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Original Article

Isolation of Cowanol from the *Garcinia oblongifolia* Plant and Evaluation of its *in vivo* Toxicity and Bone-protective Activity in Medaka Fish *(Oryzias latipes)*

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Abstract: Garcinia oblongifolia Champ. ex Benth is a plant species widely distributed in Vietnam. It contains xanthones - bioactive compounds known for their potent antioxidant, anti-inflammatory, and anticancer properties. Xanthones have also emerged as potential bone-protective agents. Here, cowanol, a common xanthone, was isolated from the branches of *G. oblongifolia* in Binh Dinh Province, with 98.4% purity. The *in vivo* toxicity and bone-protective activity of cowanol were assessed using medaka (*Oryzias latipes*), a standardized fish model in toxicity and disease research. Acute toxicity was evaluated over a 96-hour exposure period, assessing wild-type embryos (24-120 hours post-fertilization) to concentrations ranging from 2 to 50 µM and larvae (7-11 days post-fertilization) to the doses of 2 to 30 µM. Results showed that cowanol was non-toxic to embryos but exhibited dose- and time-dependent toxicity to larvae, with the lethal concentration 50 (LC_{50}) of 11.4 µM. At the doses of 2 µM or lower, cowanol was safe for medaka larvae. The bone-protective effect of cowanol was investigated using *rankl*:HSE:CFP transgenic larvae model for osteoporosis. Our findings revealed that cowanol significantly reduced bone damage in the Rankl-induced osteoporosis fish at three tested doses of 0.5, 1.5, or 2 µM with bone protection indexes reaching up to 29.38%. This study highlights the potential exploration of xanthones from Vietnamese plants for therapeutic applications.

Keywords: Acute toxicity, cowanol, medaka, osteoporosis, *rankl*:HSE:CFP.

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1. Introduction

Garcinia is the largest genus in the family Guttiferae, comprising over 400 species primarily distributed in tropical regions such as Southeast Asia, southern China, the Indian Peninsula, and equatorial Africa [1]. In Vietnam, this genus includes 29 species and is one of the most common, widely distributed plants throughout the country [2]. *G. oblongifolia*, commonly known as a small tree with a height of 8-10 meters and a diameter of 20-25 cm, has dark brown, smooth bark, a pyramidal crown, and branches that are often perpendicular to the main trunk. The bark, roots, and leaves of *G. oblongifolia* are used in traditional medicine to treat gastric and duodenal ulcers, burns, boils, and allergic pruritus [3].

Previous studies have shown that *G. oblongifolia* primarily contains xanthones [4] and benzophenones [5], which exhibit a range of biological activities, including hypoglycemic [4], anti-inflammatory [6], antioxidant and anticancer effects [7]. Notably, xanthones have recently demonstrated bone-protective and anti-osteoporotic activity [8,9,10,11]. Jeong *et al*. found that cudratrixanthone U (CTU) from *Maclura tricuspidata* inhibits RANKL-induced differentiation and function [12]. Erik Idrus and Bramma Kiswanjaya demonstrated that Mangosteen (*Garcinia mangostana* L.) peel extracts slow bone resorption and reduce bone damage in osteoporosis mouse models [11]. Five xanthone derivatives from the *Halenia corniculata* inhibit osteoclast formation *in vitro* [10]. Moreover, 3,6-diisopropoxyxanthone, a synthesized xanthone derivative reduces bone resorption in both *in vitro* and *in vivo* tests [13].

Osteoporosis is a major public health issue due to aging, unhealthy lifestyles, and drug abuse, affecting about 33% of women and 20% of men over 50 globally [14]. In Ho Chi Minh City, the prevalence is 27% in women and 13% in men aged around 60 [15].

Bone health is maintained by balance between bone formation by osteoblasts, the bone forming cells, and bone resorption by osteoclasts, the bone "eating" cells. Risk factors like decreased estrogen in postmenopausal women, a sedentary lifestyle, and corticosteroid use disrupt bone metabolism and increase bone resorption, leading to reduced bone density and increased fracture risk [14, 16].

Elevated RANKL (Receptor Activator of Nuclear Factor kappa B Ligand) levels, a main stimulator for osteoclast formation, contribute to bone destruction and are targets for osteoporosis treatments [17, 18]. Current drugs, such as bisphosphonates including alendronate, work by inhibiting bone resorption but face challenges like high costs and safety concerns [16, 19] underlining the need for research to develop more effective and safer anti-osteoporosis therapies [16, 19].

