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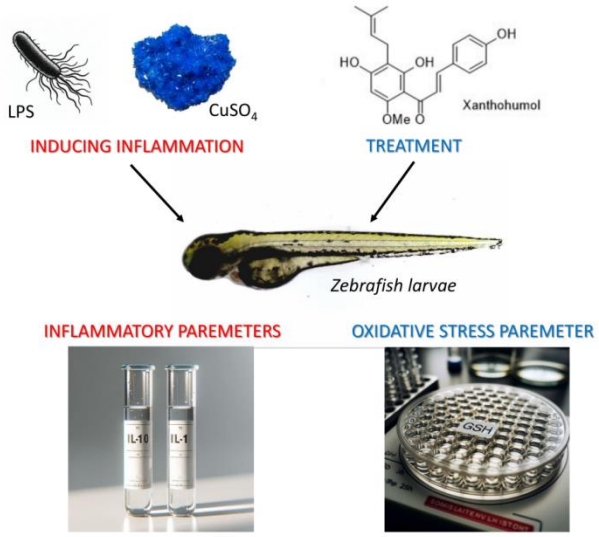
Creating a Reliable Zebrafish Model for Studying Inflammation: Exploring the Therapeutic Potential of Xanthohumol

Agata Szumlak[(1)], Dorota Luchowska-Kocot[(1)] ✉, Fares Qtaishat[(2)], Anna Boguszewska-Czubara[(1)]✉

[(1)] Department of Medical Chemistry, Medical University of Lublin, Chodzki 4A, 20-093 Lublin, Poland
[(2)] School of Medicine, University of Jordan, Queen Rania St, 11947 Amman, Jordan

✉ Correspondence to: dorota.luchowska-kocot@umlub.pl, anna.boguszewska-czubara@umlub.pl



Abstract: Inflammation is the body's natural immune response to pathogens, such as viruses, bacteria, and parasites, and is closely linked to oxidative stress caused by an imbalance in reactive oxygen species (ROS) and antioxidants. Chronic inflammation can lead to diseases and cancer. Due to the side effects of synthetic anti-inflammatory drugs such as NSAIDs and corticosteroids, natural compounds such as flavonoids are gaining interest. This study aimed to validate the zebrafish (Danio rerio) as a model for studying inflammation and to compare the anti-inflammatory effects of xanthohumol with ibuprofen and quercetin. Using lipopolysaccharide (LPS) and copper sulfate ($CuSO_4$) to induce inflammation, zebrafish larvae provided an

excellent model. The study determined the lethal doses (LD50) of several substances, with xanthohumol having an LD50 of 2.659 µg/ml, ibuprofen 15 µg/ml, and quercetin 200 µM. Exposure to xanthohumol at 2 µg/ml before LPS administration significantly improved larval survival, maintaining 80% viability compared to 25% with LPS alone. Additionally, xanthohumol reduced IL-1 levels by over twofold and increased IL-10 expression, demonstrating its anti-inflammatory effects. Xanthohumol also protected against oxidative stress induced by $CuSO_4$, significantly reducing cell damage compared to controls. These results highlight xanthohumol's strong protective and anti-inflammatory properties.

Keywords: Inflammation, Danio rerio, xanthohumol, hop extract

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Introduction

Chronic inflammation plays a pivotal role in the development of various diseases, including cardiovascular disorders, neurodegenerative diseases, and cancer [1]. Persistent inflammation correlates with elevated concentrations of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukins (IL-1β, IL-6), and reactive oxygen species (ROS), which contribute to the pathology of these conditions [2]. To reduce inflammation, medications like non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are frequently prescribed. However, prolonged use of these synthetic drugs often results in severe side effects, including gastrointestinal, cardiovascular, and hepatic complications [3]. This underscores the need for safer, more effective alternatives.

Natural products have emerged as promising candidates for inflammation management due to their minimal side effects and potent biological activity. Among these, hop extract (Humulus lupulus) has garnered significant attention for its anti-inflammatory and antioxidant properties [4]. A key bioactive compound in hops, xanthohumol, has been shown to modulate inflammatory pathways by suppressing cytokines like TNF-α and IL-6 and boosting the expression of antioxidant enzymes such as NAD(P)H oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) [5, 6]. Despite this, the potential of xanthohumol as an anti-inflammatory agent, particularly in comparison to conventional drugs, remains underexplored.

The zebrafish (Danio rerio) model is widely recognized for evaluating the anti-inflammatory potential of natural compounds due to its genetic similarity to humans and the transparency of its embryos, allowing real-time observation of inflammatory processes [7]. Lipopolysaccharide induced inflammation in zebrafish is commonly used to study the effects

of potential anti-inflammatory agents. LPS triggers a robust immune response characterized by increased macrophage and neutrophil activity and the release of cytokines such as IL-1β, IL-6, and TNF-α [8]. Studies using this model have confirmed the effectiveness of natural anti-inflammatory agents in suppressing these immune responses, positioning zebrafish as an ideal organism for such investigations. However, the efficacy of xanthohumol in mitigating LPS-induced inflammation in zebrafish has not been fully characterized, leaving a gap in understanding its therapeutic potential in this model.

Additionally, inflammation induced by environmental stressors like metals remains underexplored in the context of natural anti-inflammatory agents. Among these, copper (Cu) is an essential trace element involved in many physiological processes, such as mitochondrial respiration, antioxidant defense, and connective tissue formation [9]. However, excessive copper exposure or dysregulated copper homeostasis has been linked to inflammation and oxidative stress. Copper ions (Cu^{2+}) can promote the production of ROS, which in turn leads to lipid peroxidation, protein oxidation, and DNA damage, all of which contribute to inflammatory processes [10]. Additionally, elevated copper levels can activate pro-inflammatory signaling pathways, such as NF-κB, leading to the upregulation of cytokines like TNF-α and IL-6 [11].

