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Rutin exhibits an anti-resorptive effect in a medaka fish model of osteoporosis

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Abstract. With the increasing prevalence of osteoporosis worldwide due to the aging population, there is a substantial need for the search and development of new anti-osteoporosis substances. Rutin (quercetin-3-O-rhamnosyl glucoside) is a flavonoid glycoside found in many plants and herbal medicines, known for its potent antioxidant and potential osteoprotective properties. In this study we investigated the anti-osteoporotic effects of rutin, for the first time, using a medaka fish (Oryzias latipes) model for osteoporosis. The medaka fish model is a non-mammalian model that is increasingly preferred for drug screening. Rankl-induced osteoporosis fish larvae were treated with rutin at five different doses (10, 25, 50, 100, and 200 µM) for 96 hours starting from 7 days post-fertilization (dpf). The effect of rutin on bone damage was assessed via indexes of mineralization protection (I_P) which are based on the index of bone mineralization (I_M) of the tested fish. The results showed that rutin significantly reduced the level of Rankl-induced bone damage at concentrations of 10, 25, 50, and 100 µM, with the highest effect observed at a concentration of 10 µM. These findings provide important evidence for further studies on the bone-protective effects of rutin on medaka fish models for the development of anti-osteoporosis drugs.

Keywords: medaka fish, osteoporosis, I_M, I_P, rutin.

Classification numbers: 1.1.1, 1.2.1

1. INTRODUCTION

Bone is a dynamic organ that undergoes continuous repair and renewal through a remodeling process. In this process, osteoclasts break down old bone, while osteoblasts build new bone. Working together, osteoclasts and osteoblasts maintain a delicate balance between bone breakdown and formation. Disruption of this balance can lead to bone disorders, including osteoporosis which is caused by increased bone breakdown that exceeds bone formation [1].

Osteoporosis affects a significant number of individuals of all genders and races, particularly the elderly and postmenopausal women. The disease is characterized by low bone mass, impaired and porous bone structure, and an increased risk of fractures. According to the

International Osteoporosis Foundation, osteoporosis-related fractures affected one in three women or one in five men above the age of 50 globally in 2019 [2]. As the worldwide population continues to age, the number of affected individuals is rapidly increasing, posing a significant burden on societies. Fractures associated with osteoporosis greatly reduce the quality of life for patients, and the cost of treatment and medications is substantial [2, 3]. In Vietnam, a recent study conducted in Ho Chi Minh City surveyed 1,421 women and 652 men aged over 50 years, revealing an osteoporosis prevalence rate of 27% among women and 13% among men [4]. Given that these rates are representative of the Vietnamese population, they should impose a significant public health burden on the country.

While various approaches have been used to treat osteoporosis, bisphosphonates, specifically alendronate and risedronate, are commonly used as first-line therapy [5, 6]. Anti-osteoporosis drugs can inhibit bone resorption and/or stimulate bone formation. Nonetheless, current anti-osteoporotic drugs have limitations in terms of efficacy and safety [3]. Hence, there is a need to explore new osteoprotective agents and develop improved medications.

Receptor activator of nuclear factor kappa-B ligand (RANKL), a member of the tumor necrosis factor (TNF) superfamily, is known as a key promoter of osteoclast formation and function. An increase in RANKL expression can result in heightened bone resorption and osteoporosis [7]. Increased RANKL is known to be associated with both primary osteoporosis, caused by the natural aging process, and secondary osteoporosis, caused by underlying diseases or treatments [5, 8]. Therefore, an animal model induced by RANKL provides an advantage in reflecting both types of osteoporosis.