The medaka fish *(Oryzias latipes)*, alongside the zebrafish *(Danio rerio)*, is widely used in toxicology [20], disease research [21], and drug development due to its low cost, ease of maintenance, short generation time, external fertilization, transparent embryos, and a small genome which is highly similar to human genome [22, 23]. Medaka has been intensively used to study bone metabolism, evidenced by research using transgenic and mutant fish showing similarities between medaka and human bone processes [24-28]. Factors involved in bone metabolism with potential applications in drug development were discovered on medaka models [29].

To model osteoporosis, To *et al*. developed the *rankl*:HSE:CFP transgenic fish, which expresses Rankl, the bone resorption-stimulating factor, upon heat induction. Heatshock at 39 °C triggers Rankl expression (indicated by CFP signal), leading to osteoclast formation, bone destruction, and an osteoporosis-like phenotype in fish larvae [28]. The subline, used here, specifically shows bone damage in the neural arches, which is assessed by I_M method measuring the length of the first 15 mineralized neural arches. This enables evaluation of compounds in reducing RANKL-induced bone damage [30, 31].

In this study, we isolated cowanol, a major xanthone compound, from the branches of *Garcinia oblongifolia*. We then assessed the acute toxicity of the substance in medaka fish to establish a basis for evaluating its bone-protective effects in our transgenic osteoporosis fish models.

2. Experimental

2.1. Plant Materials

Branches of *Garcinia oblongifolia* Champ. ex Benth. were collected in June 2020 from Song Kon Forest Enterprise in Binh Dinh Province for the isolation of cowanol. The scientific identification of the branches was confirmed by Associate Professor Dr. Dang Van Son at the Institute of Tropical Biology, Vietnam Academy of Science and Technology. After harvesting, the branches were dried and ground to obtain 4.0 kg of sample material. The sample was subjected to extraction using a Soxhlet apparatus with ethyl acetate as solvents. Solvent recovery using a rotary evaporator yielded 152.3 g of ethyl acetate (EtOAc) extract.

2.2. Isolation of Cowanol

Column chromatography of the EtOAc extract (152.3 g) on silica gel using an *n*-hexane-EtOAc solvent system with increasing polarity (0-100%) yielded 50 fractions of 1 liter each. These were combined into 9 fractions, labeled BE1-9, based on thin-layer chromatography (TLC) results. The column chromatography of fraction BE8 (36.4 g) on silica gel (*n*-hexane-acetone 0-100%) yielded 8 fractions (BE8.1-8.8). Further column chromatography of fraction BE8.4 (4.7 g) on silica gel ($CHCl_3$-EtOAc 0-100%) yielded 7 fractions (BE8.4.1-8.4.7). Purification of fraction BE8.4.5 (368.7 mg) by column chromatography on silica gel (*n*-hexane-EtOAc 0-100% followed by $CHCl_3$-EtOAc 0-100%) resulted in the isolation of cowanol. The isolated compound weighed 105.2 mg. The purity of cowanol was analyzed by HPLC-UV, showing a purity of 98.4%.

2.3. Instruments and Experimental Conditions

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 instrument [500 MHz (^{1}H) and 125 MHz (^{13}C)] using $CDCl_3$ or acetone-d_6 as solvents. High-resolution mass spectrometry (HRMS) was performed on a Shimadzu LC/MS (Japan). The purity of the isolated compounds was analyzed using a Shimadzu HPLC-PDA system (Japan). Column chromatography was carried out on silica gel 60 (40-63 µm, Merck). Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (250 µm, Merck). Components on the TLC plates were detected using UV light, iodine vapor, or by spraying with $FeCl_3$/EtOH solution (for the detection of phenolic compounds).

2.4. Fish Lines and Fish Maintenance

In this study, we used wild-type and the *rankl*:HSE:CFP subline *c6* fish larvae, which was segregated in our laboratory [30] from the original *rankl*:HSE fish provided by the National University of Singapore [28]. The *c6* subline (hereafter referred to as Rankl fish/embryos/larvae) exhibits an osteoporosis phenotype characterized by damage to the mineralized neural arches [30, 32]. Experiments were conducted on hemizygous Rankl embryos/larvae, which were offspring of homozygous Rankl and wild-type fish parents. The fish were cultured and maintained as previously described, at the temperature of 28-30 °C and 14-hour light/10-hour dark cycle [28, 30]. Rankl fish embryos were screened for the fluorescent reporter CFP at 11 days post-fertilization (dpf), which is 2 days after heat shock [30]. All experiments involving fish were conducted in accordance with animal welfare laws and guidelines from the Dinh Tien Hoang Institute of Medicine, Hanoi, Vietnam (Approval number: IRB-A-2312).

