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Toxicological and immunomodulatory properties of the extract from Clerodendrum cyrtophyllum Turz

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A dissertation submitted by

NGUYEN THU HANG

in partial fulfilment of requirements

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Toxicological and immunomodulatory properties of the extract from *Clerodendrum cyrtophyllum* Turz

ABSTRACT

Clerodendrum cyrtophyllum Turcz, a plant belonging to the Lamiaceae family, is widely used in traditional medicine in Vietnam, China, India, Korea and Thailand for the treatment of certain conditions such as colds, enteritis, dyspepsia, throat inflammation, rheumatic arthritis, fever. Despite their frequent use, studies on their efficacy and safety are still limited to date. The main objective of this thesis was to provide scientific evidence of the anti-inflammatory and antioxidant efficacy and safety in support of the ethnopharmacological uses of *C. cyrtophyllum* as well as the underlying phytochemistry and molecular mechanisms of action of the leaf extract of *C. cyrtophyllum*.

In order to study the anti-inflammatory activity of *C. cyrtophyllum* leaf extract, several *in vitro* and *in vivo* tests were performed. The results showed that the treatment with the ethanolic extract of *C. cyrtophyllum* leaves (EE) protected the blood cell membrane from haemolysis and inhibited NO and TNF-α production in LPS-activated RAW264.7. Furthermore, the expression of genes related to inflammatory processes such as *cox-2, pla2, c3a,* cytokines (*il-1ß, il-8, tnf-α*), and *nf-ƙb* were downregulated in the presence of this extract.

Several experiments were also designed to prove the antioxidant activity of *C. cyrtophyllum* leaf extract. The results showed that the extract of this plant can directly scavenge free radicals and indirectly increase the activity of endogenous antioxidants, including glutathione (GSH), glutathione S-transferase (GST) and catalase (CAT). *C. cyrtophyllum* leaf extract also inhibited oxidative stress via up-regulation of *sod* and down-regulation of *gstp2, hsp70* and g*add45bb*. The antioxidant activity of this extract was shown to involve the Nrf2/keap1/ARE pathway.

In addition to the anti-inflammatory and antioxidant activity, the ethanolic extract of *C. cyrtophyllum* also protected zebrafish larvae from copper sulphate toxicity. To elucidate the protective mechanism of the extract, we used proteomics as a tool to assess its effects on protein alterations, biological processes and protein-protein interaction networks in response to $CuSO_4$. The results showed that treatment with ethanolic extract of *C. cyrtophyllum* up-regulated proteins in certain signaling pathways such as the ribosome, pyruvate metabolism and glycolysis/gluconeogenesis pathways or the regulation of copper metabolism. The up-regulation of these proteins by EE helps to restore protein synthesis and maintain normal development of zebrafish larvae.

To provide evidence for the safety of *C. cyrtophyllum*, we evaluated the toxicity of the ethanolic extract of the plant leaves in the zebrafish embryo. These results indicate the toxic effects of this plant on embryonic stages and provide insight into potential mechanisms of toxicity on embryonic development. Phytochemical analysis indicated that acteoside, cirsilineol and cirsilineol-4'-O-β-D-glucoside are the main compounds in the ethanolic and methanolic extracts of *C. cyrtophyllum* leaves. They can be considered as potential compounds for the discovery and development of drugs derived from *C. cyrtophyllum*.

Overall, our results provide scientific evidence to validate the ethnopharmacological use of this plant as well as guidance for further research to develop this plant as botanical medicines or plant-derived medicines for the benefit of human health.

RÉSUMÉ

Clerodendrum cyrtophyllum Turcz, une plante appartenant à la famille des Lamiaceae, est largement utilisée dans la médecine traditionnelle du Vietnam, de la Chine, de l'Inde, de la Corée et de la Thaïlande pour le traitement de certaines affections telles que le rhume, l'entérite, la dyspepsie, l'inflammation de la gorge, l'arthrite rhumatismale, la fièvre. Malgré son utilisation fréquente, les études sur son efficacité et sa sécurité sont encore limitées jusqu'à présent. L'objectif principal de cette thèse était de fournir des preuves scientifiques de l'efficacité et de la sécurité anti-inflammatoire et antioxydante en soutien aux utilisations ethnopharmacologiques de *C. cyrtophyllum* ainsi que la phytochimie sous-jacente et les mécanismes d'action moléculaires de l'extrait de ses feuilles.

Afin d'étudier l'activité anti-inflammatoire de l'extrait de feuilles de *C. cyrtophyllum*, plusieurs essais *in vitro* et in vivo ont été réalisés. Les résultats ont démontré que le traitement avec l'extrait éthanolique des feuilles de *C. cyrtophyllum* a protégé la membrane des cellules sanguines de l'hémolyse et a inhibé la production de NO et de TNF-α dans les RAW264.7 activés par le LPS. De plus, l'expression des gènes liés aux processus inflammatoires tels que *cox-2, pla2, c3a*, les cytokines (*il-1ß, il-8, tnf-α*), et de *nf-ƙb* était régulée à la baisse en présence de cet extrait.