The inflammatory potential of copper is particularly relevant in cases of copper-induced toxicity, where chronic exposure can lead to liver and kidney damage, as well as neuroinflammation. Copper overload is also associated with certain metabolic and neurodegenerative diseases, such as Wilson's disease and Alzheimer's disease, where inflammation is a critical pathological feature [12]. Therefore, studying copper-induced inflammation is essential for understanding its role in disease progression and for identifying potential therapeutic interventions to mitigate its harmful effects. Investigating whether xanthohumol can mitigate inflammation caused by excess copper exposure could reveal novel applications of this compound.

Moreover, other natural compounds, particularly polyphenols and flavonoids, have demonstrated significant anti-inflammatory and antioxidant effects. These compounds, found abundantly in fruits, vegetables, and grains, inhibit the production of inflammatory mediators and regulate oxidative stress, further supporting their therapeutic potential in chronic inflammatory diseases [13, 14]. In this context, hop extract and its active components, like xanthohumol, offer promising opportunities for the development of safer anti-inflammatory therapies.

This study aims to address these gaps by evaluating the anti-inflammatory effects of hop extract, specifically xanthohumol, using an LPS-induced zebrafish inflammation model,

with a focus on cytokine regulation. The study also explores the role of xanthohumol in mitigating copper-induced inflammation and oxidative stress, focusing on cellular protection. By comparing xanthohumol with standard anti-inflammatory agents such as ibuprofen and quercetin, this work seeks to elucidate the therapeutic potential of hop-derived compounds and advance their application as natural alternatives to synthetic drugs.

Results and discussion

Our findings demonstrate that exposure to xanthohumol significantly modulates inflammatory markers, suggesting its therapeutic potential in managing inflammation. Inflammation is a protective response triggered by chemical, biological, or physical stimuli, involving immune cells such as macrophages, neutrophils, and lymphocytes [15,16]. These cells produce substances that limit tissue damage and initiate healing processes, including cytokines and growth factors that attract additional immune cells to the site of inflammation [17,18]. Markers of inflammation are often used as indicators of disease severity and therapeutic efficacy [16].

Managing inflammatory conditions involves multiple strategies, including the administration of anti-inflammatory agents like non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, which act on a molecular level by inhibiting inflammatory mediators. The main drugs used for treating inflammation are synthetic compounds: non-steroidal drugs (ibuprofen, aspirin, diclofenac) and steroidal drugs (betamethasone, prednisolone, dexamethasone). Despite their efficacy, prolonged use can lead to serious side effects, such as gastrointestinal, cardiovascular, hepatic, renal, cerebral, or pulmonary complications [19]. As a result, there is a growing interest in natural substances, which offer a gentler approach to inflammation therapy [17, 20]. Sources of these compounds include grains, legumes, vegetables, fruits, and nuts [20]. These products are valuable in the development of medicinal preparations (nutraceuticals). Natural compounds with confirmed anti-inflammatory properties include flavonoids, which are bioactive molecules with anti-inflammatory, antioxidant, and immunomodulatory effects. Flavonoids have the ability to inhibit the production of certain inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, as well as chemokines from immune cells. Furthermore, flavonoids can regulate the expression of pro-inflammatory genes (doi.org) and suppress the activity of enzymes like cyclooxygenase (COX), lipoxygenase (LOX), and nitric oxide synthase (NOS) [21]. They also promote apoptosis and demonstrate anticancer potential. Owing to these properties, flavonoids hold therapeutic potential for treating a range of diseases, including atherosclerosis, coronary heart disease, and cancer [22]. One of the most well-known

flavonoids is quercetin, which is commonly found in apples, grapes, onions, and tomatoes [21, 23].

Hops (Humulus lupulus) have been used in folk and traditional medicine for centuries. Their medicinal properties have been recognized by various cultures throughout history. In traditional Chinese medicine, hops were used to alleviate symptoms of indigestion, stimulate appetite, and serve as a calming agent. It was believed that hops could help maintain energetic balance in the body. The ancient Greeks and Romans also employed hops for their medicinal properties, using them to aid digestion and treat stomach ailments. In medieval Europe, hops were primarily used as a sedative and sleep aid. Additionally, hops were incorporated into beer brewing, providing health benefits due to their antimicrobial properties. In Ayurvedic medicine, hops were used to support digestion, relieve nervousness and stress, and serve as a natural remedy for insomnia. They were also used to reduce pain and inflammation [24].

Xanthohumol is a natural flavonoid compound isolated from hops (Humulus lupulus L.). The antioxidant properties of this compound have been confirmed in numerous studies. It has been shown that xanthohumol can inhibit the production of nitric oxide and superoxide anions and neutralize hydroxyl and peroxyl radicals [25]. Xanthohumol also exhibits chemopreventive properties, inducing apoptosis in cancer cells both in vivo and in vitro, and inhibiting angiogenesis [26]. Literature data suggest that xanthohumol has anti-inflammatory properties, which have been observed in vitro in various cell types and in vivo in models of acute and chronic inflammation affecting different organs [27-30].

Inflammation research models are crucial for understanding inflammatory mechanisms and testing new therapies. In vitro, cell cultures such as macrophages or monocytes are commonly used, and these cells are stimulated by LPS to study the molecular mechanisms of the inflammatory response, including the activation of signaling pathways (NF-κB, MAPK) and the secretion of inflammatory mediators (IL-1β, TNF-α). In vivo, animal models are commonly employed, where inflammation is induced through endotoxins like lipopolysaccharide (LPS), chemical agents (e.g., carrageenan), or mechanical factors such as injuries. These models enable the evaluation of the inflammatory response at the level of the whole organism, including immune cell activation, cytokine secretion, and changes in microcirculation. In recent years, the zebrafish model (Danio rerio) has gained increasing popularity as an alternative to traditional animal models [31, 32]. The transparency of zebrafish larvae and the ease of genetic manipulation allow for real-time observation of inflammatory responses at the cellular and molecular levels, making them a valuable tool in

inflammation research. The immune system of Danio rerio exhibits significant similarity to humans, both at the molecular and cellular levels [31, 33].

