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ANABOLIC EFFECT ON BONE FORMATION OF NANO ENCAPSULATED VOLATILE OIL OF *RUTA GRAVEOLENS* LEAVES IN LARVAL ZEBRAFISH MODEL

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Keywords:

Osteoporosis, Zebrafish, Anabolic effect, Teriparatide, Alendronate, Nano-encapsulated volatile oil of *R. graveolens* (NELVORG)

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ABSTRACT: Aim: The present research aims to screen anabolic activity on bone formation and bone density in normal and Teriparatide (Rh PTH biosynthetic human parathyroid hormone 1-34) induced inhibition of osteogenesis in zebrafish larval model to alleviate osteoporosis and other bone and joint disease or disorders of the Nano encapsulated essential oil isolated from the *Ruta graveolens* (NELVORG) Family: Rutaceae using a novel, fast, economical and genetically tractable method for screening 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. The nano-encapsulated volatile oil of *R. graveolens* leaves (NELVORG) was prepared by solvent displacement method, and particle size was standardized by scanning electron microscope (SEM). 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. Zebrafish larvae (n=6) at 3dpf were taken in 24 well plates containing embryo medium. 30ng/ml teriparatide was made in 20mM sodium dihydrogen phosphate, 0.9% sodium chloride, and 2.13% g/l mannitol were added to the well from 3 d.p.f to 6 d.p.f to induce catabolic effects in bones. At 6 d.p.f embryo medium containing 30ng/ml Teriparatide were removed and replaced with the combination of 30ng/ml teriparatide + NELVORG in triplicates till 9 d.p.f.10µg/ml of Alendronate used as a standard drug and 0.1% DMSO was a vehicle control. Zebrafish larvae were collected from the well at 9dpf and subjected to alizarin red staining for quantification and area of stained portion were analysed using image pro plus to calculate the density of staining. **Results:** The result showed NELVORG significantly protected the catabolic effect of teriparatide and rescue bone loss and density and comparable to the standard drug alendronate in zebrafish larval 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.

INTRODUCTION: Disease of bone loss like osteoporosis contributes to the major worldwide health problem with an estimated 100 million people at the risk of developing disease [1, 2].

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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.

It was reported that intermittent exposure of parathyroid hormone (PTH) in humans results in dose-dependent increases in the formation of mineralized bone, whereas continuous exposure to PTH results in net bone loss [3]. Only fewer therapies are prone to be effective in increasing bone mass and improving the defects in bone micro-

architecture to establish advanced osteoporosis or for fracture healing improvement.

In our study, the 3Rs ethical principle (Replacement Refinement Reduction) was implemented to minimize harms to vertebrate animals used in science. Recently zebrafish is becoming an established model of several human disease states including for investigating developmental aspects of bone formation [4, 5.] Further, the zebrafish 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 [4].

Volatile oil (VO) is a valuable natural product found applications in many areas including pharmaceuticals, cosmetics, perfumes, phyto-therapy, spices, *etc*. The 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 a new direction on plant applications and a new perspective on the potential therapeutic use of natural products. The volatile oil is a complex mixture comprising of many single components.

Many researchers worked on the development of drug formulations which mainly focuses on the framing of delivery systems, targets for the delayed and sustained release after administration. These kinds of formulations are known as Modified drug delivery system and have advantages over conventional preparations. Some advantages are improved efficacy, reduced toxicity, improved patient compliance and convenience and optimization of the delayed drug release from these formulations. Controlled drug release is a method which allows controlling time and the site of drug release at a specific rate. The colloidal system plays an important role in the pharmaceutical research field, among the controlled drug release. Thus, a colloidal particle ranges in size on the nanometric scale and known as Nanoparticles. Nanoparticles are designed by taking the following consideration, to achieve site-specific action of the drug at a therapeutic rate and dose regimen control of particle size, morphology, the release of bioactive chemicals.

Nanoparticles were first developed during the 1970s for carrying of vaccines and anti-cancer drugs. Controlling the release of drug molecules, Nanocarriers protect essential oils against thermal or photodegradation which provides, increased stability, flavor, and function and extending the final product shelf life.

The family Rutaceae consists of the 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 a citrus family [6, 7]. 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, and abortifacient. The local use of this drug along with honey is good for the treatment of paralysis, tremors, joint pains, nervine disorder [8]. 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 [9].