Plusieurs expériences ont également été conçues pour prouver l'activité antioxydante de l'extrait de feuilles de *C. cyrtophyllum*. Les résultats ont montré que l'extrait de cette plante peut directement piéger les radicaux libres et indirectement augmenter l'activité des antioxydants endogènes, notamment le glutathion (GSH), la glutathion S-transférase (GST) et la catalase (CAT). L'extrait de feuilles de *C. cyrtophyllum* a également inhibé le stress oxydatif via la régulation positive de *sod* et la régulation négative de *gstp2, hsp70* et *gadd45bb*. Il a été prouvé que l'activité antioxydante de cet extrait impliquait la voie Nrf2/keap1/ARE.

Outre l'activité anti-inflammatoire et antioxydante, l'extrait éthanolique de *C. cyrtophyllum* a également protégé les larves de poisson zèbre contre la toxicité du sulfate de cuivre. Pour élucider le mécanisme de protection de l'extrait, nous avons utilisé la protéomique comme outil pour évaluer ses effets sur les altérations des protéines, les processus biologiques et les réseaux d'interaction protéine-protéine en réponse au $CuSO_4$. Les résultats ont montré que le traitement avec l'extrait éthanolique de *C. cyrtophyllum* a régulé à la hausse les protéines de certaines voies de signalisation telles que le ribosome, le métabolisme du pyruvate et les voies de la glycolyse/gluconéogenèse ou la régulation du métabolisme du cuivre. La régulation positive de

ces protéines par l'EE aide à restaurer la synthèse des protéines et à maintenir le développement normal des larves de poisson zèbre.

Pour fournir des preuves de la sécurité de *C. cyrtophyllum*, nous avons évalué la toxicité de l'extrait éthanolique des feuilles de la plante chez l'embryon de poisson zèbre. Les résultats indiquent les effets toxiques de cette plante sur les stades embryonnaires et donnent un aperçu des mécanismes potentiels de toxicité sur le développement embryonnaire. L'analyse phytochimique a indiqué que l'actéoside, le cirsilineol et le cirsilineol-4'-O-β-D-glucoside sont les principaux composés de l'extrait éthanolique et de l'extrait méthanolique des feuilles de *C. cyrtophyllum*. Ils peuvent être considérés comme des composés potentiels pour la découverte et le développement de médicaments dérivés de *C. cyrtophyllum*.

Dans l'ensemble, nos résultats fournissent des preuves scientifiques pour valider l'utilisation ethnopharmacologique de cette plante ainsi que l'orientation pour des recherches ultérieures afin de développer cette plante en tant que médicament botanique ou médicaments dérivés de plante au profit de la santé humaine.

LIST OF ABBREVIATIONS

ABTS	2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)
ATOX1	Antioxidant copper chaperone
C3a	Complement 3a
CAT	Catalase
COX17	Cytochrome c oxidase assembly protein 17
COX-2	Cyclooxygenase-2
CTR1	Copper transporter 1
DEP	Differentially expressed proteins
dpf	Day post fertilization
EE	The ethanol extract from *Clerodendrum cyrthophyllum* Turcz leaves
FRAP	Ferric Reducing Antioxidant Power
gadd45bb	Growth arrest and DNA-damage-inducible, beta b
GO	Gene ontology
GPX	Glutathione peroxidase
GSH	Glutathione
GST	*G*lutathione S-transferase
H2DCFDA	2′, 7′-dichlorodihydrofluorescein diacetate
HPTLC	High performance thin layer chromatography
hsp70	Heat shock protein
Il-1	Interleukin 1
IL-10	Interleukin 10
Il-6	Interleukin 6
IL-8	Interleukin 8
KEGG	The Kyoto Encyclopedia of Genes and Genomes database
LD	Lethality concentration
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinases

ME	Methanol extract
MPO	Myeloperoxidase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric oxide
NOAEL	No observed-adverse-effect level
PLA2	Phospholipase A2
PPI	Protein-protein interaction
QE	Quercetin
qPCR	Quantitative polymerase chain reaction
RAW 264.7	Murine macrophage cell line
RBC	Red blood cell
ROS	Reactive Oxygen Species
SOD	Superoxide dismutases
TI	Teratogenic index
TNF-α	Tumor necrosis factor alpha

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SECTION I

Chapter 1

General introduction

1. Inflammatory

Inflammation is a response of the immune system. It protects the body from injurious stimuli such as pathogens, damaged cells, toxic compounds, or irradiation and initiates the healing process [1]. An inflammatory response is characterized by several processes: 1) the recognition of pathogens; 2) the activation of inflammatory pathways; 3) the release of inflammatory mediators; 4) the recruitment of inflammatory cells; 5/ the resolution of inflammation.

1.1. The recognition of pathogens

Microbial or damaged cells can trigger the inflammatory response through activation of germline-encoded pattern-recognition receptors (PRRs)[1]. Signaling through PRRs activates intracellular signaling cascade that leads to nuclear translocation of transcription factors, such as activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB).

