisolone was removed and replaced with the combination of 10µg/ml prednisolone and NELVORG (0.1ml/ml) in triplicate till 9 d.p.f was taken as a test group. Alendronate (10µg/ml) and (0.1%) DMSO was taken as a standard and control group respectively. Zebrafish larvae were collected from the well at 9 d.p.f and subjected to alizarin red staining for

labeling of the skeleton.

Skeletal Staining

Bone mineralized matrix deposition was evaluated using Alizarin red S staining, which is a dye gets attached to the calcium salts, stain them and can be easily observed and measured. At 9 d.p.f zebrafish larvae was stained with 0.1% alizarin red s in 0.1% KOH for 2hrs and then can be easily observed under stereomicroscope [40, 20].

Quantifications

Stained zebrafish head and cranial bone portions were observed using stereomicroscope and images was captured using a color view camera. The area and integral optical density (IOD) of the stained portion were quantified using color threshold using image pro plus image analysis software version (6.0) [13, 25]. Statistical analysis was calculated using one way ANOVA by SPSS version 16. Data are presented as mean ± standard deviation, if the p<0.01 statistical difference were considered significant.

RESULTS

Phytochemical analysis of volatile oil: Identification of compounds present in the volatile oil of leaves by GC-MS analysis.

The GC-MS analysis of the isolated V.O indicated the presence of 42 constituents with the presence of 2-Decanone, 2 – Pentadecanone, Eugenol etc. It was reported that the presence of 2-undecanone as the major constituent in the volatile oil of *R. graveolens* leaves which was absent in my analysis. Below compounds were identified.

Table 1: Phytoconstituents present in the VO of *R. graveolens* leaves.

SI No.	Retention Time	Compound
1	5.07	Cyclohexane, 4- isopropeneyl-1-methoxymethoxy methyl-
2	8.37	Cyclohexene,3,4-diethenyl-3-methyl-
3	8.90	2-Decanone
4	9.96	2- Decanone
5	11.74	2 –Pentadecanone
6	12.16	1,7-octadiene,2,7-dimethyl-3,6-bis(methylene)-

7	14.22	Cyclooctane, 1,2-dimethyl-
8	14.73	Eugenol
9	14.90	2- Nonadecanone
10	15.78	Caryophyllene
11	16.53	2- Hexadecanol
12	16.79	Cis-alpha-bisabolene
13	17.14	2-Dodecanone
14	17.71	3-buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-
15	19.05	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl-(1S-Cis)
16	19.22	1,3- benzodioxole, 4-methoxy-6-(2-propenyl)-
17	19.67	Oxirane – decyl
18	20.16	Cyclohexanemethanol, 4-ethenyl-alpha., 4-trimethyl-3-(1-methylethenyl)-
19	20.55	15-octadecenal
20	20.89	Caryophyllene oxide
21	21.79	Hexadecane
22	22.28	Cubenol
23	22.80	.tau.-cadinol
24	23.19	.alpha.-cadinol
25	23.60	Formic acid, 2-phenylethyl ester
26	25.30	Benzofuran,2,3- dihydro-2,2,5,6-tetramethyl-
27	25.77	Phenanthrene
28	26.51	Tetradecanoic acid
29	27.16	Bicycle [2,2,1]hept-2-en-7-ol,7-(4-methoxy phenyl)-,syn-
30	27.69	2-pentadecanone,6,10,14-trimethyl
31	28.97	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
32	29.50	Hexadecanoic acid, methyl ester
33	29.96	1,2-benzenedicarboxylic acid, butyl octyl ester
34	30.86	n-Hexadecanoic acid
35	31.59	1-(2-methoxy-5-methyl-benzyl)-3-nitro-1H-(1,2,4) triazole
36	32.84	Trans-13-octadecenoic acid, methyl ester
37	34.33	Octadecanoic acid
38	39.46	10-Heneicosene
39	41.08	2-methy octacosane
40	41.45	2(1H)-phenanthrene,3,4,49,9,10,10a-hexahydro-6-methoxy-1,1,4a-trimethyl-7-(1)
41	42.18	2-amino-7,10-dimethyldibenzo(b,f)(1,4) oxazepine-11(10H)-one tms
42	42.58	2-methyl octacosane

