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Essential oil of *Citrus limetta* Risso.(Rutaceae) leaf inhibited prednisolone induced osteoporosis in zebra fish larvae model

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ABSTRACT

The present study aims to investigate anti-resorptive activity to treat osteoporosis and other bone and joint disorders of the essential oil isolated from the *C.limetta* leaves 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.The essential oil was isolated from the leaves of *C.limetta* EOCLL and extracted EO was subjected to GC-MS Analysis. 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. Osteoporosis (OP) was induced by immersing zebrafish larvae from 3dpf to 6dpf (day post fertilisation) in embryo medium containing 10µg/ml prednisolone. At 6 dpf, the E3 medium containing prednisolone was removed and replaced with the combination of 10µg/ml prednisolone and EOCLL in triplicate till 9 dpf was taken as a test group. Alendronate (10µg/ml) and (0.1%) DMSO was taken as a standard and control group respectively. At 9 dpf zebrafish larvae subjected to alizarin red staining for the labelling of the skeleton and the area of stained portion was analyzed using image pro plus analysis to calculate the density of staining. Results: The result showed EOCLL significantly prevented Prednisolone induced bone loss and density and comparable to the standard drug Alendronate in zebrafish larval (ZF) model *in vivo*. So, EOCLL may be developed as a novel nontoxic potential candidate for the prevention or treatment of osteoporosis.

Keywords: *Prednisolone, leaf essential oil, Osteoporosis, Citrus limetta, Zebrafish larvae model.*

1. 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 [1,2]. 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 [3]. 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 [4,5]. 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 [6,7]. 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[6]. 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. Essential 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 [8,9]. A variety of plants of this family used in traditional system of medicine worldwide. *Citrus limetta* (Rutaceae) is popularly known, kolumichai in Tamil, It was reported that the GC-MS analysis of the isolated E.O from the leaves indicated the presence of following constituents D.Limonene, α –pinene, α-terpineol, citronelal, α– bisbolol, camphene, β- bisabolene, nonanal, borneol, geranial, linalool, bergamol.

The survey of literature on *C.limetta* also reveals that leaves contain various phytoconstituents like alkaloid especially flavonoid compounds. Leaves of *C.limetta* is traditionally known to be useful for the treatment of wide panel of diseases like epileptic seizures, hypertension, prostate disorder, giddiness, rheumatism, wound infection, cough, sore throat, fever, wounds, tooth decay, gastric ulceration, reproductive problems, etc. Various scientific investigation of the leaves showed and as antioxidant, anti- platelet aggregation, antimicrobial, sedative, digestive, hemostatics, diuretics, analgesics, anti-inflammatory and cardiovascular diseases. anti-diabetic activity, antiulcer, hepato- protective, antihypertensive and wound healing activity. Root traditionally used for liver diseases, tuberculosis, asthma, diabetes, hypertension, toothaches, anaemia, etc. Fruit is used for malaria, stomach pain, liver and spleen enlargement, anaemia, to induce lactation, to increase lactation, to lower hypertension and as haemapoitic, anti- microbial, sedative, diuretic and digestive, expectorant, vermifuge, etc. Researches proved its anticancer, immunomodulatory, erythropoitic,diuretic, antifungal, anti-bacterial hypertensive effect, anti-diabetic, hepatopro- tective, cardiac activities, anti-inflammatory effect etc. [10-13]

These initiated us to investigate the leaves especially essential 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 essential oil of this plant on bones which have not been studied.

2. MATERIALS AND METHODS

The leaves of the healthy plant *C.limetta* Risso. selected for our study was collected from Annavasal, Pudhukkottai Tamil Nadu, India, during the month of Jun 2017 and was authenticated by Dr. Stephen, Department of Botany, American college, Madurai.

2.1. Isolation of essential oil from the leaves

The leaves were dried at room temperature under shade, powdered, sieved (60mesh) and stored in a well closed container. From the dried plant EO is

isolated (EOCLL) by hydro distillation using Clevenger apparatus and analyzed by GC-MS.

2.2. Identification of compounds present in the essential oil of the leaves by GC-MS

JEOL GC MATE 11 model used, Column HP 5ms, carrier gas high pure helium gas with flow rate 1ml/mt, oven temp 50-250 deg/min, Mass analyzer quadrupole with double focusing, with photon multiplier tube.

2.3. Acute toxicity study using brine shrimp (*artemia nauplii)* lethality bioassay (bsla)

In order to study the toxicity of the EOCLL we performed Brine Shrimp Lethality Bioassay which based on the ability to kill laboratory cultured brine shrimp (*Artemia nauplii*). The brine shrimp assay is a useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, pesticides and cytotoxicity testing of dental materials. [14,15]

2.4. Toxicity assessment of eocll of the leaf

Ten free swimming hatched out *nauplli* were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5ml brine solution containing various concentration of EO (ppm) was added to 4.5 ml of brine solution and maintained at room temperature for 24 hrs under the light then surviving larvae were counted. Experiment was conducted along with control (vehicle treated), different concentrations of the EO (100-1000 ppm) in a set of three tubes per dose. The percentage lethality was determined by comparing the mean surviving larval of the test and control tubes. LC50 value was obtained from the best – fit line, plotted concentration verses percentage lethality. Podophyllotoxin was used as a positive control in the bio assay.