1.2. Inflammatory pathways

Nuclear factor kappa B (NF-κB) pathway regulates pro-inflammatory cytokine production and inflammatory cell recruitment. Under physiological conditions, inhibitor of NF-κB (IκB) proteins bind and inhibit NF-κB. In response to inflammatory stimuli, PRRs activate IκB kinase (IKK), IKK regulates NF-κB pathway activation through IκB phosphorylation. IκB phosphorylation results in nuclear translocation of NF-κB. NF-κB activates the transcription of interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor- α (TNF-α) along with cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), cell adhesion molecules.

Mitogen-activated protein kinase (MAPKs) regulate proliferation, stress responses, apoptosis, and immune defense. MAPKs include extracellular-signal-regulated kinase ERK1/2, p38 MAP Kinase, and c-Jun N-terminal kinases (JNK)[1]. Inflammatory stimuli and stress activate the JNK and p38MAPK pathway leads to phosphorylation and activation of p38 transcription factors which initiates the production of cytokines.

1.3 Inflammatory mediators

In response to tissue injury, inflammatory cells are activated and release inflammatory mediators such as eicosanoids, proinflammatory cytokines, acute-phase proteins, chemokines, complement components, proteases, nitric oxide and reactive oxygen metabolites. These substances bind to receptors on the cells and lead to increase vascular permeability, promote neutrophil chemotaxis, stimulate smooth muscle contraction and result in the symptoms of inflammation such as pain, heat, redness, swelling and loss of function.

Cytokines: Cytokine are secreted by immune cells**.** They include both pro-inflammatory cytokines and anti-inflammatory cytokines**.** Functions of cytokines are shown in Table 1. Cytokines modulate the immune response to infection or inflammation.

Table 1. Summary of cytokines and their functions [1]

Cytokine	Main sources	Function
IL-1β	Macrophages, monocytes	Pro-inflammation, proliferation, apoptosis, differentiation
IL-4	Th-cells	Anti-inflammation, T-cell and B-cell proliferation, B-cell differentiation
IL-6	Macrophages, T-cells, adipocyte	Pro-inflammation, differentiation, cytokine production
IL-8	Macrophages, epithelial cells, endothelial cell	Pro-inflammation, chemotaxis, angiogenesis
IL-10	Monocytes, T-cells, B-cells	Anti-inflammation, inhibition of the pro-inflammatory cytokines
IL-12	Dendritic cells, macrophages, neutrophils	Pro-inflammation, cell differentiation, activates NK cell
TNF-α	Macrophages, NK cells, CD4+ lymphocytes, adipocyte	Pro-inflammation, cytokine production, cell proliferation, apoptosis, anti-infection
Transforming growth factor-β (TGF-β)	Macrophages, T cells	Anti-inflammation, inhibition of pro-inflammatory cytokine production
Interferon gamma (IFN-γ)	T-cells, NK cells, NKT cells	Pro-inflammation, innate, adaptive immunity anti-viral

Lipid Derived Mediators

The cell damage leads to the activation of phospholipase A2 (PLA2). PLA2 causes the release of arachidonic acid from membrane phospholipid. Via cyclooxygenase (COX) pathway or lipoxygenase (LOX) pathway, prostaglandins (PGs), leukotrienes (LTs) and lipoxins (LXs) are produced. Prostaglandins induce a variety of effects on blood vessels, nerve endings. Leukotrienes are associated with neutrophil activation, bronchoconstriction and alterations in vascular permeability.

1.4. Inflammatory cells

At sites of tissue injury, damaged epithelial and endothelial cells release factors that trigger the inflammatory cascade, along with chemokines and growth factors, which attract neutrophils and monocytes. The first cells attracted to a site of injury are neutrophils, followed by monocytes, lymphocytes (natural killer cells [NK cells], T cells, and B cells), and mast cells. Monocytes can differentiate into macrophages and dendritic cells and are recruited via chemotaxis into damaged tissues.