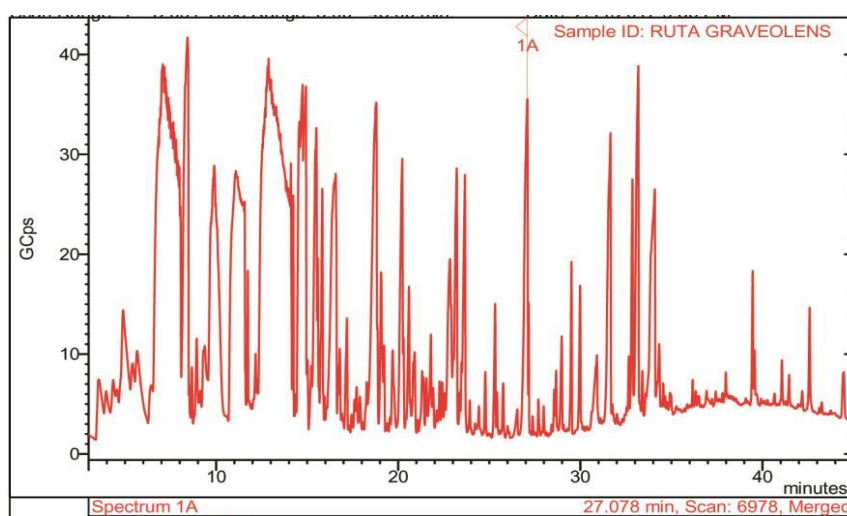


Figure 1: GC-MS PROFILE OF THE V O OF THE *R. graveolens* leaves.

Nanoencapsulation

The nanoparticles formed were measured to ascertain their size distribution by

intensity. SEM analysis depicted the size of nanoparticles to be 162nm with quite uniformity in Nano size.

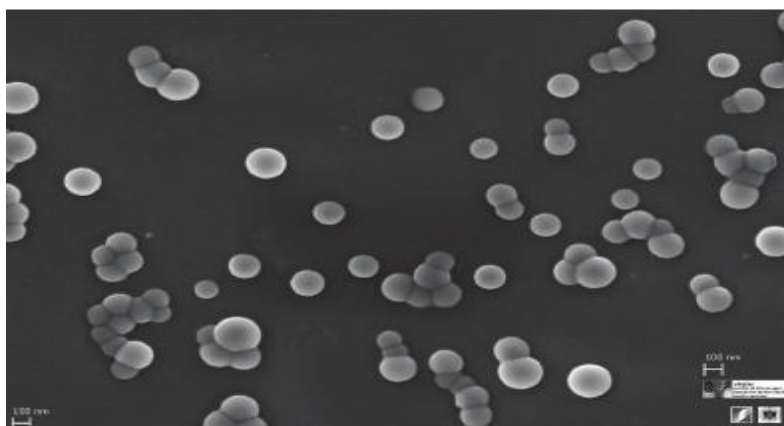


Figure 2: NELVORG under SEM.

TOXICOLOGICAL STUDIES

Whole Embryo Toxicity Study

In our study, the 3Rs ethical principle (Replacement Refinement Reduction) was implemented to minimize harms to vertebrate animals used in science. Effects of VO on developmental stages revealed no malformations and incidence of mortality up to 1µl/ml concentration level but medium to strong edema, eye malformations, bent tail, weak undulated notochord, and twisted notochord were observed at 2µl/ml concentration till 80hpf. No mortality was observed at this concentration level. Total mortality was observed in the standard podophyllotoxin at 0.01µg/ml [34]. These observations

showed no pronounced retardation in zebrafish embryo development when exposed to a normal concentration which indicated that volatile oil would pose no hazard to early stages of *Danio rerio*.

Larval Toxicity Study

Zebrafish larval toxicity study revealed that no mortality was observed up to 1µl/ml but 5% mortality was observed at 2µl/ml concentrations. No mortality was observed in both the controls. 100% mortality was found at podophyllotoxin at 0.1µg/ml concentrations (Fig. 2). This study showed no significant mortality or malformation in zebrafish larvae at 24 hours of exposure in normal concentration.