In previous studies, zebrafish have been shown to be an appropriate model for inflammatory diseases, as they display similarities in the structures and functions of cytokines present in mammals, such as TGF-β, IFN-β, IL-17, IL-8, IL-10, IL-1β, and IL-6 [34].

Our research focused on a comprehensive approach to analyzing inflammation using the zebrafish model (Danio rerio). We conducted experiments utilizing two different methods of inflammation induction: biological, through exposure to lipopolysaccharide (LPS), and chemical, using copper sulfate. This allowed us to examine various aspects of inflammatory responses triggered by both pathogens and toxic factors.

Lipopolysaccharide (LPS) is an endotoxin that forms complexes in the outer membrane of Gram-negative bacteria. LPS is a potent activator of the inflammatory response and a trigger for cytokine release from macrophages [35]. Additionally, it increases the production of reactive oxygen species (ROS) and nitric oxide (NO). LPS-induced inflammation is widely used in anti-inflammatory drug research [31]. In animal studies on species like mice, pigs, and cattle, cytokine functions produced during LPS-induced inflammation have been investigated. The toxic effect is primarily due to the secretion of IL-1β, IL-6, and TNF-α [17]. Similarities between the inflammatory response to LPS in mammals and Danio rerio have been demonstrated. In mammals, the TLR4 (toll-like receptor) complex plays a crucial role in recognizing this substance, but the mechanism in Danio rerio is not well understood. In zebrafish models, high doses of LPS can induce inflammation leading to sepsis or death [36]. Inflammation can be induced non-invasively by immersing larvae in LPS-containing media or invasively by injecting LPS into the yolk [31]. Studies using the zebrafish model with LPS-induced inflammation have confirmed the anti-inflammatory effects of compounds such as phillyrin, a component of Forsythia suspensa Vahl fruits, and extracts from the leaves of Chimonanthus nitens Oliv [37, 38].

Copper is another chemical agent that can induce inflammation and is the third most abundant transition metal in the human body. Imbalances in copper levels contribute to disorders such as Alzheimer's disease, Parkinson's disease, as well as genetic disorders like Wilson's disease, Huntington's chorea, and Menkes disease. Studies have shown that zebrafish embryos tolerate low concentrations of copper, but higher doses can increase mortality rates [39]. Inflammatory responses can be triggered by an excess of copper sulphate ($CuSO_4$) from the external environment [31]. Copper acts as a catalyst in ROS production. Cu^{2+} ions are reduced to Cu^{+}, which can break down H_2O_2, leading to the

release of hydroxyl radicals. Additionally, copper reduces glutathione levels, inhibiting the reduction of Cu^{2+} to Cu^{+} and participating as a substrate in ROS removal processes [39]. Inflammation can be induced by exposing Danio rerio embryos to copper (II) sulfate [31]. Zebrafish show similarities to humans in copper metabolism, possessing two genes encoding copper-transporting ATPases: atp7a and atp7b. The expression of the atp7a gene is present in the neural cord of embryos, while the atp7b gene is expressed in the liver and brain up to the fifth day post-fertilization. Studies have shown that silencing atp7b expression induces oxidative stress in embryos and results in smaller brain and liver sizes [40]. The zebrafish model with $CuSO_4$-induced inflammation is used in anti-inflammatory drug research. A common marker used is the accumulation of neutrophils in neuromasts [31]. Natural products with confirmed inhibitory effects on $CuSO_4$-induced inflammation include squalene ($C_{30}H_{50}$), skipjack tuna (Katsuwonus pelamis) enzymatic peptide (SEP), and triterpene glycoside from the root of Sanguisorba officinalis [33, 41, 42]. These substances inhibit neutrophil accumulation. A similar effect, in both the $CuSO_4$ and LPS models, is shown by the compound PP7, derived from Paris polyphylla [43]. Research has also demonstrated that an extract from the leaves of Clerodendrum cyrtophyllum Turcz reduces oxidative stress and the levels of inflammatory cytokines such as IL-1β, IL-8, IL-10, and TNF-α [44].

The first stage of the research involved analyzing the lethal doses (LD50) of the substances used in the experiment to determine the concentration range to be applied in the study.

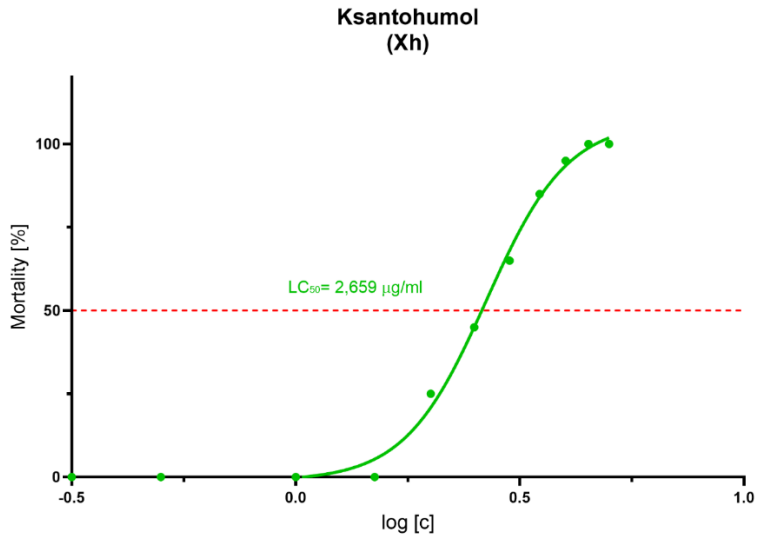

Fig. 1. Mortality of zebrafish larvae as a function of xanthohumol concentration. Determined LD50 = 2.659 µg/ml.