1.5. The resolution of inflammation

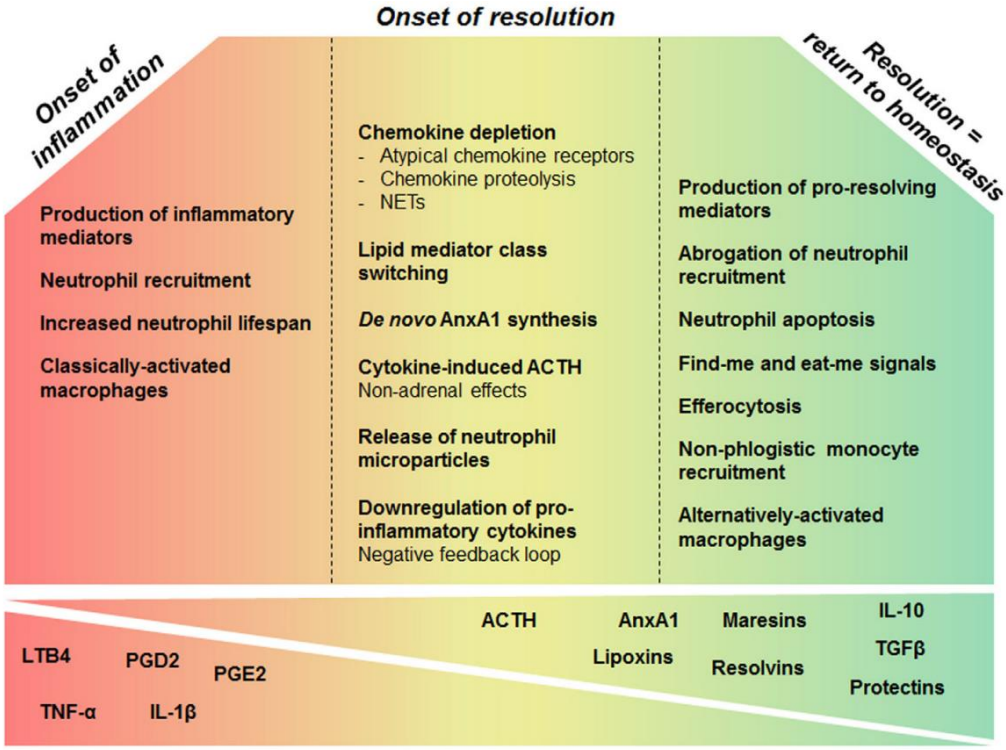

Figure 1. Overview resolution of inflammation process [2]

While the inflammatory response grows, several mechanisms enable the initiation of the pro-resolving response leading to the return to tissue homeostasis. The resolution process starts

shortly after the onset of the inflammatory response. Chemokine depletion by proteolysis or neutrophil extracellular traps switch off neutrophil recruitment. The production of cytokines is downregulated. The switch from pro-inflammatory lipid production to the synthesis pro-resolving mediators such as lipoxins and other pro-resolving lipids. Resolution mediators activate apoptosis of leukocytes. In response to local mediators, proinflammatory macrophages switch to resolution-phase macrophages[2]. These events will re-establish tissue homeostasis. In most cases, the inflammatory response eventually subsides.

2. Oxidative stress

Inflammation and oxidative stress are two closely related pathophysiological processes, one of which can readily be caused by the other (Figure 2). At the site of inflammation, phagocytosis of bacteria or foreign particles is associated with an increase in oxygen uptake by neutrophils, called a respiratory burst. During this phase, high amounts of reactive oxygen species (ROS) such as superoxide anion ($\cdot O2^{-}$), hydroxyl radical ($HO\cdot$), and hydrogen peroxide (H_2O_2) are produced. ROS can initiate intracellular signaling pathways and promote proinflammatory gene expression. An increase in the expression of phospholipase A2, 5-lipoxygenase (5-LOX), and cyclooxygenase-2 (COX-2) inducible nitric oxide synthase (iNOS) and other ROS generating enzymes such as NADPH oxidase, xanthine oxidase, and myeloperoxidase are observed. Overproduction of radicals and peroxides activate Nrf2/Keap1/ARE pathway, Nrf2 will be released from a complex with Keap1. It then binds to promoters contain an antioxidant response element (ARE) and initiate the cellular defense by induction of genes for phase II detoxifying and antioxidant enzymes, *e.g.* heme oxygenase-1 (Ho-1), glutathione peroxidase(Gpx), superoxide dismutase (Sod), and catalase (Cat) [3]- Figure 2. As a result, the ability of cells to scavenge ROS is enhanced, and thus the injury of cells, tissues, and organs induced by oxidative stress is reduced[4]. So anti-inflammatory and anti-oxidant drugs play an important role in the therapeutic strategy of chronic diseases. Steroids, nonsteroid anti-inflammatory agents and immunosuppressants are now used to control and inhibit inflammation [5]. However, drug resistance and the serious side effect of current medications are major problems that need to be solved for conventional anti-inflammatory drugs. Finding new medicines to replace or support conventional drugs is an important purpose of drug discovery and development.