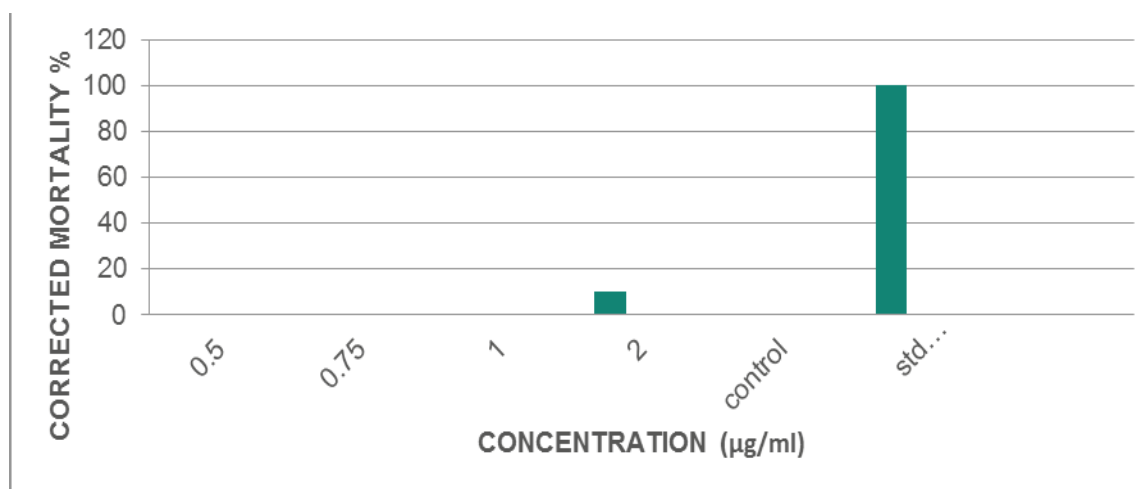


Figure 3: Zebra fish larval toxicity study

Effect on GC induced inhibition of osteogenesis:

The present study revealed that NELVORG can prevent GC induced inhibition of osteogenesis on zebrafish larval model. 10µg/ml prednisolone elicited a significant decrease in area and IOD of the stained larval skull from 3 to 9

dpf when compared to the control. Alendronate 10µg/ml and NELVORG showed increased stained area and IOD ($p < 0.01$) Figure (4, 5, 6), so that can prevent GC-induced inhibition of osteogenesis. It indicates that NELVORG attenuates prednisolone induced inhibition of bone mineralization in ZF larvae.