Based on the mortality of larvae across all trials, the lethal doses (LD) of the substances used in the experiment were determined. For lipopolysaccharide (LPS), the LD50

was 49.44 µg/ml, for $CuSO_4$, it was around 41.65 µg/ml, and for xanthohumol, it was 2.659 µg/ml. The toxic doses for ibuprofen and quercetin were found to be 15 µg/ml and 200 µM, respectively. Detailed data are presented in Figures 1 and 2.

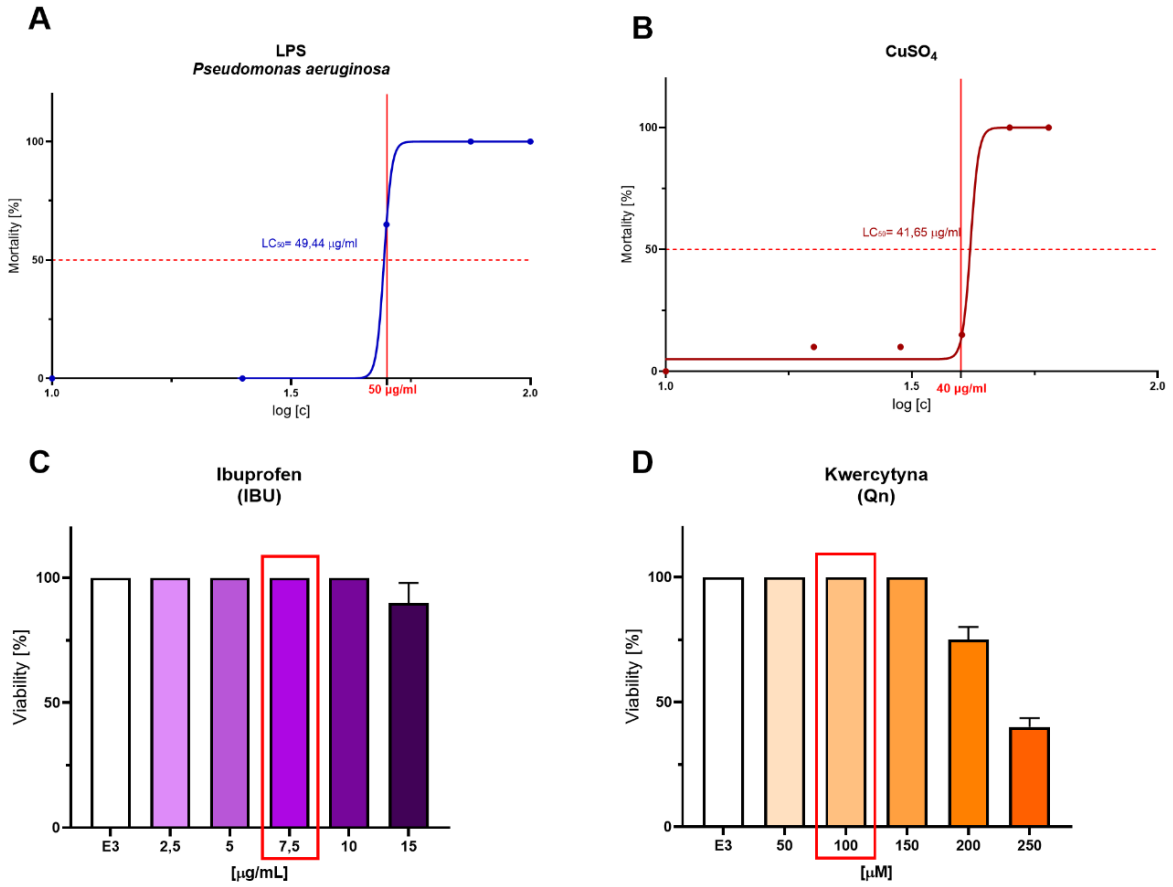

Fig. 2. Mortality of zebrafish larvae as a function of the concentrations of substances used to validate the experimental model of inflammation induced by LPS and $CuSO_4$. A. Graph showing the toxicity of LPS with the determined LD50 = 49.44 µg/ml. B. Graph showing the toxicity of $CuSO_4$ with the determined LD50 = 41.65 µg/ml. C. Effect of ibuprofen on the viability of D. rerio larvae. D. Effect of quercetin on the viability of D. rerio larvae.

One of the most important findings was that exposing larvae to xanthohumol before administering LPS significantly increased larval survival. Exposure to LPS caused a mortality rate of 75%, while administration of xanthohumol at a concentration of 2 µg/ml maintained larval viability at 80%. Lower doses of xanthohumol even resulted in up to 100% survival, suggesting strong protective effects, likely due to its antioxidant and anti-inflammatory properties. For comparison, co-administration of ibuprofen at a dose of 7.5 µg/ml with the dose of LPS that caused 75% of death, prevented mortality in zebrafish larvae. Detailed data are presented in Figure 3. The results confirm the significant role of xanthohumol,

comparable to well-known anti-inflammatory substances such as ibuprofen and quercetin, in protecting against cell damage caused by inflammation.