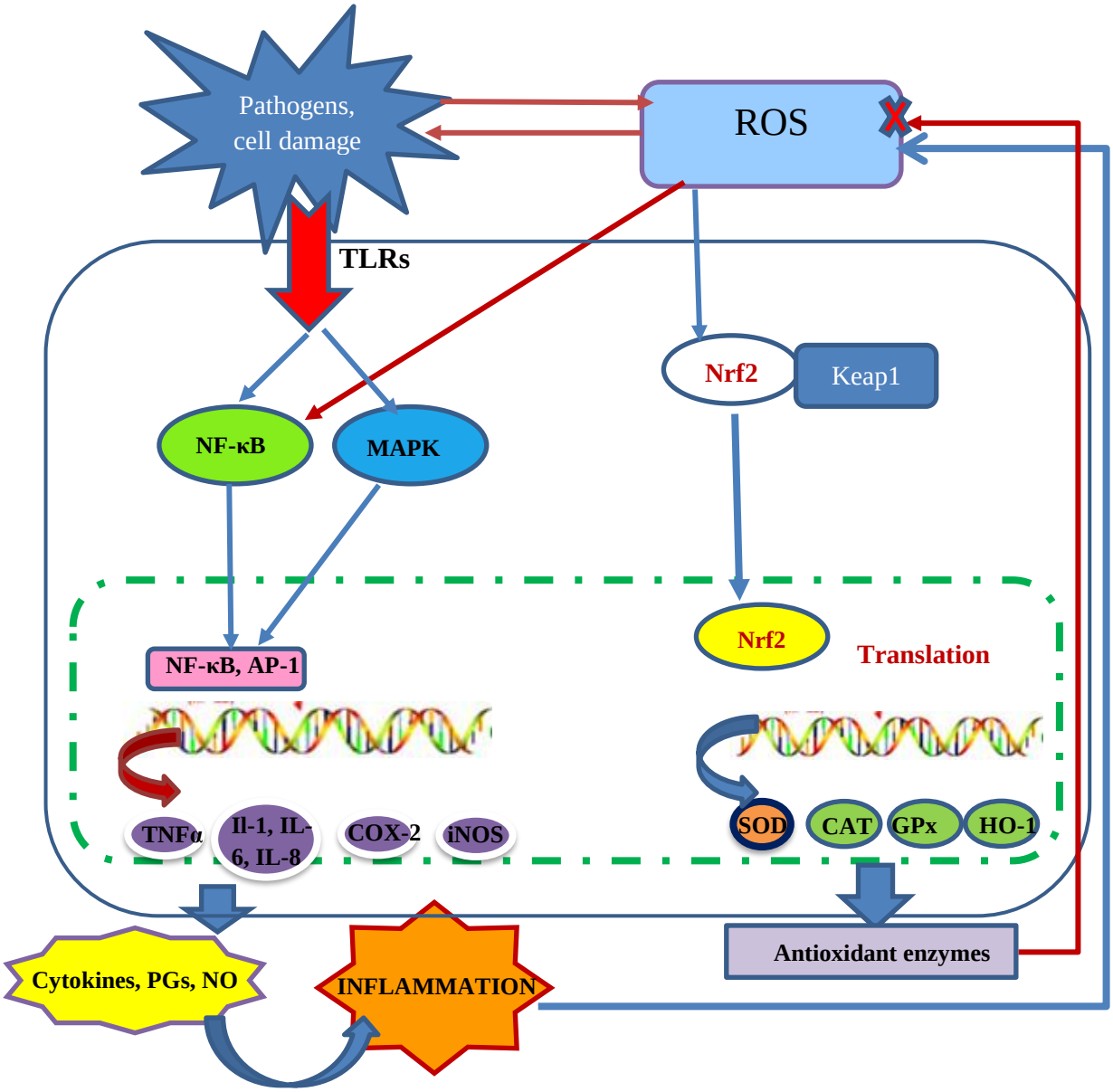

Figure 2. The relationship and dependence between oxidative stress and inflammation.

3. Drug development from the medicinal plants

New medicines can be developed from synthetic compounds but also from bioactive compounds present in medicinal plants (herbal medicines). Medicinal plants have been used as a source of medicine for thousands of years and they have produced a positive impact on the progress of human civilization. About 80% of the population in developing countries depend on traditional medicine for their primary health care. About 48% population in Australia, 31% in Belgium, 70% in Canada, 49% in France and 42% in the United States are using medicinal plants to prevent, treat disease and improve quality of life [6]. Recent studies showed that phytochemicals (flavonoids, triterpenoids, polyphenolic compounds, limonoids…) are potential antioxidant and anti-inflammatory compounds [7]. Therefore, medicinal plants are

important sources for anti-inflammatory and anti-oxidant drug development. There are some preparations for traditional medicine. The medicinal plants have been used in folk medicine in decoction, powdering, and extraction with alcohol [8], [9]. This is still the common method for using traditional medicines, especially in developing countries. Recently, with the development of the modern pharmaceutical techniques, there are some improvements in the administration routes, preparation methods, and application of traditional medicines. The dry raw herbs can be ground into powder and are mixed into pills. Pills are convenient to use, transport, store, reduce wastage of herbs and ideal for herbs that have a strong smell or an unpleasant taste. Syrups are made by adding sugar to a liquid herbal decoction. The dry raw herbs also can be cut into small pieces and make into herb teas. Using modern techniques, the ingredients are extracted, concentrated, combined, turned into powdered herbal extracts and make into pills, tablets and capsules[8]. These products are very popular in the drug market nowadays. The other way for drug development from the medicinal plant is that scientists find the single active compound responsible for the therapeutic effect from medicinal plants. It is one way for scientists in the discovery and development of the new drug. Medicinal plants have provided an endless source of new drugs. The medicinal plant is extracted, fractionated and purified for active compounds [10]. Isolated bioactive compounds then are used for evaluating efficacy and safety based on pharmacological models before moving into the clinical phase in humans. Figure 2 describes the necessary steps for this process. Undoubtedly, this approach greatly improved medical care, human health, thus extending human life. Nearly half of the FDA-approved drugs from the 1940s to the end of 2014 was derived from natural products[11]. A comprehensive review of human drugs suggested that, of 847 small molecule-based drugs, 43 were natural products, 232 were derived from natural products (usually semisynthetically), and 572 were synthetic molecules. However, 262 of the 572 synthetic molecules had a natural product–inspired pharmacophore or could be considered natural product analogs [12]. Approximately one-quarter of the best-selling drugs worldwide in 2001 – 2002 were natural products or derived from natural products [13].