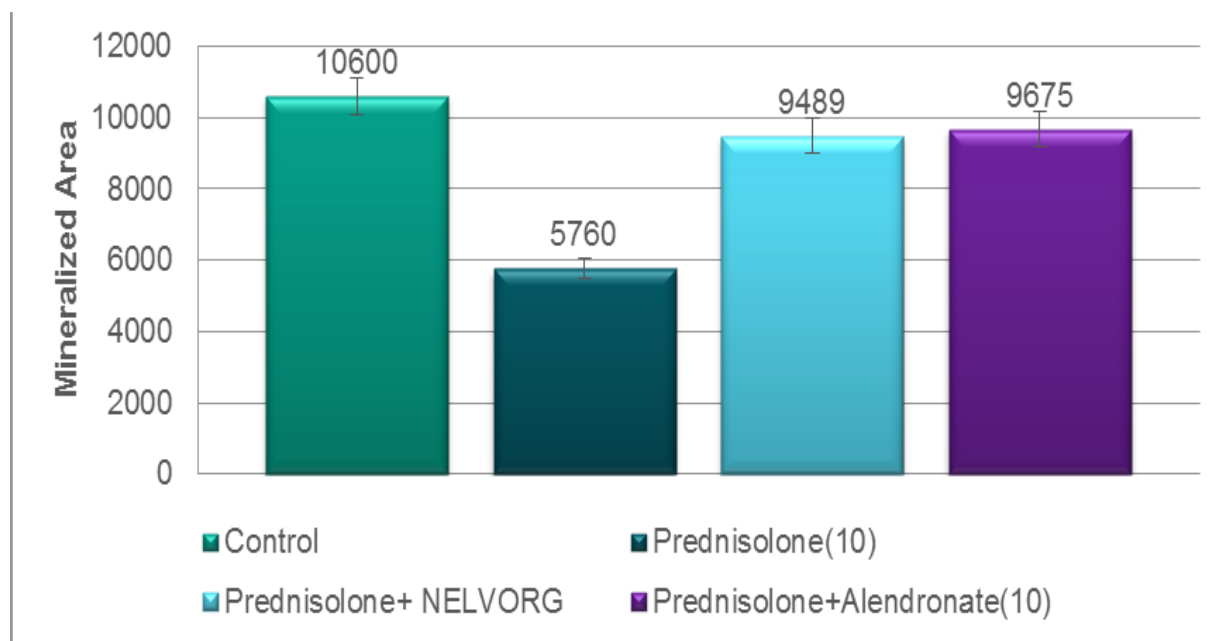


Figure 4: Effect of bone area on Prednisolone induced bone loss

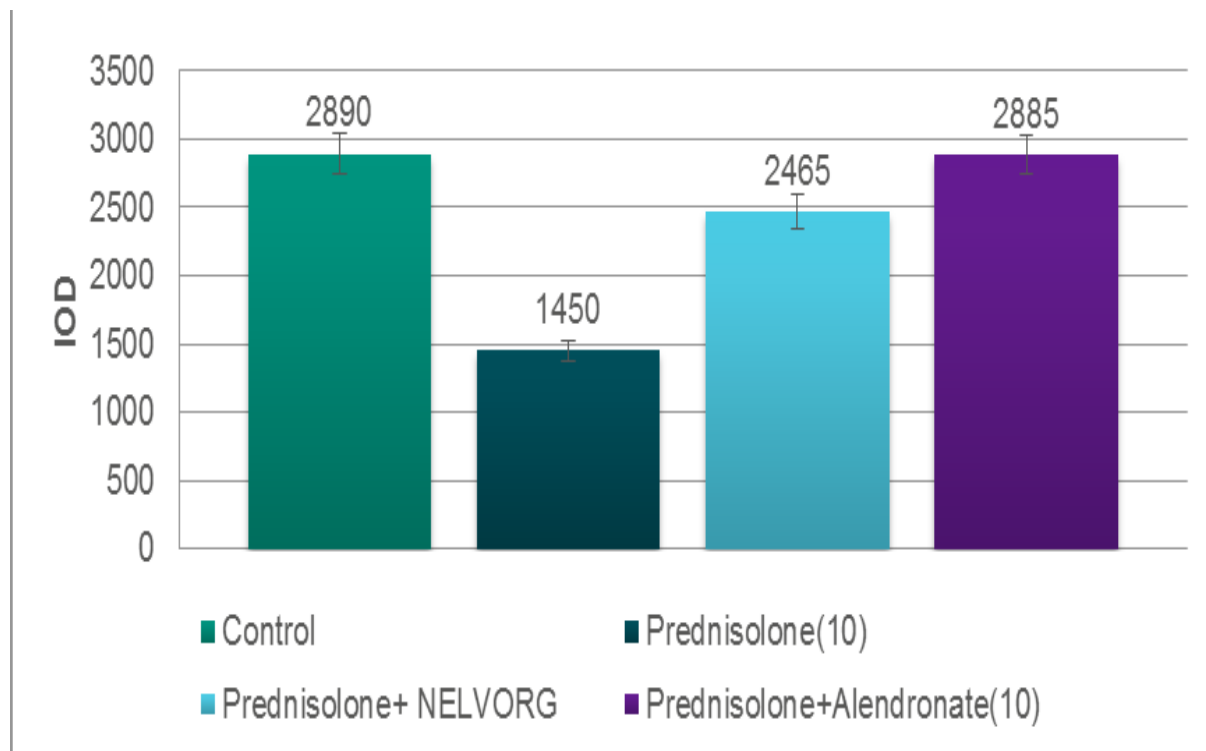
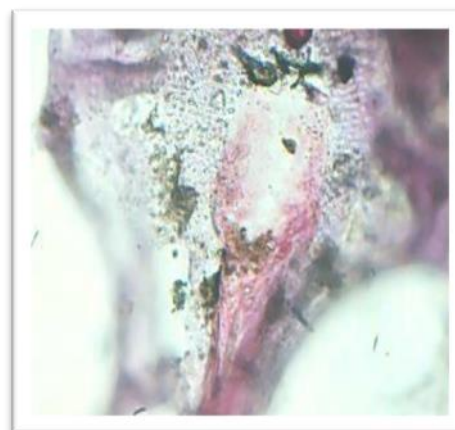


Figure 5: Effect of bone density on Prednisolone induced bone loss



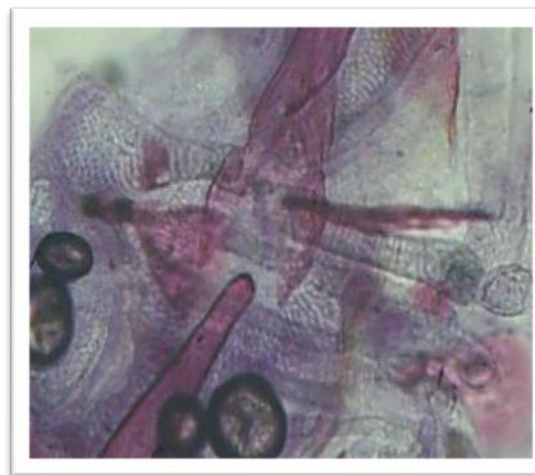
a) Control: 0.1% DMSO



b) Prednisolone treated (10µg/ml)



b) NELVORG



d) Standard Alendronate (10µg/ml).