It was reported that the leaves possess antinociceptive [10], antiparasitic [11], wound healing [12], antifungal [13], antiulcer [14], antiarrhythmic [15], anti-*H. pylori* [16]. The aerial parts showed relaxant activity [17], antioxidant [18] anti-Alzheimer's [19], antimicrobial [20], antibacterial and cytotoxicity [21], antiviral activity [22] and hepatoprotective activity [23]. These initiated us to investigate the leaves especially volatile oil of the plant with the 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 a bone which has not been studied.

MATERIALS AND METHODS:

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

FIG. 1: HABIT AND HABITAT OF *R. GRAVEOLENS*

Extraction of Volatile oil from *R. graveolens*: The fresh leaves of *R. graveolens* hydrodistilled in Clevenger apparatus under cold water circulation for 4 h. The pale yellow volatile oil was collected in brown vials and stored at 4 ºC 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.

Nanoencapsulation of Volatile Oil of the *R. graveolens* Leaves (NELVORG): The following steps were involved in the production of nano-encapsulated *R. graveolens* leaves.

- 100 mg of ethyl cellulose dissolved in 8 ml of DMSO using a magnetic stirrer.
- 0.5ml of VO of *R. graveolens* was dissolved in 1ml of DMSO using magnetic stirrer.
- Then both the ethylcellulose solution and VO solution mixed.
- Then this solution was added dropwise to 30 ml of distilled water by a syringe with constant stirring with the help of magnetic stirrer.
- Formation of nanoparticle was observed using microscope [24, 25, 26, 27].

Measurement of Nanoparticles:

Scanning Electron Microscope: SEM is a method for high-resolution imaging of the 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 nanoparticles were subjected to SEM analysis under ambient conditions to study a particle size [24].

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 mammalians studies, drug screening assay using zebrafish is becoming increasingly popular [28, 29].

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 ºC in embryo medium. The development of blastula eggs was monitored at a specified time points (12, 36, 60, & 80 h) under a microscope. Endpoints used for assessing the effect of the drug during the major organ is visibly 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.

Anabolic Effect on Bone Formation:

Assay on Anabolic Effect of Bone Formation and bone density in normal and Teriparatide Induced Inhibition of Osteogenesis in Larval zf Mode: Zebrafish larvae (n=6) at 3 days post fertilization (d.p.f) were taken in a 24 well plates

containing embryo medium (5mM NaCl, 0.17mM Kcl, 0.33mM $CaCl_2$, 0.33mM Mg_2SO_4, methylene blue). 30ng/ml teriparatide was made in 20mM sodium dihydrogen phosphate, 0.9% sodium chloride, and 2.13% g/l mannitol were added to the well from 3 d.p.f to 6 d.p.f to induce catabolic effects on bones. At 6 d.p.f embryo medium containing 30ng/ml, teriparatide was removed and replaced with the combination of 30ng/ml teriparatide + NELVORG in triplicates till 9 d.p.f. 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 that 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 2 h and then can be easily observed under stereomicroscope [30, 31].

Quantifications: Stained zebrafish head and cranial bone portions were observed using stereo-microscope and images was captured using a color view camera. The area and integrated optical density (IOD) of the stained portion were quantified using color threshold using image pro plus image analysis software version (6.0) [3, 32].

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*. Soleimani *et al*., 2009, Melnyk *et al*., 2015 reported the presence of 2-undecanone as the major constituent in the volatile oil of *R. graveolens* leaves which was absent in my analysis.

GC-MS Profile of the Volatile Oil of the *R. graveolens* leaves:

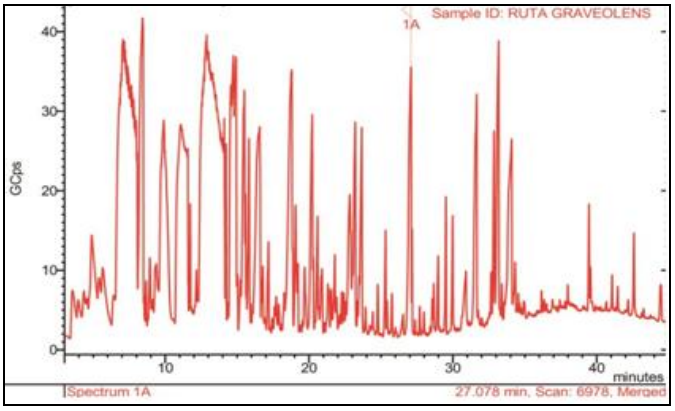

FIG. 2: GC-MS PROFILE

TABLE 1: PHYTOCONSITUTIENTS PRESENT IN THE VOLATILE OIL OF *R. GRAVEOLENS* LEAVES

S. 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(methlene)-
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