4. Advantages and disadvantages of plant-derived drugs versus herbal medicine?

Should we continue using folk medicine or change to modern medicine? Which is the best medical practice for herbal medicine. It is still a difficult question for the scientists. Each of them displays both advantages and disadvantages in the treatment of human disease.

Herbal medicines have been used for thousands of years for preventing and treat disease. However, because of the lack of safety and efficacy validation as well as concerns on poor

standardization and quality control, traditional medicines are not well accepted by medical practitioners and by the pharmaceutical industry [14]. The complexity of resupply, difficulty in isolation and identifying active ingredients, lack of reproducibility, and interference from compounds in complex mixtures bring a challenge to the development of drugs from herbal medicines[12]. Lack of evidence for safety and efficacy is a major problem of herbal medicine. In the recent review of 1000 herbal medicines, only 156 herbal medicines had published studies supporting specific pharmacological activities and therapeutic applications[15]. One-fifth of the plants in this survey had phytochemical studies but no efficacy evidence was available. Also, among traditional medicine used in Western countries, 12% of them had no published scientific studies and about 1 in 200 were toxic or allergenic[10],[15]. It is difficult to determine modes of action of traditional medicine because modern pharmacology is not designed to study complex mixtures of substances. Other factors such as variable content in different batches of plant materials, and inconsistent use of extraction methods and formulations also contribute to the limitation of using herbal medicine. Indeed, due to the complexity of plant extracts, developing an evidence-based herbal medicine is a difficult task that requires analytical effort as well as manufacturing skills in order to produce well-defined, standardized herbal preparations.

Because of the complexity of plant extracts, it still is common practice for scientists to investigate medicinal plants just to find the single chemical substance responsible for the therapeutic effect [16]. Using a single molecule is easier for the scientist to study its pharmacokinetic and pharmacodynamic properties, mechanisms of action, interactions, and adverse effects. Plant-derived drugs have dominated in human pharmacopeia for thousands of years. However, the biological activity may be the result of the combination of several compounds, the isolation process may cause its loss or reduction. Information from trials showed that, in many cases, the potency of whole plant extracts is stronger than isolated fractions or single compounds[15]. Modern medicine suggested that an ideal therapeutic intervention should act on single, specific targets. However, diseases with multifactorial etiology as well as with a high rate of resistance, the combination of drugs with different targets increases treatment efficacy. Therefore, it is not surprising that the plant extract with a mixture of compounds can bring a better treatment effect than a single compound because it can affect multiple targets [12]. Despite disadvantages, both herbal medicine and plant-derived drugs still are important medicine sources for the treatment of human diseases. They need to continue to develop and standardize to make people’s health care better and safer.