Figure 6: Effect on bone mineralization of *zf* larval skull-glucocorticoid-induced osteoporosis.

DISCUSSION

A previous study reported containing 34 components in the volatile oil of the leaves of *R. graveolens* [29]. As a polyphenolic compound show an anti-oxidative effect and is thought of as one of the most effective natural product of antioxidants. Increasing pharmacological data have indicated that volatile oil has the following properties such as antibacterial, an effective fungicide, anti-tumor properties, have the ability to prevent transmission of some drug resistance strains of pathogens antispasmodic, diuretic, antiseptics, local anesthetic, in dentistry and aromatherapy. GC-MS Analysis of volatile oil of leaves showed important constituents presence

and was compared with the instrument library.

The toxicological study reveals that the volatile oil of the leaves of *R. graveolens* is nontoxic and safe. Encapsulation of biologically active components represents a feasible and efficient method to modulate drug release, increase the physical stability of the active components, protect from interaction with the environment, decrease their volatility, enhance their bioactivity, and reduce toxicity and convenience.

Modified drug delivery system has advantages over conventional preparations

like reduced toxicity, convenience and optimization of the delayed drug release at a specific rate additionally due to the subcellular size, they may tend to increase cellular absorption mechanism and increase bio-efficacy. Volatile oil loaded Nano delivery system designed to possess a number of special features for therapy like sustained and controlled release drug locally, cellular uptake and subcellular trafficking, deep tissue penetration etc. We prepared Nano encapsulated volatile oil (NELVORG) and its size was determined using SEM.

Zebrafish is a new type of ideal model which possesses several advantages, including extra-uterine development, small size, short generation time, optically transparent embryos, strong regeneration ability, and genomic conservation between zebrafish and humans [15]. By contrast, rodent animals such as rats, mice, and rabbits have too many limitations including long cycles, large expense, high labor-intensity, limited sensitivity, and unsuitable testing for trace ingredients [33, 34]. In particular, zebrafish had high similarity with humans in terms of bone architecture, bone cells, matrix proteins, and molecular signaling, suitable for the screening of agents to prevent and treat osteoporosis [5, 23]. Moreover, the cranial bone of zebrafish larvae develops in approximately 1 week from 3- dpf to 9-dpf with two kinds of osteogenesis similar to humans, including endochondral and intramembranous ossification [17].

This investigation showed that NELVORG can prevent GC induced inhibition of osteogenesis in the presence of prednisolone. According to the results shown in (Fig. 4, 5, 6,) prednisolone 10µg/ml elicited a significant decrease of the area and IOD stained with alizarin red in larval skull compared with the control and Alendronate standard drug, reversed the decrease of the stained area and IOD. NELVORG hampered the inhibition of prednisolone on the area and IOD of the

cranial bone in larvae as a climax comparable to the standard drug. It indicates that NELVORG attenuates the prednisolone induced inhibition of bone mineralization in zebrafish larvae ($p < 0.001$)

CONCLUSIONS

Our data showed NELVORG significantly prevented the glucocorticoid-induced bone loss and density and comparable to the standard drug alendronate in ZF larval model in vivo.

Nano-encapsulated volatile oil of the leaf of *R. graveolens* may be developed as a potential candidate for the prevention or treatment of osteoporosis.

In conclusion, the leaves of *R. graveolens* may be further investigated for the development as novel nontoxic preventive/to be an effective therapy in increasing bone mass and improving bone microarchitecture characteristic in established and advanced osteoporosis or for accelerating fracture healing alternative to the existing bisphosphonates.

But to confirm our findings further investigations of this effect to the mammalian model is necessary. Further pharmacokinetic studies are also required to understand the post metabolism ingredients along with the clinical efficacy and safety in human.

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CONFLICT OF INTEREST

I have no conflict of interest to declare.

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