Medaka and zebrafish have recently emerged as powerful non-mammalian models for studying human diseases, including bone diseases, due to their numerous experimental advantages [9]. In 2012, To et al. generated the *rankl*:HSE:CFP transgenic medaka fish to model osteoporosis. In this fish, the expression of ectopic Rankl and the cyan fluorescent protein (CFP) can be induced by heat-shock. Upon exposure to a heat-shock at 39^{o}C, the fish expresses exogenous Rankl, which stimulates osteoclastogenesis and bone resorption, leading to an osteoporosis-like phenotype even at the early larval stage [10]. The original Rankl fish was then segregated into sublines with varying levels of mineralization damage. For our study, we utilized the sublines that exhibit bone damage only on neural arches to assess the anti-osteoporosis ability of compounds [11, 12]. A method to quantify the level of bone mineralization based on the lengths of mineralized neural arches, known as the Index of bone mineralization (I_M), was established. Using this method, the level of bone mineralization I_M, the level of bone damage in the fish I_D, and the level of bone protection of substance I_P can be calculated [13].

Rutin (quercetin-3-*O*-rutinoside) (Figure 1) is a natural flavonoid glycoside found in many plants, for example buckwheat, green tea, *Citrus* plants [14]. In Vietnam, this substance can be easily extracted from "hòe hoa" -*Styphnolobium japonicum* (L.) Schott, "mạch ba góc"-*Fagopyrum esculentum* Moench, "bát giác liên" – *Podophyllum tonkinense* Gagnep. [15]. Rutin has been shown to be a potent natural antioxidant and possesses diverse pharmacological activities including antibacterial, antitumor, anti-inflammatory, and antihypertensive [16]. Recently, some studies *in vitro* or *in vivo* in mouse models showed that rutin exhibits some osteoprotective potential [14, 17 - 19]. Here, for the first time, we tested the anti-resorptive ability of rutin using the d1d1 subline of the *rankl*:HSE:CFP transgenic medaka model of osteoporosis to initiate the study on bone protective effects of the compound on new valuable non-mamalian animal models.

2. MATERIALS AND METHODS

2.1. Materials

rankl:HSE:CFP transgenic fish [10] provided by the Winkler's Laboratory, National University of Singapore were maintained and segregated in our laboratory for many generations. Rutin (quercetin-3-*O*-rutinoside) (Figure 1) with the purity of 96 % checked by HPLC, was isolated from the plant *Podophullum tonkinense* Gagnep [20]. DMSO (Dimethyl sulfoxide) was purchased from Kanto Chemicals. Other reagents were purchased from Sigma-Aldrich Co.

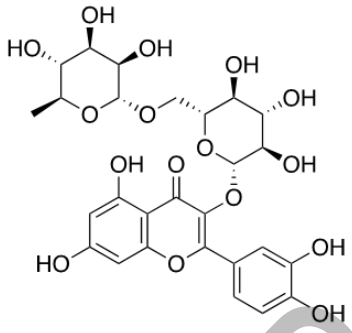

Figure 1. Chemical structure of rutin**.**

2.2. Method

2.2.1. Fish lines and fish maintenance

d1d1 subline of the *rankl:*HSE:CFP transgenic fish that exhibits an osteoporosis-like phenotype at neural arches was used [10 - 11]. We crossed homozygous d1d1 rankl:HSE:CFP fish with wild-type fish to obtain hemizygous offspring, hereafter named Rankl fish/embryo(s)/larva(e) to evaluate the anti-resorptive effect of rutin. Fish were bred and maintained at controlled temperature range of 28-30^{o}C under a circadian cycle of 14 h light: 10h dark following established procedures. Embryos were raised in embryo medium E3 containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgCl_2$ at 30 ^{o}C [10 - 13]. All fish experiments were performed in accordance with the animal welfare laws and guidelines from Dinh Tien Hoang Institute of Medicine, Ha Noi, Viet Nam (Approval number: IRB-AR.002).