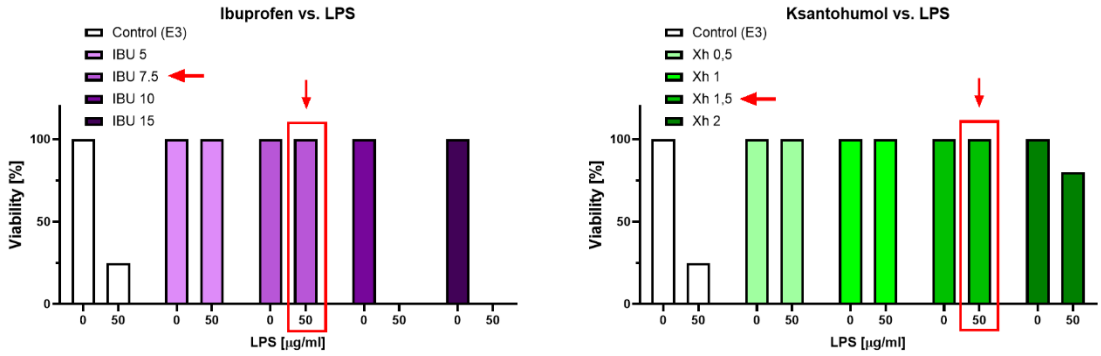

Fig. 3. Viability of larvae (%) after 30 minutes of incubation in E3 solution (negative control), ibuprofen (A), or xanthohumol (B) at selected doses (µg/ml) followed by 24 hours of incubation in LPS at a dose of 50 µg/ml.

Further experiments demonstrated that xanthohumol, similar to the well-known antioxidant quercetin, possesses the ability to protect cells from damage induced by $CuSO_4$. Propidium iodide staining revealed a reduced number of cells exhibiting inflammatory responses in the groups pre-incubated with xanthohumol (1 µg/ml) and quercetin (100 µM) prior to exposure to $CuSO_4$ (Figure 4). This indicates that both xanthohumol and quercetin can effectively safeguard cells against oxidative stress caused by copper, significantly decreasing the number of damaged cells compared to the control samples.

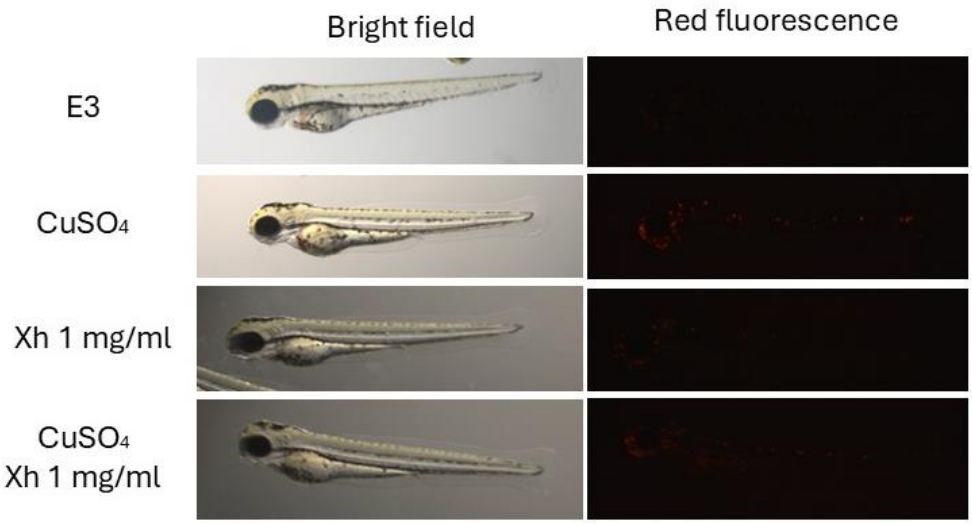

Fig. 4. The effect of xanthohumol (Xh) at the dose of 1 mg/ml on $CuSO_4$-induced oxidative injuries in Danio rerio. Representative fluorescence images of various experimental groups after propidium iodide (PI) staining.

Substances serving as markers of inflammation, such as cytokines and acute-phase proteins, act as indicators of disease activity and treatment efficacy. Cytokines, including

interleukins (IL-1, IL-6, IL-8), tumor necrosis factors (TNF-α, TNF-β), and interferons, play a crucial role in regulating the inflammatory response. For instance, IL-1β and TNF-α are pivotal in response to bacterial and viral infections, and their excessive production can lead to autoimmune diseases and cancers. The results obtained from our studies regarding the levels of cytokines IL-1 and IL-10 also provided valuable insights into the action of these substances (Figure 5). Exposure of larvae to LPS resulted in an increase in IL-1 levels, which is characteristic of pro-inflammatory responses. However, pre-incubation with ibuprofen (7.5 μg/ml) and xanthohumol (1.5 μg/ml) before LPS administration led to a more than twofold reduction in IL-1 expression, indicating the ability of these substances to inhibit inflammatory responses. Conversely, IL-10, an anti-inflammatory cytokine, exhibited increased expression in response to xanthohumol and ibuprofen, suggesting that both substances may support anti-inflammatory mechanisms. Notably, xanthohumol demonstrated a stronger effect in elevating IL-10 levels compared to ibuprofen. These findings align with literature data confirming that xanthohumol reduces the production of inflammatory markers, including TNF-α, IL-6, IL-8, and IL-12 [27, 28]. Furthermore, xanthohumol has been shown to have a protective effect against inflammation and liver fibrosis in mouse models and to inhibit the progression of osteoarthritis [28, 29]. Studies have also demonstrated that xanthohumol suppresses the expression of inflammatory cytokines and lowers oxidative stress levels in mice with acute lung injury induced by LPS exposure [30].

Xanthohumol, a flavonoid found in hops, has been extensively studied for its anti-inflammatory properties. Research indicates that xantohumol can significantly decrease the production of proinflammatory cytokines like TNF-α, IL-6 and NO, especially in cell models of inflammation, such as chondrocytes treated with IL-1β. This effect is primarily mediated through the inhibition of key inflammatory signaling pathways, including C/EBPβ, and the upregulation of HO-1, a protein with protective functions in inflammatory responses. These mechanisms align with those of well-established anti-inflammatory drugs like ibuprofen, which further supports XH's potential as a therapeutic agent for managing inflammation and oxidative stress. Additionally, studies have highlighted that XH provides protection against various forms of induced inflammation and oxidative damage, positioning it as a promising candidate for future clinical applications [45].