2.5. Effect on prednisolone 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 dpf) 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 $Mg2SO_4$, methylene blue). OP was induced by immersing zebrafish larvae from 3dpf to 6dpf in E3 medium containing 10µg/ml prednisolone. At 6 dpf the E3 medium containing prednisolone was removed and replaced with the combination of 10µg/ml prednisolone and EOCLL (0.1ml/ml) in triplicate till 9 dpf 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 dpf 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 dpf zebrafish larvae was stained with 0.1% alizarin red s in 0.1% KOH for 2hrs and then can be easily observed under stereomicroscope [16,17]

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) [3,18]. 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.

3. RESULTS

Phytochemical analysis of essential oil: Identification of compounds present in the essential oil of leaves by GC-MS analysis. GC-MS Profile of the EOCLL showed the presence of d-limonene, β-pinene, α-pinene, α-terpineol, Citronellal, α-Bisabolol, Camphene, β-Bisabolene, Nonanal, Borneol, Geranial, Linalool, Bergamol.

In continuation of our efforts to verify the safety of EO, we performed Brine shrimp lethality assay (BSLA) using free swimming hatched out *Artemia nauplii* which based on the ability to kill laboratory cultured brine shrimp. It was observed that 100% of mortality above 800 ppm for EOCLL. LC50 for EOCLL was about 419ppm in 24hrs.100% mortality was observed at 3ppm for podophyllotoxin positive control. This prescreen showed safety of the EOCLL without any symptoms of toxicity.

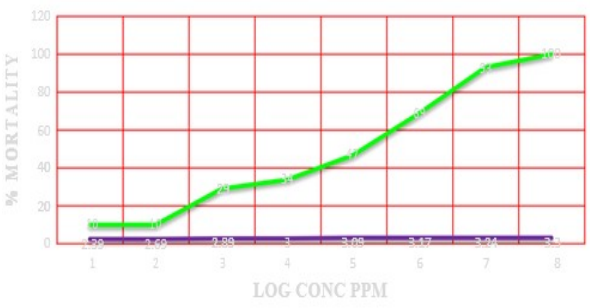
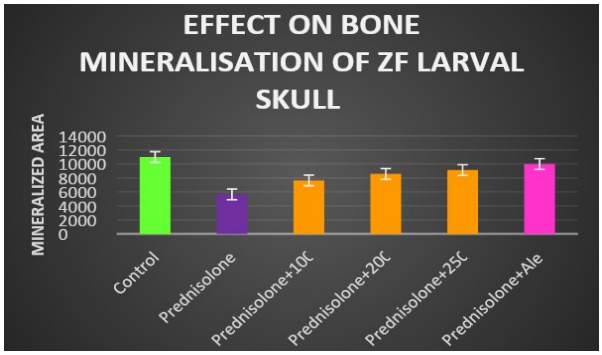


Figure -1: Acute toxicity study of eocll

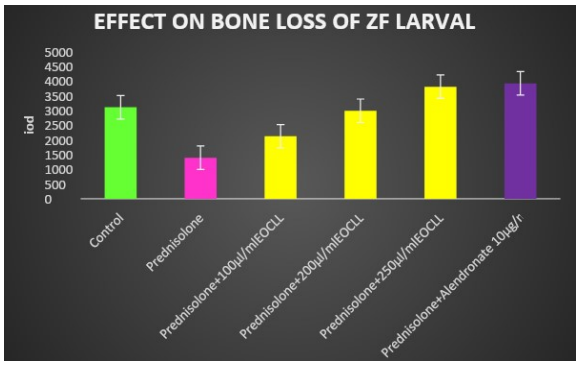
3.1. Effect on prednisolone induced inhibition of osteogenesis

The present study revealed that EOCLL can prevent prednisolone 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 EOCLL showed increased stained area and IOD ($p<0.001$) Figure (4, 5, 6), so that can prevent prednisolone induced inhibition of osteogenesis. It indicates that EOCLL attenuates prednisolone induced inhibition of bone mineralization in ZF larvae.

It was observed that the bone mineralisation area control 10987, prednisolone 5655, prednisolone+100µl/ml EOCLL 7639, prednisolone+200µl/ml EOCLL 8578, prednisolone+250µl/ml EOCLL 9123, prednisolone+alendronate 10µg/ml 9989.



Bone density IOD was control 3101, prednisolone 1390, prednisolone+100µl/ml EOCLL 2119, prednisolone+200µl/ml EOCLL 2982, prednisolone+250µl/ml EOCLL 3798, prednisolone+alendronate 10µg/ml 3912.



4. DISCUSSION

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 essential oil of leaves showed important constituents presence and was compared with the instrument library. The toxicological study reveals that the essential oil of the leaves of *C.limmeta* is nontoxic and safe. 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 [6]. 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 [19,20]. 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 [21,22]. 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 [23]. This investigation showed that EOCLL can prevent inhibition of osteogenesis in the presence of prednisolone. According to the results 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. EOCLL 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 EOCLL attenuates the prednisolone induced inhibition of bone mineralization in zebrafish larvae ($p<0.001$)

5. CONCLUSIONS

Our data showed EOCLL significantly prevented the prednisolone induced bone loss and density and comparable to the standard drug alendronate in ZF larval model *in vivo.* Essential oil of the leaf of *C.limetta* may be developed as a potential candidate for the prevention or treatment of osteoporosis. In conclusion, the leaves of *C.limetta* 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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