2.2.2. Rutin treatment

Rutin treatment was conducted following the established drug treatment procedure on Rankl fish [13]. A rutin stock solution was prepared by dissolving 4.9mg rutin in 100 µl DMSO 100 % and then diluted in E3 solution to achieve final concentrations of 10, 25, 50, 100, and 200 µM (with a final concentration of DMSO of 0.1 %). Hemizygous d1d1 *rankl*: HSE:CFP larvae (Rankl larvae) were randomly divided into groups, each treated with rutin at one of these five doses or with DMSO 0.25 % as a solvent control or with alendronate (Sigma A4978) 25 µg/mL as a positive control or with E3 as a non-treated control. The sample size for each treated or non-treated group is 30 larvae. The treatment was carried out for 96 hours, from 7 days post fertilization (dpf) to 11 dpf. At 9 dpf, the larvae were heat-shocked at 39°C for 90 minutes to induce an osteoporosis-like phenotype. Wild-type fish were used as a control to assess normal bone mineralization.

2.2.3. The I_M method for bone mineralization quantification

11 dpf fish larvae were fixed with PFA 4% and stained with alizarin red 0.5% to visualize the mineralized bone matrix as previously reported [10, 21]. Specimens were then mounted and imaged by Zeiss Axioplanz microscope equipped with Optika B3 Camera at 10x magnification. Image processing was performed using ImageJ software.

Mineralized bone damage in the neural arches, (as indicated by green arrows in Figure 2, where neural arches of Rankl larvae appear shortened or completely degraded compared to wild-type fish), is a previously reported characteristic of Rankl fish [10-12]. Therefore, neural arches were selected as the representative bone structure for analyzing the level of bone mineralization and bone damage using the established I_M method [13].

The lengths of the 15 first mineralized neural arches were measured on the lateral image of the bone-stained fish using Image J software (refer to supplemental data for detailed procedures). The total length of the 15 first neural arches of each larva was calculated as the Index of bone mineralization (I_M), where 'k' represents the ordinal number and 'L' is the length of each arch [13]. The I_M is inversely proportional to the level of mineralized bone damage; thus, the lower the I_M value, the higher the level of bone damage in the fish.

$$\boldsymbol{I_M = \sum_{k=1}^{15} L} \qquad (1)$$

When the I_M of wild-type ($I_{M(WT)}$) and of Rankl fish group ($I_{M(Rankl)}$) are determined, the Index of mineralization damage (I_D) of the Rankl fish group can be calculated by the following formula:

$$\boldsymbol{I_D = \frac{I_{M(WT)} - I_{M(Rankl)}}{I_{M(WT)}} x\ 100\%} \qquad (2)$$

The Index of bone mineralization protection (I_P) of a tested substance can be calculated based on I_M by the following formula:

$$\boldsymbol{I_P = \frac{I_{M(+Rankl+S)} - I_{M(+Rankl-S)}}{I_{M(-Rankl-S)}} x\ 100\%} \qquad (3)$$

(where "± Rankl" indicates with/without Rankl; "± S" indicates with/without tested substance), bespeaks the anti-resorptive efficacy of a tested substance [15, 23].

2.2.4. Statistical analysis

Unpaired student t-test or one-way ANOVA followed by Tukey's multiple comparison test was used to compare groups and check whether disproportion was statistically significant with Prism 9 software (GraphPad Software Inc., San Diego, CA). Differences when a $p < 0.05$ (marked with one asterisk *), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****) were considered significant. Results are presented as mean ± S.E.M.

3. RESULTS AND DISCUSSION

3.1. Osteoporosis-like phenotype of d1d1 *rankl*:HSE:CFP larvae

Since intergenerational variation over time in expression and function of a transgene is an existing concern in transgenic animal-based research [22], we first checked whether the larval offspring of the d1d1 Rankl fish ensure suitable phenotypes for drug treatment. Rankl larvae were heat-shocked at 39 ^{o}C for 90 minutes at 9 dpf and fixed and stained with alizarin red at 11 dpf. Fifteen first vertebrae were then captured and their representative images are presented in Figure 2.