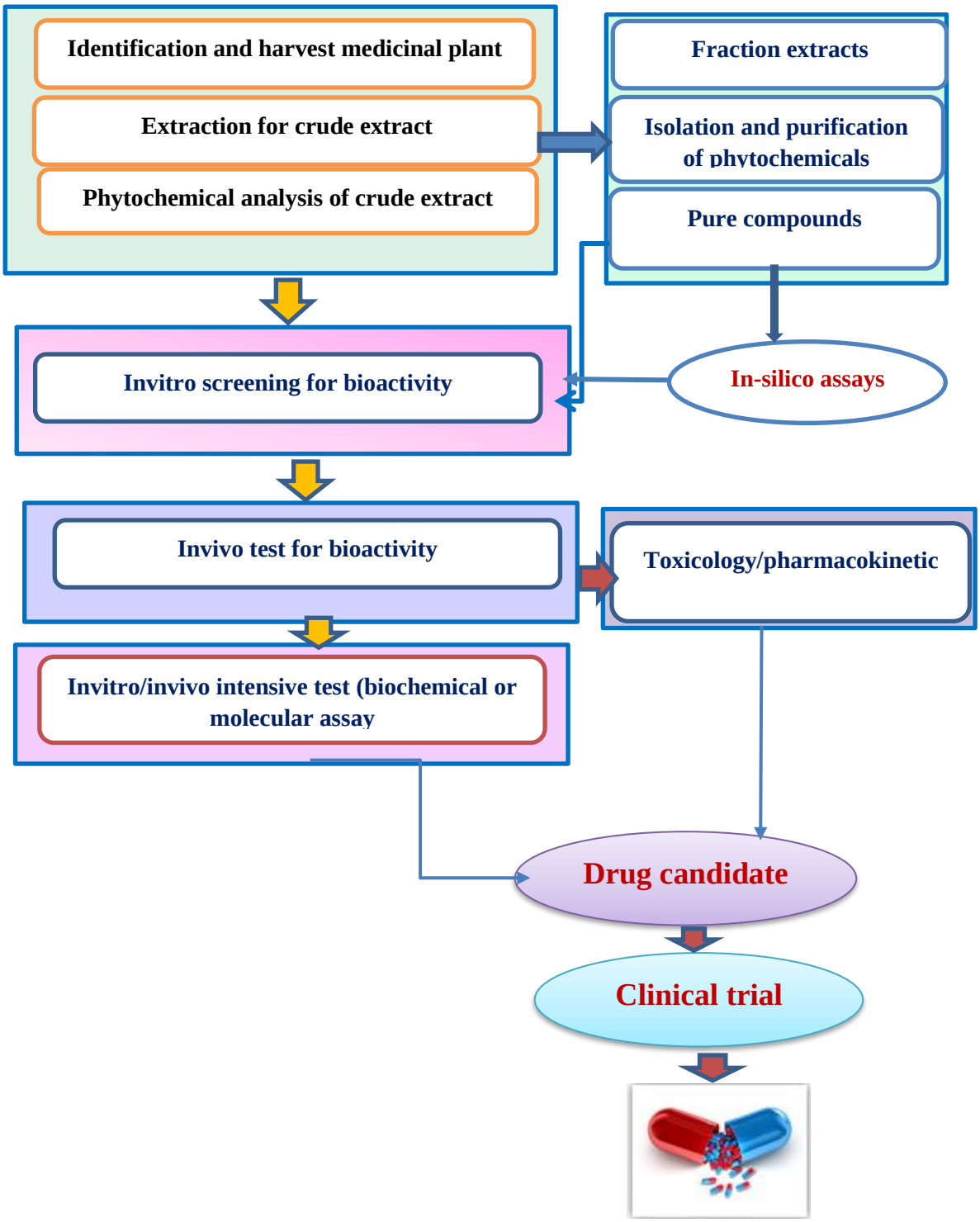

Figure 3. A systematic approach for drug discovery from the medicinal plant

5. Review of pharmacology models for the evaluation of anti-inflammatory and anti-oxidant activities

5.1 *In-silico* models

The *in- silico* method has been developed in the last decade and applied in the pharmacology field. *In silico* are biological experiments conducted on a computer. It uses virtual screening tools to make predictions about the biological activity and toxicity of compounds. In this way, we can save time and cost, increasing the success of *in-vitro* evaluations[17]. W.Utami et al (2020) used molecular docking to predict anti-inflammatory ability of 11 compounds from *Ficus religiosa* [18]. *In silico* model also has been used for screening anti-inflammatory activity of 412 lichen compounds by molecular docking with human Cox-2 enzyme [19]. This model is simple and economic. However, *in silico* model only is suitable for pure compounds. Therefore, it is only suitable at the phase after the phytochemicals are isolated.

5.2 *In vitro* models

In vitro models often use cells or tissues for the evaluation of drug action or toxicity. In this way, the drug can affect directly targets such as the enzymes, receptors and the inflammatory mediator. The model is used widely in the discovery and development of new drugs because of its rapidity, sensitivity, reproducibility, reliability and requires small quantities of the test samples, collection of data simpler than animal models [20]. It can be readily adaptable to high-throughput screening of huge compound libraries[21]. Depending on the strategy of the company, *in vitro* models can be used for the initial screening, or to characterize the molecular mechanism of a lead compound. However, cells are cultured out of the body, their response is different than in an intact organism. *In vitro* data is difficult to extrapolate to intact organisms. Results obtained *in vitro* often poorly correlate with *in vivo* mechanisms. Moreover, cell lines are usually transformed, exhibiting different gene expression and cell cycle profiles than those of cells in the living organism.

5.2.1 *In vitro* models currently use in the evaluation of anti-inflammatory activity

HRBC membrane stabilization: The lysosomal membrane plays an important role in preventing the release of lysosomal constituents and limiting the inflammatory response. The erythrocyte membrane is similar to the lysosomal membrane. So the drugs that can stabilize the erythrocyte membrane have also an anti-inflammatory effect. In this model, hemolysis can be induced by heat (54°C) or hypotonicity, and the measurement of hemoglobin release helps to evaluate the membrane stabilization [22].

Advantages and limitations: This model can be used for screening many extracts and is relatively cheap however the lysosomal membrane is not a specific target for assessing the anti-inflammatory effect. Only 37 papers used this model among a total of 24899 papers from Pubmed which deal with the anti-inflammatory activity of plant extracts.

Hyaluronidase inhibitory assay

Hyaluronidases are a family of enzymes that catalyze the degradation of hyaluronic acid (HA), a constituent of the connective tissues. Hyaluronidase inhibitors are effective in suppressing inflammation[23]. The anti-inflammatory ability of extracts or pure compounds can be evaluated through their effects on hyaluronidases. This model is cheap, can be screened for many extracts however hyaluronidase is not the major target for anti-inflammatory activity. Among the 24899 papers, only 96 papers used this model.