2.5. Toxicicological Test Procedures

Acute toxicity of cowanol was assessed on wild type medaka at both embryonic and larval

stages following the Organisation for Economic Co-operation and Development (OECD) guidelines (Test Guideline (TG) 236 (2013).

Based on the results of preliminary tests (data not shown) to identify the dose range between the highest dose that showed no lethal effect and the lowest dose that caused lethal effects on the embryos, a series of concentrations including 2, 4, 6, 8, 10, 12, 16, 30, and 50 µM were chosen for the acute toxicity test of cowanol on medaka embryos over a period of 96 hours (4 days). Embryos at 1 dpf were grouped into sets of 10 embryos, each set was placed in a well of a 6-well cell culture plate. Each well contained 4 mL of E3 medium supplemented with cowanol at each of the tested concentrations and 0.1% DMSO. Two additional groups of embryos were maintained in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) [22, 28] as a normal control and in E3 medium with 0.1% DMSO as a solvent control. Mortality rates and morphological abnormalities were recorded, dead embryos were removed, and the solutions renewed daily until 4 dpf. The acute toxicity test was conducted in triplicate, with n = 30 embryos per group.

For the larval stage, acute toxicity was tested using a similar procedure as for the embryos, but with cowanol concentrations of 2, 4, 6, 8, 10, 12, 16, and 30 µM and sets of 5 larvae per well. The procedure began with larvae at 7 dpf and continued until the larvae reached 11 dpf.

2.6. Determination of LC_{50}

Non-linear dose-response curve and the median lethal concentration (LC_{50}), defined as the dose that is lethal to 50% of the animals, were computed using GraphPad Prism 10 [33].

2.7. Heatshock for Generation of Osteoporosis Phenotype in Rankl Fish Embryos

A heat shock at 39 °C for 90 minutes was applied to Rankl fish embryos at 9 dpf to induce an osteoporosis-like phenotype. Wild-type control fish were subjected to the same heat shock procedure, as described previously [28, 30].

2.8. Cowanol Treatment

Cowanol treatment was conducted according to the procedures outlined in a previous study [30].

Based on the results from cowanol's toxicity tests on larvae, it is considered safe to evaluate cowanol's effects at doses of 2 µM or lower. A 50 mM stock solution of cowanol was prepared in anhydrous DMSO and diluted in E3 solution to final concentrations of 0.5, 1, 1.5, or 2 µM, with the final DMSO concentration being 0.1%. Rankl hemizygous larvae were randomly assigned to groups and raised in a 24-well cell culture plate with a total solution volume of 1 mL. The experimental groups included: (+Rankl +cowanol) with the respective cowanol concentrations, a DMSO control group (+Rankl + DMSO) containing 0.1% DMSO, and a positive control group (+Rankl +Alen) treated with 50 µg/mL of alendronate (Sigma A4978; with molecular weight of 325.12 g, 50 µg/mL of alendronate is equivalent to 150 µM) [30]. At 9 dpf, larvae were heat-shocked at 39 °C for 90 minutes. Following a 2-hour recovery period at 28 °C, the drug-containing medium was replaced, and larvae were maintained until 11 dpf. At this stage, larvae were screened for CFP expression before being fixed in 4% paraformaldehyde (PFA) for bone staining with alizarin red S. Additionally, a group of heat-shocked wild-type larvae (WT group) was included as a control for bone development without any drug treatments. The experiment was repeated until an adequate number of larvae from each group were available for statistical analysis.

2.9. Staining of Mineralized Bone Structures and Imaging

To visualize the mineralized bone matrix in 11 dpf fish larvae, the specimens were fixed and stained with alizarin red S (Sigma A5533) following previously established protocols [30].

2.10. Imaging

Imaging of alizarin red bone-stained fish larvae was performed as described previously [28,30] using an Axiovert 100M microscope (Carl Zeiss, Germany).