As expected, while their wild-type (WT) siblings had intact bone structures (yellow arrow in Fig. 2A), all d1d1 Rankl larvae showed partly or complete destruction in many mineralized neural arches (green and light green arrows in Fig.2B). Levels of bone mineralization of fish larvae of the two groups (n = 15 for each group) were then determined by the mean value of Indexes of the mineralization I_M as 2400.30 (WT) and 891.16 (Rankl). Student's t test was used to compare these groups with p-value < 0.0001 (Fig. 2C). Afterward, the Index of bone mineralization damage I_D of the Rankl fish was calculated as 62.87 %, meaning these larvae had lost about 62.87 % of their mineralized neural arches (Fig. 2D). Moreover, the homogeneity observed in the bone destructed phenotype of these Rankl-induced larval individuals indicated that the d1d1 fish line was suitable for experiments in this study.

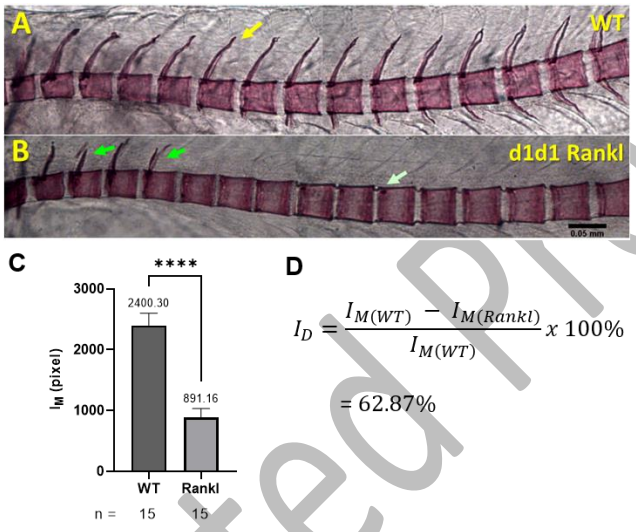

Figure 2. Images of mineralized-structure staining of 15 first vertebrae of a wild-type (WT) larva (A) and a d1d1 *rankl*:HSE:CFP larva (B). Yellow arrows indicate intact neural arches, green arrows indicate partly destructed neural arches and light green ones indicate completely destructed neural arches. (C) Mean values of mineralization index (I_M) of WT and Rankl fish. (D) Index of mineralization damage of d1d1 Rankl fish.

3.2. Rutin shows anti-resorptive effect at doses of 10, 25, 50, and 100 µM in Rankl-induced osteoporotic larvae

To assess the ability of rutin to protect mineralization structures from Rankl-induced bone damage, we first tested rutin at doses of 25 and 100 µM chosen based on previous *in vitro* studies [18, 23 - 26] and then extended the concentration range by adding two polar and one intermediate concentration (10, 200, and 50 µM, respectively).

Representative images of alizarin red-stained fish in the treated and control groups are shown in Figure 3. While mineralized bone structures remained intact in the wild-type larvae of all groups, all Rankl larvae showed damage in neural arches at various levels. Among the Rankl larvae of the DMSO control groups, the most severe destruction was observed, with fractured, shortened, or completely destructed arches. Statistical analysis revealed that the mean I_M values, which represent the level of mineralization in neural arches of the DMSO groups, fluctuated slightly in the three experiments (n = 30 in each group for one experiment; I_M = 801.36, 819.51, and 707.39 for DMSO groups of experiments 1, 2, and 3, respectively). Lateral images of the

non-treated group (E3) exhibited a similar pattern of bone loss phenotype as those of the DMSO group. This was statistically confirmed when no significant difference was found between these groups, indicating that DMSO 0.25% did not affect bone mineralization in this experimental setting.

Larvae treated with rutin at doses of 25 and 100 µM appeared to have milder bone damage than their corresponding non-treated or solvent control (Fig. 3B-C). One-way ANOVA statistical analysis revealed that mean I_M of Rutin 25 µM-treated Rankl fish (1092.13, n = 30) is significantly higher than the DMSO (1) and E3(1) groups (801.36, n = 30, $p<0.05$ and 713.28, n = 30, $p < 0.01$, respectively). Moreover, 100 µM rutin-treated larvae also had a mean I_M value (1356.4, n = 30) significantly higher than that of both the DMSO (2)-treated group and the E3(2) group (819.51 and 833.69 respectively, $p < 0.05$) (Fig.3B'-C'). Thus, rutin at doses of 25 and 100µM reduces the level of mineralized bone damage in the transgenic rankl:HSE:CFP osteoporosis fish model.