Nano-Encapsulation: The nanoparticles formed were measured to ascertain their size distribution by intensity. SEM analysis depicted the size of nanoparticles to be 162 nm with quite a uniformity in nano size.

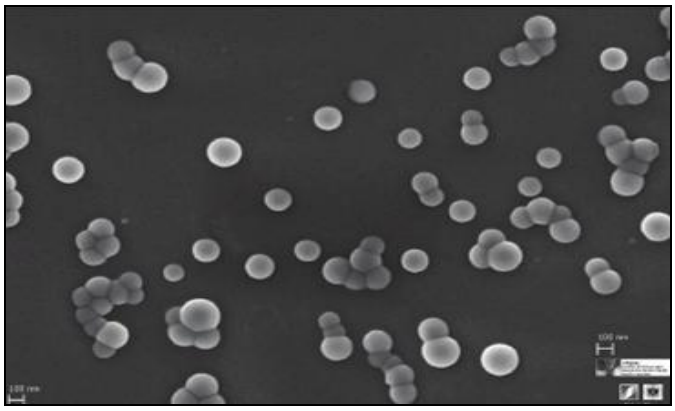

FIG. 3: NELVORG UNDER SEM

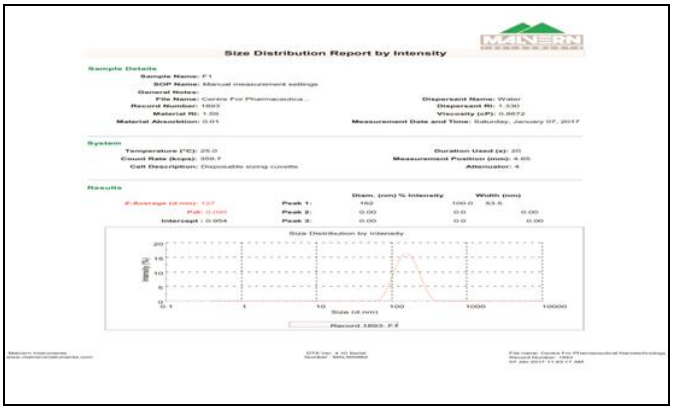

FIG. 4: SIZE DISTRIBUTION REPORT OF NANO PARTICLES

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 Volatile oil of *R. graveolens* 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 [16].

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. 5**. This study showed no significant mortality or malformation in zebrafish larvae at 24 h exposure in normal concentration.

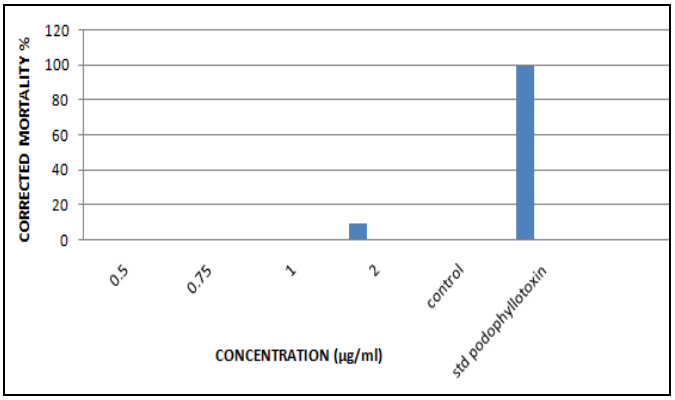

FIG. 5: ZEBRAFISH LARVAL TOXICITY STUDY

Anabolic Effect on Bone Formation:

Assay on Anabolic Effect of Bone Formation and Bone Density in Normal and Teriparatide Induced Inhibition of Osteogenesis in the Larval Zebrafish Model: An anabolic effect of NELVORG has been evaluated in continuous exposure of Teriparatide induced bone loss in zebrafish larvae. Teriparatide (30µg/ml) is a drug used for the treatment of anabolic bone formation at intermittent exposure, while continuous exposure leads to bone demineralization. Zebrafish larvae exposed continuously to Teriparatide (30µg/ml) from 3 d.p.f to 9 d.p.f showed a marked decrease in bone demineralization or loss and IOD showed a dose-dependent reduction in bone mineralization when compared to control (0.1% DMSO).