Oxidative stress is strongly associated with inflammation, arising from an imbalance between the generation of reactive oxygen species (ROS) and the body's capacity to neutralize them. Excessive ROS production causes molecular damage and amplifies inflammatory responses, contributing to the onset of chronic diseases. Antioxidant enzymes, including superoxide dismutase (SOD), glutathione (GSH), and catalase, play a critical role in

detoxifying ROS, and their deficiency can exacerbate inflammation. A key aspect of the study was evaluating the protective effects of xanthohumol and quercetin against oxidative stress induced by $CuSO_4$. Results from the FRAP (Ferric Reducing Antioxidant Power) assay demonstrated that pre-incubation with xanthohumol (1 µg/ml) and quercetin (40 µg/ml) effectively mitigated the reduction in oxidative potential observed in samples exposed solely to $CuSO_4$. Furthermore, pre-incubation with these compounds helped preserve intracellular antioxidant glutathione (GSH) levels, which were otherwise diminished by $CuSO_4$ exposure. These findings confirm the protective effects of xanthohumol, comparable to those of quercetin, in counteracting oxidative stress and enhancing cellular antioxidant defenses.

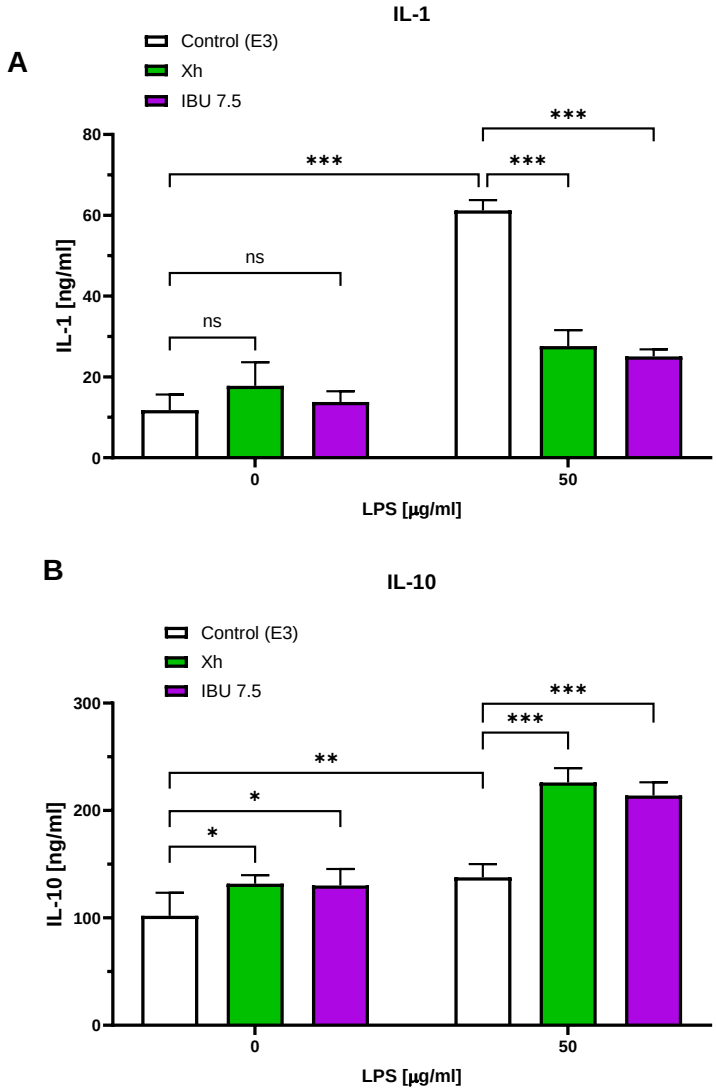

Fig. 5. The effect of xanthohumol (Xh) at a dose of 1.5 µg/ml and ibuprofen (IBU) at a dose of 7.5 µg/ml on selected inflammatory markers in homogenates of zebrafish (Danio rerio) with LPS-induced inflammation (50 µg/ml). A. Graph representing interleukin 1 (IL-1) levels in D. rerio homogenates; Two-way ANOVA, Tukey's test: ns – not significant, ***p<0.001. B. Graph representing interleukin 10 (IL-10) levels in D. rerio homogenates; Two-way ANOVA, Tukey's test: *p<0.05, **p<0.01, *p<0.001.

Research on anti-inflammatory and antioxidant properties of xanthohumol in *Danio rerio* experimental model has been unexplored. Its potential have been confirmed in numerous in vitro studies, particularly in macrophage and epithelial cell models. These include its ability to modulate oxidative stress, suppress NF-κB activation, and reduce the production of pro-inflammatory mediators like IL-6, TNF-α, and IL-1β in LPS-stimulated macrophages [46]. Only fewer studies have explored its effects in vivo, particularly in broader animal models focusing on general inflammation. Existing research has predominantly addressed specific conditions, such as LPS-induced lung inflammation [47] or oxazolone-induced dermatitis in mice [48], where xanthohumol has been shown to suppress the release of pro-inflammatory cytokines and inhibit pathways like STAT-1 and IRF-1. These findings underscore xanthohumol's potential in mitigating inflammation and reducing cytokine-driven damage. This aligns with its broader immunomodulatory role, including enhancing antioxidant enzyme activities (e.g., Nrf2 activation) and promoting cellular redox balance. Our findings are consistent with the existing body of research on xanthohumol, further confirming its anti-inflammatory and antioxidant properties in both in vitro and in vivo models. Similar to previous studies that have demonstrated xanthohumol's ability to suppress pro-inflammatory mediators like IL-6, TNF-α, and IL-1β in LPS-stimulated macrophages, our research shows a significant reduction in IL-1 and an increase in IL-10 levels in zebrafish exposed to LPS. These findings are consistent with its established role in modulating cytokine-driven inflammation and enhancing anti-inflammatory pathways. Additionally, the antioxidant effects observed in our study, such as the preservation of glutathione (GSH) levels and reduction in oxidative stress markers, align with xanthohumol's known role in activating antioxidant pathways like Nrf2. This supports its dual function as an anti-inflammatory and antioxidant agent, which has been highlighted in prior research. While much of the previous work has focused on specific inflammatory conditions or isolated in vitro systems, our research expands this understanding by demonstrating xanthohumol's efficacy in a versatile zebrafish model. Moreover, our findings demonstrate the utility of zebrafish as a model for evaluating the anti-inflammatory properties of natural compounds as well as xanthohumol's potential for therapeutic application in chronic inflammatory diseases.