Next, we proceeded to assess the impact of rutin at concentrations of 10, 50, and 200 µM alongside alendronate, which served as a positive control (n = 30 for each group). Alendronate has been widely employed as a standard drug in the treatment of osteoporosis and has been shown to exhibit potent anti-resorptive effects at a dosage of 25 µg/mL in this Rankl-induced fish model [15, 18]. Among the Rankl groups, the alendronate-treated group exhibited the least destruction in neural arches and the highest index of mineralization value (I_M = 1113.73, $p < 0.001$). Following this, the group treated with rutin at a concentration of 10 µM (I_M = 1043.29, $p < 0.05$) and rutin at a concentration of 50 µM (IM = 957.73, $p < 0.05$) showed comparatively lower damage. Interestingly, the highest dose of rutin (200 µM, I_M = 700.18) appeared to have no effect on bone resorption, as evidenced by a comparable damage pattern to the DMSO(3) and E3(3) control groups, whose I_M values were 707.39 and 635.12, respectively. This observation was further supported by statistical analysis (Fig.3D-D').

Taken together, our results indicate that rutin has the potential to decrease damage to mineralized structures in Rankl fish at four different doses (10, 25, 50, and 100 µM). The effectiveness of rutin at these doses, along with alendronate, was compared using the Index of mineralization protection (I_P) shown in Fig. 3E. Alendronate at 25 µg/mL demonstrated the highest I_P value at 23 %. Interestingly, rutin at the lowest concentration of 10 µM exhibited a higher I_P value (19 %) compared to all other rutin doses (18 %, 14 %, and 17 % for 25, 50, and 100 µM rutin, respectively). Taking into consideration both cost and treatment effectiveness, it is recommended that further experiments investigate the underlying mechanism of rutin's anti-osteoporosis ability using a dose of 10 µM.

3.3. Discussion

The findings of this study demonstrate that rutin when administered at doses of 10, 25, 50, and 100 µM, can reduce mineralized bone damage in a Rankl-induced medaka fish model of osteoporosis. The bone protection index for rutin at these doses was observed to be 19 %, 18 %, 14 %, and 17 %, respectively. As a positive control, alendronate, a standard anti-osteoporosis drug, at a concentration of 25 µg/ml, exhibited a protective index value of 23 %. It is worth noting that the highest tested dose of rutin, at 200 µM, did not display any significant effects. Therefore, the anti-resorptive effect of rutin in this fish osteoporosis model is demonstrated to be dose-dependent.