Alendronate used as standard drug reversed the decrease of the stained area and IOD. Image analysis of the stained area of NELVORG demonstrated an increase in mineralized area. The result was statistically significant ($p<0.001$) **Fig. 6, 7**. Hence, NERVORG showed a statistically significant reduction in bone loss by preventing the inhibition of osteogenesis.

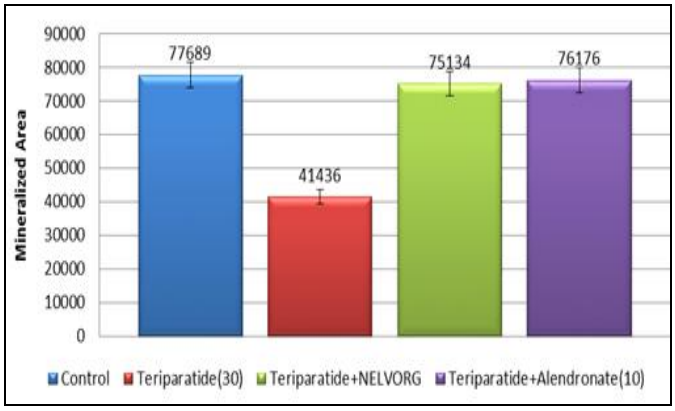

FIG. 6: EFFECT ON BONE AREA ON TERIPARATIDE INDUCED BONE LOSS

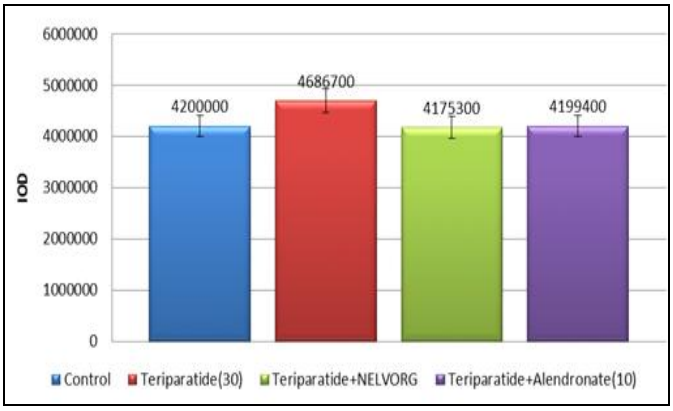

FIG. 7: EFFECT ON BONE DENSITY ON TERIPARATIDE INDUCED BONE LOSS

Effect on Bone Mineralisation of zf Larval Skull-Teriparatide Induced Catabolism: Areas of calcified matrix stained red- ossification seen in the perichordal sheath and coracoids processes.