In summary, the research findings unequivocally confirm that xanthohumol, to a degree comparable to ibuprofen and quercetin, can play a key role in protecting cells from the toxic effects of exposure to LPS and $CuSO_4$. Its anti-inflammatory and antioxidant properties suggest that it may be a valuable substance in the context of preventing cellular

damage induced by oxidative stress and inflammation. This work also underscores the potential of *Danio rerio* in complementing mammalian models to investigate inflammation mechanisms and screen therapeutic candidates.

Material and methods

Toxicity tests

Ten larvae at 3-dpf (days post fertilization) were added to each well of 12-well plates, followed by the addition of fresh E3 medium (34.8 g NaCl, 9.78 g $MgCl_2 \cdot 6H2O$, 5.8 g $CaCl_2 \cdot 2H_2O$, 2.6 g KCl). E3 solution is a commonly used medium for rearing zebrafish embryos that mimics the natural aquatic environment and provides the necessary ions and pH for optimal zebrafish development. The LPS was dissolved in E3 medium to prepare stock solution of 1mg/ml. To the first plate, an appropriate amount of LPS was added to each well to achieve final concentrations of 5, 25, 50, 75, and 100 µg/ml, with a total volume of 2 ml in each well. The $CuSO_4$ was dissolved in E3 medium to prepare stock solution of 1mg/ml. In the second plate, an appropriate amount of $CuSO_4$ was added to achieve final concentrations of 20, 30, 40, 50, and 60 µg/ml. The ibuprofen was dissolved in E3 medium to prepare stock solution of 1mg/ml. The third plate received ibuprofen at concentrations of 2.5, 5, 7.5, 10, and 15 µg/ml. The quercetine was dissolved in DMSO to prepare stock solution of 10mM/ml. The fourth plate was treated with quercetin at concentrations of 50, 100, 150, 200, or 250 µM/ml. The xenthohumol was dissolved in DMSO to prepare stock solution of 1mg/ml. Finally, the fifth plate received xanthohumol at concentrations of 0.5, 1, 1.5, or 2 µg/ml. After 24 hours of incubation at 28.5°C under consistent light/dark cycle conditions. the viability of the fish was assessed. For each experimental group, three replicates were performed.

Investigating the effect of xanthohumol on LPS-induced toxicity in zebrafish

Ten larvae at 3-dpf were added to each well of 12-well plates along with 2 ml of E3 solution. Xanthohumol (0.5, 1, 1.5, or 2 µg/ml) or ibuprofen (7.5 or 10 µg/ml) was added to the wells and subjected to incubation for one hour at 28.5°C. Following this, LPS solution (50 µg/ml) was introduced, and the plates were incubated for 24 hours at 28.5°C. The viability of the larvae was then checked.

Investigating the effect of xanthohumol on $CuSO_4$-induced toxicity in zebrafish

In a 6-well plate, a solution of xanthohumol at a concentration of 1 µg/ml and a quercetin solution at 100 µM were sequentially added, followed by E3 solution to achieve a final volume of 6 ml. The remaining wells were filled with 6 ml of E3 solution. Three nets

were prepared, each containing 10 larvae, and these were placed in the first three wells. The plate was incubated for one hour at 28.5°C. After an hour, the nets were transferred to the remaining wells for rinsing. Subsequently, a new 6-well plate was prepared, where the first three wells received 60 µl/ml of $CuSO_4$ solution along with E3 solution to bring the final volume to 6 ml, and the nets were placed in them. The remaining wells received E3 solution. The plate was incubated for 20 minutes at 28.5°C. After incubation, the nets were transferred to the remaining wells. The larvae were placed into three wells, and 2 ml of E3 solution and 20 µl of 1mg/ml solution of propidium iodide (PI) in water were added. After a 5 minutes, the liquid was collected, and 1 ml of E3 solution with tricaine was added. The larvae were visualized using a SteREO Discovery V8 fluorescence microscope.

Analysis of inflammatory markers

Samples' preparation

Twenty larvae at 3-dpf were added to each well of 6-well plates along with 3 ml of E3 solution. In the first plate, ibuprofen (7.5 µg/ml) or xanthohumol (1.5 µg/ml) was added to the wells. After 30 minutes of incubation at 28°C, LPS solution (50 µg/ml) was added, followed by a second incubation at the same temperature. After 24 hours, tricaine solution was added, and the larvae were frozen at -20°C. In the second plate, quercetin solution (10 µl/ml) or xanthohumol (1 µg/ml) was added to the wells. The plate was incubated for one hour at 28°C, followed by the addition of CuSO4, and the plate was incubated again at the same temperature. After 20 minutes, tricaine solution was added, and the larvae were frozen at -20°C. After thawing, the samples were homogenized and centrifuged (15 minutes, 4°C, 8000 rcf). The supernatant was collected and placed in separate tubes. The samples were stored at -20°C.

Protein quantification

After thawing, the samples were diluted with PBS at a ratio of 1:10. Samples for the standard curve and working reagent (WR) were prepared using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions. Standards (range 250-2000ng/ml) and test samples (obtained as described in Section 4.1) were added to a 96-well plate, followed by the addition of WR to each well. The plate was incubated at 37°C for 30 minutes. Absorbance values were measured using the BioTek Synergy H1 Multimode Reader (AGILIENT TECHNOLOGY) at a wavelength of λ=562 nm. For further analyses, homogenates were diluted with PBS (phosphate buffered saline) to contain the same amount of protein per ml.