2.11. Quantification of Level of Bone Mineralization and Bone Mineralization Damage

I_M method [30] was used to assess the level of bone mineralization via Index of bone mineralization (I_M). I_M is the sum of length of the first 15 mineralized neural arches ($I_M = \sum_{k=1}^{15} L$, where k is the ordinal number of neural arch and L is the length of each arch; I_M is indicated in pixel (px)). Cowanol at doses that have significantly higher I_M values than that of DMSO control is considered to have antiresorptive and bone protective effect in this fish model; the index of mineralization protection I_P (%) of each dose was calculated by the formular: I_P = 100% x ($I_{M\ (+Rankl\ +cowanol)}$ - $I_{M\ (+Rankl\ -\ cowanol)}$)/ $I_{M\ (-Rankl-cowanol)}$.

2.12. Statistical Analysis

Student's t-tests (two-tailed, unequal variance) or one-way ANOVA followed by Tukey's multiple comparison test were used to compare groups and determine significance, using Prism 5 (GraphPad Software Inc., San Diego, CA). Differences with a p-value of less than 0.05 were considered statistically significant. Results are presented as means ± standard error of the mean (S.E.M.).

3. Results and Discussion

3.1. Results

3.1.1. Isolation of Cowanol

Cowanol was obtained as a yellow gum and gave a positive reaction with $FeCl_3$/EtOH reagent. HRMS analysis showed a molecular ion peak $[M+H]^+$ at m/z = 495.2375, corresponding to the molecular formula $[M+H]^+$ ($C_{28}H_{33}O_5^+$, m/z = 495.2382).

The ^{1}H NMR spectrum showed resonances corresponding to a chelated hydroxyl group [δ_H 13.90 (s, 1-OH)], two isolated aromatic protons [δ_H 6.82 (1H, s, H-5); 6.36 (1H, s, H-4)], a methoxy group [δ_H 3.79 (3H, s, H3-7-OCH_3)], an isoprenyl group including an olefinic proton [δ_H 5.35 (1H, m, H-12)], a benzyl methylene group [δ_H 3.44 (2H, d, H2-11)], a primary alcohol [δ_H 4.30 (2H, s, H2-14)], and an allyl methyl group [δ_H 1.73 (3H, s, H3-15)]. Additionally, a geranyl side chain was observed, consisting of two olefinic protons [δ_H 5.27 (1H, m, H-21) and 5.03 (1H, m, H-17)], a benzyl methylene group [δ_H 4.13 (2H, d, H2-16)], two allyl methylene groups [δ_H 2.09 (2H, m, H2-20); 1.98 (2H, m, H2-19)], and three allyl methyl groups [δ_H 1.52 (3H, s, H3-23); 1.56 (3H, s, H3-25); and 1.83 (3H, s, H3-24)]. The ^{13}C NMR spectrum showed resonances corresponding to 29 carbons, including a conjugated carbonyl carbon [δ_C 182.5 (C-9)], 12 aromatic carbons, a methoxy group [δ_C 61.5 (7-OCH_3)], a geranyl side chain with two C=C double bonds [δ_C 125.1 (C-21); 131.3 (C-22); 124.9 (C-17); and 135.4 (C-18)], three methylene groups [δ_C 26.5 (C-16); 40.2 (C-19); and 27.0 (C-20)], and three methyl groups [δ_C 17.5 (C-23); 16.3 (C-24); and 25.5 (C-25)]. Additionally, an isoprenyl side chain was observed, including a C=C double bond [δ_C 124.6 (C-12); 134.8 (C-13)]; a methylene group [δ_C 21.4 (C-11); 61.1 (C-14)]; and a methyl group [δ_C 21.8 (C-15)]. Based on these spectral data, combined with comparison to reference data [34], the isolated compound was identified as cowanol (Figure 1).

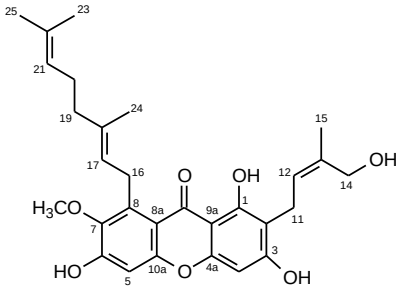

Figure 1. Chemical structure of cowanol.

3.1.2. Acute Toxicity of Cowanol on Medaka Embryos and Larvae

Results of acute toxicity tests on medaka embryos and larvae are presented in Figure 2 and Table 1. The procedures for the tests are depicted in Figure 2A.