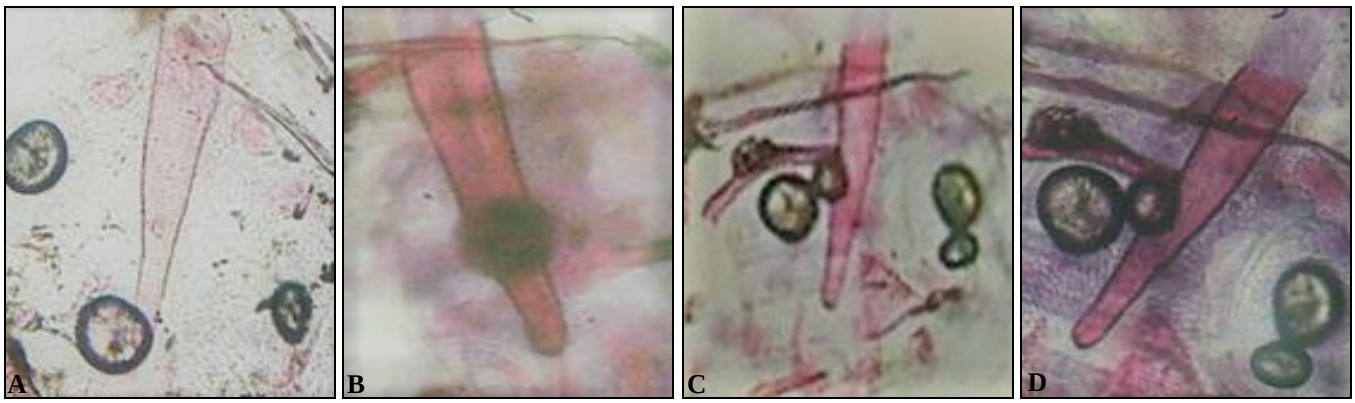

FIG. 8: AREAS OF CALCIFIED MATRIX STAINED RED- OSSIFICATION SEEN IN THE PERICHORDAL SHEATH AND CORACOIDS PROCESSES. A - Continuous exposure of larval zebrafish at 9 days postfertilization (dpf) to parathyroid hormone (PTH) Teriparatide results in demineralization of bone. B - Control shows areas of the calcified matrix are stained red. C - A significant anabolic effect was observed with NELVORG; there is an increase in the density of staining, which reveals an increase in mean mineralized area. D - Standard drug Alendronate.

DISCUSSION: The previous study reported containing 34 components in the volatile oil of the leaves of *R. graveolens* [43]. As a polyphenolic compound show an antioxidative 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, can prevent transmission of some drug resistance strains of pathogens antispasmodic, diuretic, antiseptics, local anesthetic, in dentistry and aromatherapy. GC-MS Analysis of the 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 non-toxic 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 bioefficacy. Volatile oil loaded Nano delivery system designed to possess several 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 extrauterine development, small size, short generation time, optically transparent embryos, strong regeneration ability, and genomic conservation between zebrafish and humans [4]. By contrast, rodential 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 [35, 36]. 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 intra-membranous ossification [37].

Zebrafish bones resemble mammalian bones, with both intramembranous and endochondral ossification being seen in the craniofacial skeleton [38, 39]. The bones are vascularized, innervated, and contain cavities filled with adipose tissue [40]. Osteoblasts and osteocytes are also seen in larval zebrafish bone. Hence, larval zebrafish bones contain the necessary cells for both bone formation and resorption activity.

Studies on the action of PTH on bone mass have produced conflicting results [43]. It has been demonstrated that PTH has an anabolic or OP-rescuing effect when given as a single high dose but has a catabolic effect when given at a lower dose for a longer duration [44, 45]. Clinically, once-daily administration of PTH stimulates new bone formation, but continuous exposure has been likened to hyperparathyroidism, stimulating bone resorption more than formation [46]. We have examined the role of PTH in our model (Zebrafish) by continuous exposures. A catabolic effect was observed at all doses when PTH (teriparatide) was administered continuously. Our results demonstrate that the mineralized area gives the most robust readout of anabolic changes, whereas optical density measurements are most informative for studying bone loss. The viability of zebrafish was unaffected by the continuous presence of the highest dose of PTH.

Continuous exposure to PTH resulted in a striking decrease in mineralized bone after 6 days of treatment (A), when compared to control (B). Image analysis of mineralized area revealed only a statistically significant loss of bone at 30 ng/ml PTH whereas IOD analysis revealed a significant dose-dependent reduction in mineralization ($p<0.01$). NERVORG showed a statistically significant reduction in bone loss by preventing the

inhibition of osteogenesis (C) and the results were compared with the standard bisphosphonates (Alendronate) (D). In conclusion, we have demonstrated that larval zebrafish provide a valid assay system for bone anabolism, with similar effects to mammals being seen following administration of Teriparatide. NELVORG effectively protected the magnitude of the decrease in mineralization of bone

CONCLUSION: The present investigation highlights the pharmacognostical, phytochemical and pharmacological studies of the leaves of *R. graveolens* L. family Rutaceae and potential protective effect of Nano encapsulated oil on anabolic bone formation in zebrafish larval model. NELVORG protected the catabolic effect of teriparatide (Rh PTH, biosynthetic human parathyroid hormone 1-34) after continuous exposure during bone formation on *in-vivo* larval zebrafish.

The 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: Author have no conflict of interest to declare.

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