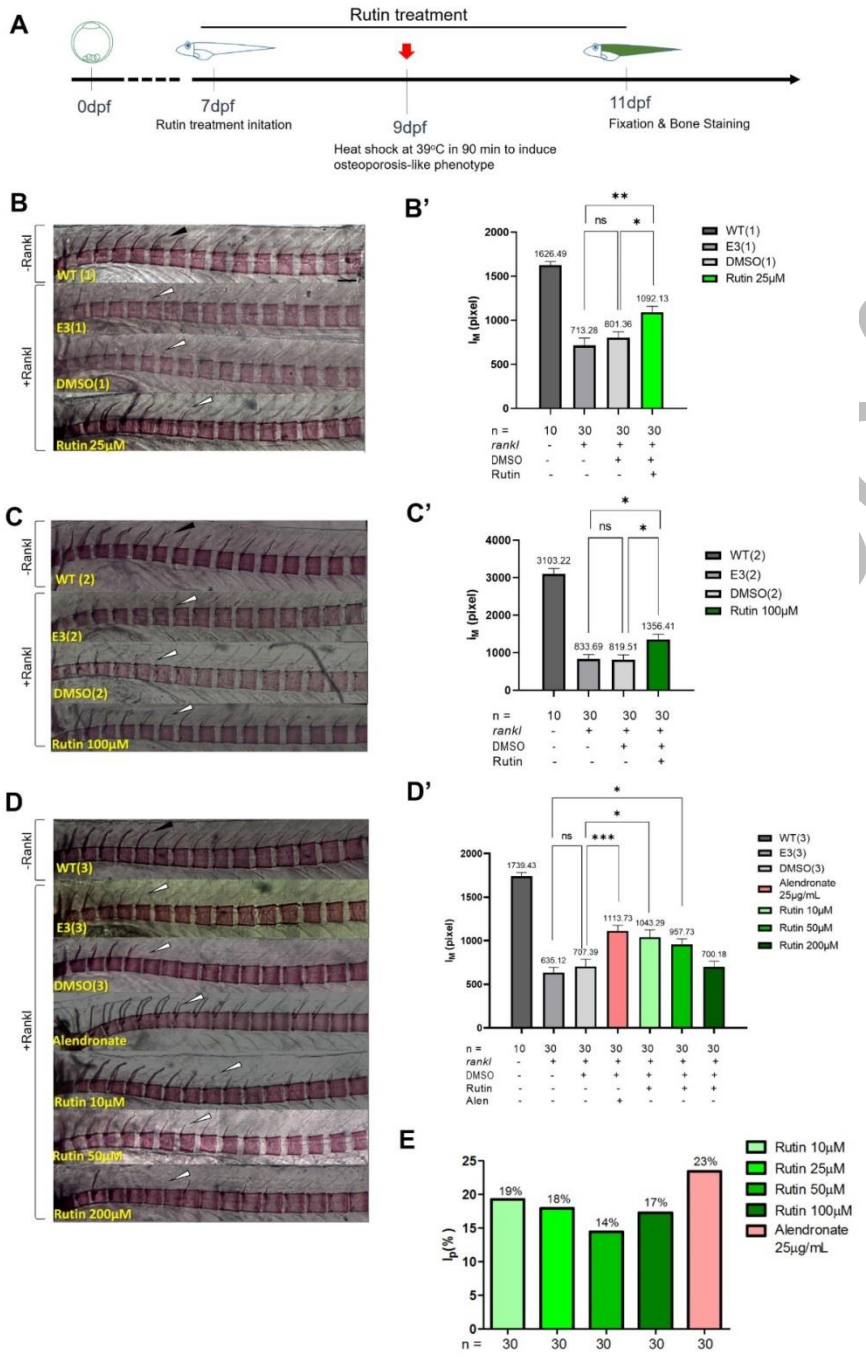

Figure 3. Rutin protects mineralized bone of neural arches from resorption in Rankl-induced osteoporotic fish. (A) Rutin treatment procedure with red arrow indicating heat-shock induction and fish in green symbolizing one with heat-shock induced Rankl. (B-B') Representative image of fish treated with rutin 25µM, DMSO(1), E3(1), and WT(1) control groups and corresponding mean values of mineralization index (IM). (C-C') Representative image of fish treated with rutin 100µM, DMSO(2), E3(2), and WT(2) control groups and corresponding mean values of mineralization index (IM). (D-D') Representative image of fish treated with alendronate 25µg/mL, rutin 10, 50 and 200µM, DMSO(3), E3(3) and WT(3) control groups and corresponding mean values of mineralization index (IM). Black arrowheads indicate intact

bone structure while white arrowheads indicate damaged neural arches. n: number of embryos in corresponding group. + or – indicates the presence or absence of corresponding factors written on the left side for each fish group. +/-Rankl indicates fish larvae with or without ectopic Rankl expression. Bars indicate S.E.M. Scale bar: 0.05mm. (E) Index of mineralization protection (I_P) of rutin at effective doses and alendronate.

The fact that rutin is effective at lower doses but not at the highest tested dose is explainable. Bioactive substances may not exhibit efficacy at high doses due to either a hormetic effect, where high doses can cause toxic effects on the tested organisms [26], or receptor saturation resulting in a saturation effect [27]. When compared to the effect of alendronate, which at a concentration of 25 µg/ml is equivalent to 75 µM (based on the molecular weight of alendronate – Sigma A4978 is 325.12), rutin demonstrates a lower bone protective effect but at lower doses.