Determination of inflammatory markers induced by lipopolysaccharide in zebrafish homogenates

IL-1

Quantitative measurement of interleukin 1 (IL-1) concentration was performed using the BIOASSAY TECHNOLOGY LABORATORY (BT LAB) Fish Interleukin 1 ELISA Kit (E0060FI) according to the manufacturer's instructions. Solutions for a standard curve (standard solutions) were prepared within a concentration range of 4-128 ng/ml. Fifty µl of standard solutions and 40 µl of test samples (obtained as described in Section 4.1) were added to a coated 96-well plate. Ten µl of IL-1 antibody solution was added to the samples, followed by 50 µl of streptavidin conjugated with horseradish peroxidase (Streptavidin-HRP) in each well. This enzyme catalyzes a reaction that produces a colored product, which can be measured spectrophotometrically.Absorbance values were measured using the BioTek Synergy H1 Multimode Reader (AGILIENT TECHNOLOGY) at a wavelength of λ=450 nm. Concentration results were calculated based on the standard curve.

IL-10

Quantitative measurement of interleukin 10 (IL-10) concentration was performed using the BIOASSAY TECHNOLOGY LABORATORY (BT LAB) Fish Interleukin 10 ELISA Kit (E0096FI) according to the manufacturer's instructions. Solutions for a standard curve (standard solutions) were prepared within a concentration range of 40-1280 ng/ml, according to the instructions included with the kit. Fifty µl of standard solutions and 40 µl of test samples (obtained as described in Section 4.1) were added to a coated 96-well plate. Ten µl of IL-10 antibody solution was added to the samples, followed by 50 µl of streptavidin conjugated with horseradish peroxidase (Streptavidin-HRP) in each well. This enzyme catalyzes a reaction that produces a colored product, which can be measured spectrophotometrically. Absorbance values were measured using the BioTek Synergy H1 Multimode Reader (AGILIENT TECHNOLOGY) at a wavelength of λ=450 nm. Concentration results were calculated based on the standard curve.

Determination of Total Antioxidant Status (TAS) and oxidative stress markers induced by CuSO4 in zebrafish homogenates

Total Antioxidant Status (TAS) assessment by FRAP method

The total antioxidant status was assessed using the Ferric Reducing Antioxidant Power (FRAP) method to evaluate the antioxidant properties of xanthohumol in zebrafish larvae with $CuSO_4$-induced inflammation. Solutions for the standard curve and the FRAP solution were prepared according to the protocol. Standards made using $FeSO_4$ and H_2O, as

well as test samples (obtained as described in Section 4.1), were added to a 96-well plate. An appropriate amount of distilled water and then FRAP solution consisting of acetate buffer (pH=3.6), TPTZ (2,4,6-tris(2-pyridyl)-1,3,5-triazine dissolved in HCl), and $FeCl_3$ solution (10% solution in distilled water) was added to each well. After mixing, the plate was incubated in the dark at room temperature for 30 minutes. Absorbance values were measured using the BioTek Synergy H1 Multimode Reader (AGILIENT TECHNOLOGY) at a wavelength of λ=593 nm.

Glutathione (GSH)

Quantitative measurement of reduced glutathione (GSH) was performed using the BIOXYTECH GSH-420 Kit. Samples obtained by the methods described in Section 4.1 were utilized. A standard curve was prepared according to the protocol and tested samples were added to a 96-well plate. Subsequently, Buffer, Reducing Agent, Chromogen, and Color Developer were sequentially added to all wells. The plate was mixed after each reagent addition. The plate was incubated in the dark at room temperature for 30 minutes. Absorbance values were measured using the BioTek Synergy H1 Multimode Reader (AGILIENT TECHNOLOGY) at a wavelength of λ=420 nm.

Malondialdehyde (MDA)

Malondialdehyde (MDA) concentration was quantified using the BIOXYTECH LPO-586 Kit. Samples obtained as described in Section 4.1 were diluted 1:10 and 200 µl of samples were mixed with 200 µl of the reagent. The mixture was incubated for one hour at 45°C. Absorbance values were measured using the BioTek Synergy H1 Multimode Reader (AGILIENT TECHNOLOGY) at a wavelength of λ=586 nm.Tahoma 11p, 1,5 interline).

Conclusions

The results of our study demonstrate that Danio rerio (zebrafish) is a valuable model for studying inflammation. This was verified by using ibuprofen and quercetin as positive controls, both of which effectively modulated inflammatory responses in the zebrafish model. These well-established anti-inflammatory agents confirmed the suitability of zebrafish for inflammation research.

Furthermore, the findings indicate that zebrafish is an appropriate model organism for inducing inflammation through lipopolysaccharide (LPS) and for modeling oxidative stress caused by copper sulfate. This dual induction approach allowed us to explore both pathogen-triggered and chemically-induced inflammatory responses, highlighting the versatility of the zebrafish model in studying different pathways of inflammation and stress.

Our results also suggest that zebrafish can serve as a reliable screening platform for evaluating natural compounds with potential anti-inflammatory effects. Specifically, the study showed that xanthohumol, the primary active compound in hop extract, significantly reduced IL-1 levels and increases IL-10 and exhibited antioxidant properties, highlighting its potential as a therapeutic agent. This finding points to the potential use of xanthohumol in the development of therapeutic formulations with anti-inflammatory and antioxidant benefits. However, it is important to note that while xanthohumol has demonstrated these beneficial properties, it is not currently approved as a drug by the U.S. FDA. Instead, it is available as a dietary supplement and a component in functional foods, often studied for its therapeutic potential [49].

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