Cowanol at all tested doses ranging from 2 to 50 µM showed no visible negative effects on the growth and development of fish embryos after 96 hours of exposure. Although at high concentrations, cowanol adhered heavily to the chorion (Figure 2B), the fertilization membrane of the embryos, all embryos survived and exhibited normal behavior and bodily functions, like the control group (Figure 2B, Table 1). This suggests that cowanol does not result in observable toxicity with four-day administration to medaka fish embryos.

For fish larvae exposed to cowanol at doses of 2-30 µM from 7 to 11 dpf, time- and dose-dependent toxicity effects were evident (Figure 2C-1). At the lowest tested dose of 2 µM, no mortality was observed after 96 hours of exposure. Also, a one-day exposure to cowanol at all eight tested doses did not affect larval survival. Mortality was only observed after 48 hours of exposure, with the number of dead larvae reflecting a dose- and time-dependent relationship. Among the eight tested doses (2, 4, 6, 8, 10, 12, 16, and 30 µM), only the highest dose of 30 µM resulted in 100% mortality after 96 hours.

The median lethal concentrations (LC_{50}) of cowanol for fish larvae at 48, 72, and 96 hours of exposure are estimated in Table 1. The LC_{50} value at 96 hours is 11.44 µM, with a 95% confidence interval ranging from 9.768 to 13.45 µM. This value is significant as it provides a basis for determining the dose range for evaluating bioactivity of the substance. At lethal concentrations, most fish larvae, prior to death (black arrow, Figure 2C-3), displayed a loss of motility and remained motionless while still maintaining respiration (red arrow, Figure 2C-3). This symptom is indicative of severe toxicity affecting the vitality of the fish larvae. No such signs were observed in surviving larvae exposed to cowanol at 4 µM, suggesting that this concentration or lower doses are relatively safe for the larvae.

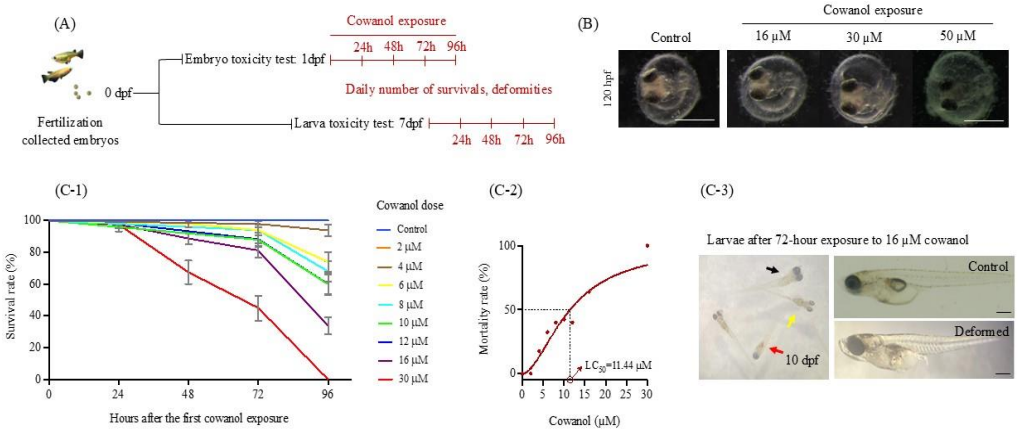

Figure 2. The acute toxicity effect of cowanol on fish embryos and larvae. (A) Procedure of the experiments for assessment of acute toxicity of cowanol to medaka embryos and larvae. (B) Images of 120 hours post fertilization (hpf) (5 dpf) embryos after 96-hour exposure to cowanol at 16, 30, 50 µM. (C-1) Daily survival rate of larvae during 96-hour exposure to cowanol at tested doses of 2 to 30 µM; Bars represent standard errors. (C-2) Representative dose response curve of the 11 dpf larvae after 96-hour exposure to cowanol at all tested doses and the corresponding estimated LC_{50} value. (C-3) Representative images of 10 dpf medaka larvae after 72-hour exposure to cowanol of 16 µM showing normal one (yellow arrow, control), one with malformations and loss of motility (red arrow), dead one (black arrow). Scale bar represents 500 µm.

Table 1. Toxicological indices of cowanol on medaka embryos and larvae

LC50 values \ hpe	24 hours	48 hours	72 hours	96 hours
LC_{50} for embryos (µM)	N/A	N/A	N/A	N/A
LC_{50} for larvae (µM)	N/A	39.58	28.47	11.44
LC_{50} 95% CI for larvae (µM)	N/A	32.15 - 67.99	24.52 - 35.76	9.768 - 13.45

CI: Confidence interval; hpe: hours post exposure; N/A: Not applicable, meaning LC50 values were not identified as cowanol at studied doses showed no lethal effect on embyos/larvae with corresponding exposure time.