With the increasing number of people worldwide affected by osteoporosis due to an aging population, the search for bone-protective compounds is of utmost importance in the development of better drugs and medications. Rutin has been reported to possess a wide range of biological abilities, including anti-oxidative, anti-viral, and anti-inflammatory properties [14]. Several studies conducted on cell and mouse models have demonstrated the inhibitory effect of rutin on osteoclast activity by reducing the expression of key elements in NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway, such as TNF-α (tumor necrosis factor alpha), IL-1β (interleukin-1 beta), and IL-6 [17, 18, 27]. Furthermore, a study by Xiao et al. in 2019 suggests the role of rutin in down-regulating FNCD1 (fibronectin type III domain-containing protein) may protect against trabecular bone loss in ovariectomized mice and induce bone marrow mesenchymal stem cell autophagy via the Akt/mTOR signaling pathway [19]. This data provides valuable evidence for further investigation of rutin as a potential bone-protective agent.

Apart from various mammalian animals, medaka (as well as zebrafish) has emerged as a highly effective non-mammalian model for studies in the field [29, 30]. Our study represents one of the initial reports on this particular fish model, providing evidence that supports previous research findings. Additionally, our fish model is induced by the overexpression of Rankl, a key factor whose increased expression is linked to both primary and secondary osteoporosis [7]. Thus, the finding that rutin can lessen Rankl-induced bone damage suggests its potential applicability in the treatment of both types of osteoporosis. However, the mechanisms that underlie the anti-resorptive effect of rutin in this fish model remain to be further elucidated. Furthermore, as the osteoporosis phenotype of the fish was induced by increased Rankl, rutin may likely inhibit the Rankl/Rank pathway. Consequently, it is suggested that the analysis of factors involved in these pathways, including TRAF6 (tumor necrosis factor receptor associated factor 6), NFATc1 (nuclear factor of activated T-cells, cytoplasmic 1), and c-Fos [6], be conducted to investigate the molecular mechanisms underlying rutin's effects.

Rutin has also been reported to exhibit bone anabolic potential by promoting osteoblast formation and mineralization *in vitro* [23, 25, 31]. These findings suggest the need for further investigation using medaka fish models to explore the potential bone anabolic effects of this substance.

The mode of how rutin can penetrate medaka fish larvae aged 7 to 11 days requires further investigation. However, absorption through the skin and via ingestion are the two probable routes of drug absorption, as reported in zebrafish larvae, a similar fish model to medaka [32, 33]. To understand the pharmaceutical effects of bioactive substances, it is important to

understand their metabolism and bioavailability in tested animals. Rutin, when orally administered to rats, has been reported to be metabolized into quercetin sulfates and quercetin glucuronides, and to be present in the blood [34]. However, there are still no studies on the metabolism and bioavailability of rutin in fish in general or in medaka fish in particular, suggesting that this issue needs to be elucidated. This is a relatively new area of research, and further studies are warranted to compare how medaka fish metabolize active natural compounds compared to those used in standard *in vivo* testing. Since many natural compounds require metabolism to become active (prodrugs), while others become inactive after this process, understanding these variations across models is crucial. While the medaka fish shares many similar biological processes with mammals [8,9], confirmation through metabolic studies would strengthen its validity as a reliable model that correlates well with commonly used animals.

4. CONCLUSIONS

Rutin (quercetin-3-*O*-rutinoside) exhibited an anti-resorptive effect in the *rankl:* HSE:CFP medaka fish model of osteoporosis at doses of 10, 25, 50, and 100 µM, resulting in the preservation of 18 %, 14 %, and 17 % of the bone against resorption, respectively. This study provides the first evidence of the bone protective potential of the compound in a non-mammalian model. These findings suggest the need for further research using medaka models to explore the potential of rutin for drug development in the treatment of osteoporosis.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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