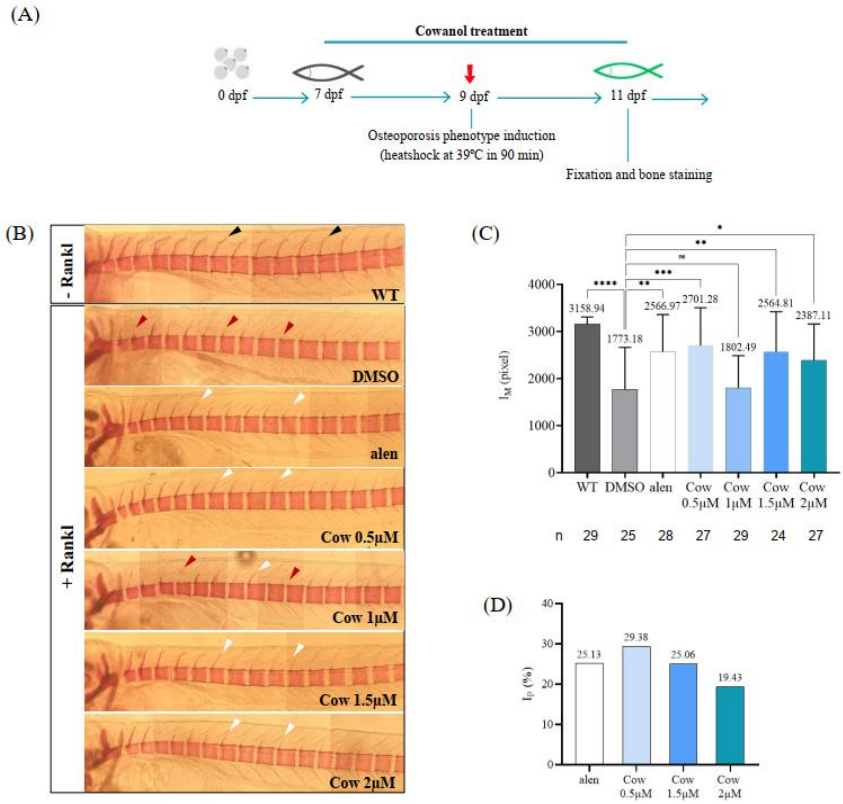

Figure 3. Cowanol reduces mineralized matrix bone damage in Rankl-induced osteoporosis medaka larvae.

(A) Procedure to assess the effect of cowanol on bone damage induced by Rankl in Rankl fish; green fish is Rankl-induced fish, the red arrow indicates heat shock induction; (B) Representative images of alizarin red stained of the first 15 vertebrae of control groups: wild-type larva (WT, -Rankl), DMSO (+Rankl), alen (+Rankl +alen) and four cowanol-treated groups of 0.5, 1, 1.5, or 2 µM. Black arrows mark intact neural arches in wildtype fish; red arrow heads indicate severely destructed neural arches, white ones mark mildly destructed neural arches; (C) The mean values of bone mineralization index (I_M) indicate the level of bone mineralization in fish larvae in the cowanol-treated groups (0.5, 1, 1.5, 2 µM) and control groups (WT, DMSO, alen). (*) indicates statistically significant difference in I_M values between groups ((*):$p < 0.05$; (**):$p < 0.01$; (***):$p < 0.001$; (****): $p < 0.0001$). n: the number of larvae in the corresponding group; (D) Index of mineralization protection (I_P) of cowanol at effective doses of 0.5, 1.5, or 2 µM compared with alendronate (alen) 50 µg/mL.

3.1.3. Cowanol Reduced Mineralized Bone Damage in the Rankl Fish Model for Osteoporosis

To evaluate the ability of cowanol to reduce Rankl-induced osteoporosis bone damage, we treated Rankl larvae (7 to 11 days post-fertilization) with cowanol and heat-shocked them at 9 days post-fertilization, following a previous study [30]. Given that cowanol was safe at 2 µM based on toxicity tests, we used four doses for the experiments: 0.5, 1, 1.5, or 2 µM. Details of the experimental procedure are provided in the "Cowanol treatment" section and illustrated in Figure 3A. At 11 dpf, fish larvae from all groups were fixed and stained with alizarin red to visualize mineralized bone structures. The first 15 vertebrae of the stained larvae were imaged and assessed levels of bone mineralization and damage using the I_M method [30]. The results are shown in Figures 3B and 3C.

As illustrated in Figure 3B, wild-type larvae had intact mineralized bone matrix in the neural arches (black arrowheads). In contrast, Rankl-induced fish larvae, including those treated with 0.1% DMSO as a solvent control (+Rankl+DMSO), alendronate as a positive control (+Rankl+alen), and the four doses of cowanol (+Rankl+cowanol+DMSO), exhibited varying degrees of damage in the mineralized neural arches (red and white arrowheads). Notably, the extent of bone damage in the alendronate-treated group and the cowanol-treated groups (0.5, 1.5, and 2 µM) appeared less severe compared to the DMSO control group (Figure 3B). The I_M values, representing the mean of total length of the first 15 mineralized neural arches, confirmed these observations. Alendronate and the three cow-treated groups (0.5, 1.5, and 2 µM) had significantly higher mean I_M values compared to the DMSO control group (Alendronate 50 µg/mL: 2566.97; n=28; cowanol 0.5 µM: 2701.28; n=27; cowanol 1.5 µM: 2564.81; n=24; cowanol 2 µM: 2387.11; n=27 vs. DMSO: 1773.18; n=25) with $p<0.001$, $p<0.01$, and $p<0.05$, respectively (Figure 3C). Thus, cowanol at these three doses can protect the mineralized neural arches of heat-shocked Rankl fish from Rankl-induced bone damage. The protection indexes (I_P) for cowanol at these doses are 29.38%, 25.06%, and 19.43%, respectively. These values are comparable to the protection index value of the positive control drug alendronate (50 µg/mL), which is 25.13%. Notably, cowanol at 1 µM showed no significant effect on bone damage (I_M = 1802.49; n=29 vs. DMSO I_M = 1773.18; n=25). Therefore, cowanol demonstrates an anti-resorptive effect in Rankl-induced fish, with the 0.5 µM dose showing the highest efficacy.

3.2. Discussion

In this study, we isolated cowanol from *G. oblongifolia* and assessed its acute toxicity and bone protective activity on medaka fish models.

The chemical structure of isolated cowanol was verified using HRMS, as well as ^{1}H and ^{13}C NMR spectroscopies [34]. Moreover, the cowanol compound that was produced exhibited a remarkably high level of purity, reaching 98.4%.

Regarding acute toxicity, cowanol was evaluated at concentrations ranging from 2 to 50 µM to medaka embryos and from 2-30 µM larvae after 96 hours of exposure. Our results indicate that cowanol was not toxic to medaka embryos aged 24 to 120 hours post-fertilization (hpf). However, it exhibited dose- and time-dependent toxicity effects on medaka larvae from 7 to 11 days post-fertilization (dpf). Specifically, cowanol was found to be safe at a concentration of 2 µM, with an LC_{50} value - the concentration that causes 50% mortality - of 11.44 µM (Figure 2).

While there is no existing *in vitro* or *in vivo* data on the toxicity of cowanol, toxicity has been reported for some extracts and xanthone compounds. Notably, *α*-mangostin, a major xanthone found in mangosteen (*G. mangostana* Linn.) fruits, has been studied extensively. *α*-mangostin is well-known for its antioxidant

and anticancer properties, leading to increased interest in its toxicity [35, 36].

The acute toxicity of *α*-mangostin have been investigated in several studies using zebrafish, another non-rodent fish model commonly employed in toxicity research. Kittipaspallop et al. [37] reported that α-mangostin caused mortality and developmental abnormalities in zebrafish embryos after 72 hours of exposure, with an LC_{50} value of 5.75 ± 0.26 µM. The study also observed teratogenic effects such as axis malformation, bent tail, pericardial edema, yolk sac edema, sluggish circulation, and heart malformation [37]. Further research by Wittaya Pimtong et al., [38] demonstrated that *α*-mangostin caused mortality in zebrafish embryos at doses ranging from 0.25 to 4 µM over 4 to 120 hours post-fertilization, with a LC_{50} value of 1.48 ± 0.29 µM at 120 hpf. The study also noted teratogenic effects on embryonic development, particularly impacting hepatogenesis [38]. A study conducted by Fazry et al., in 2018 [35] investigated the acute toxicity of xanthone crude extract (XCE), which contains *α*-mangostin and other xanthones isolated from mangosteen peel. The study found that XCE caused embryonic mortality, with a 100% mortality rate observed at concentrations of 62.5 µg/mL and above. Pure *α*-mangostin caused similar effects at concentrations of 50 µg/mL and above. Notably, pure *α*-mangostin appeared to be more toxic, as surviving embryos treated with *α*-mangostin exhibited maldevelopment, whereas this was not observed in embryos treated with XCE [35]. This suggests that different xanthones have varying levels of toxicity, and other xanthones or bioactive compounds in XCE may mitigate the toxicity of *α*-mangostin.

Our study provides the first evidence that cowanol, a common xanthone, exhibits acute toxicity in medaka fish larvae but not in fish embryos. This difference can be attributed to the protective role of the chorion, the fertilization membrane covering the embryos until they hatch, which occurs around 7 dpf [22]. The chorion may function like a barrier to limit the penetration of cowanol into embryos. In contrast, zebrafish hatch much earlier (around 2 dpf), so this may explain the reason for the difference in toxicity of a compound or xanthone to medaka and zebrafish embryos.

The acute toxicity data from this study are crucial for our subsequent research on the bone protective effects of cowanol. They indicate that doses of 2 µM and lower should be used for these experiments.

We next evaluated the bone-protective activity of cowanol across four different concentrations (0.1, 1, 1.5, or 2 µM) in a Rankl fish model. Among these doses, only the 0.5, 1.5, or 2 µM doses demonstrated a significant effect on reducing bone damage, and the 1 µM dose did not show effectiveness (Figure 3B, C). The bone protection indexes for the 0.5, 1.5, or 2 µM doses were comparable to that of alendronate with the index of bone protection reaching up to 29.38% for the 0.5 µM cowanol dose (Figure 3D). As alendronate is a widely used anti-resorptive drug, this highlights the potential of cowanol for drug development. The lack of efficacy observed at the 1 µM dose suggests a non-linear dose-response relationship, possibly due to the agent's effectiveness being apparent only at certain concentrations. Further investigation is needed to elucidate the underlying mechanisms.

To date, there are no studies addressing the effects of cowanol on bone health. However, emerging evidence suggests that other xanthones may possess bone-protective properties. In 1997, Pifferi *et al.,* reported that synthetic 3,6-diisopropoxyxanthone, modified from ipriflavone - a known anti-osteoporotic drug - significantly reduced bone resorption in both *in vitro* and *in vivo* models [13]. In 2008, Zhang *et al.*, isolated 11 xanthones from *H corniculata* and found that five of them inhibited osteoclast formation and differentiation *in vitro* [10]. In 2016, Idrus and Kiswanjaya demonstrated that mangosteen peel extracts reduced bone resorption and bone damage in osteoporosis mouse models induced by lipopolysaccharide

(LPS) [11]. Jeong *et al.*, (2020) isolated cudratrixanthone U (CTU) from *Maclura tricuspidata* and reported its *in vitro* inhibitory effects on RANKL-induced osteoclast differentiation and function [12]. Most recently, in 2022, Zhang *et al.* revealed that α-mangostin inhibits LPS-induced bone resorption by targeting RANKL-induced osteoclastogenesis via NF-κB and MAPK signaling pathways *in vivo* [39]. Additionally, Xiao Ling *et al.*, found that tetrahydroxanthone dimers produced by gut bacteria exhibited anti-osteoporosis activity in prednisolone-induced osteoporotic zebrafish [40].

Taken together, our study provides the first evidence of the bone-protective effects of cowanol, consistent with the bone-protective activities reported for other xanthones both *in vitro* [10] and *in vivo* [11]. Our findings, showing that cowanol can reduce bone damage induced by Rankl in the Rankl fish model, suggest its potential to inhibit RANKL-induced osteoclast formation and function, in line with previous studies on α-mangostin and other xanthones [12, 39].

4. Conclusion

We have isolated cowanol from *G. oblongifolia* in Vietnam, demonstrating its dose- and time-dependent toxicity and bone-protective effects in reducing bone loss induced by RANKL in a transgenic medaka model. This non-rodent model is valuable for studies on toxicity and diseases. Our findings underscore the potential of local herbs in therapeutic applications and provide important data on the bioactivity of xanthone compounds for further research of their therapeutic potential.

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