Inhibition NO production in LPS - induced RAW264.7 cells

NO is considered a key pro-inflammatory mediator in inflammation response. Therefore, NO inhibitors represent an important therapeutic advance in the treatment of inflammatory illnesses [24]. This is the most popular model in screening the anti-inflammatory activity because it is cheap, fast, and NO is a potential target for anti-inflammatory activity. A total of 24034 papers used this model for screening the anti-inflammatory activity of plant extracts.

In vitro models for investigating the mechanisms of action

In inflammatory response, the inflammatory cells (macrophage, basophils, dendritic cells) are activated, they release a lot of mediators: prostaglandins, cytokines (IL-1, IL-6, IL-8, TNF-α). [25]. The effects of the extracts or pure compounds on the release of inflammatory mediators as well as on the enzyme activity (phospholipase A2, cyclooxygenase (COX-2)) are measured using ELISA kits. We also can use q-PCR to evaluate the effect of the extracts on the expression of genes involved in the inflammatory response: *il-1, il-6, il-8, nf-κB, mpo, c3a, tnf-α, cox-2, pla2.* Western blot also can be used to detect specific protein molecules such as p38 MAPK, JNKs, ERK, Akt, IKKα/β, and IkB-α or target proteins in signal pathways related to inflammatory processes.

5.2.2 *In vitro* models used for the evaluation of antioxidant activity

Test-tube antioxidant assays

Several test-tube tests are traditionally used to evaluate the antioxidant activity. These assays are based on chemical reactions between different reagents. Test-tube assays are in general rapid, simple, low cost and allow the screening of a huge number of compounds. Methods are divided into two categories according to the HAT (hydrogen atom transfer) or SET (single-

electron transfer) reaction mechanism they involve. HAT measures the capacity of a compound to quench free radicals by hydrogen atom donation, whereas SET detects the capacity of a compound to reduce another compound by transferring one electron[26]. Table 2 is a summary of some test-tube antioxidant assays. A compound can display many antioxidant mechanisms. Antioxidant activity should not be concluded based on a single test [27]. Among methods, DPPH is the most frequently used because it is rapid, simple and inexpensive in comparison to other models. However, all these assays are performed in a noncellular environment, and no information is available about the actual effect on living systems. For these reasons, the information provided by test tube assays is usually retained under the term of antioxidant capacity (AC)[26].

Principles of some assays

DPPH scavenging activity assay: The method is based on the scavenging of DPPH radical by antioxidants, which change the color of the DPPH solution from purple to yellow- Figure 4 [28].

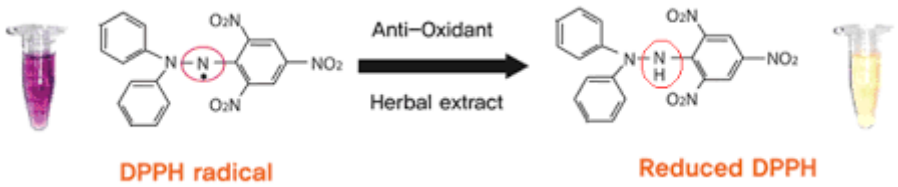

Figure 4. Principle of DPPH radical scavenging capacity assay

ABTS radical scavenging assay: The assay is based on the discoloration of a preformed ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical by antioxidant compounds, thus reflecting the amount of ABTS radicals that are scavenged within a fixed period – Figure 5[28].

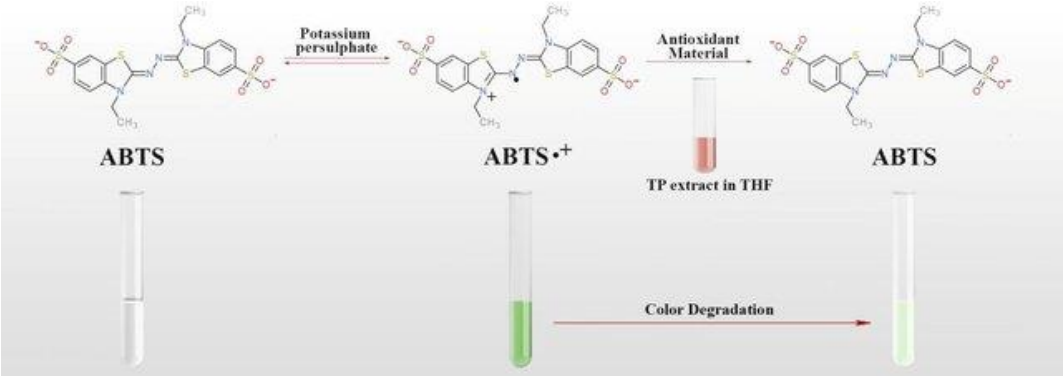

Figure 5. Principle of ABTS radical scavenging assay

Ferric reducing-antioxidant power (FRAP) assay: The FRAP assay is used to measure the antioxidant power of plant extracts in their ability to reduce Fe^{3+}-tripyridyltriazine to Fe^{2+}-tripyridyltriazine- Figure 6 [28].

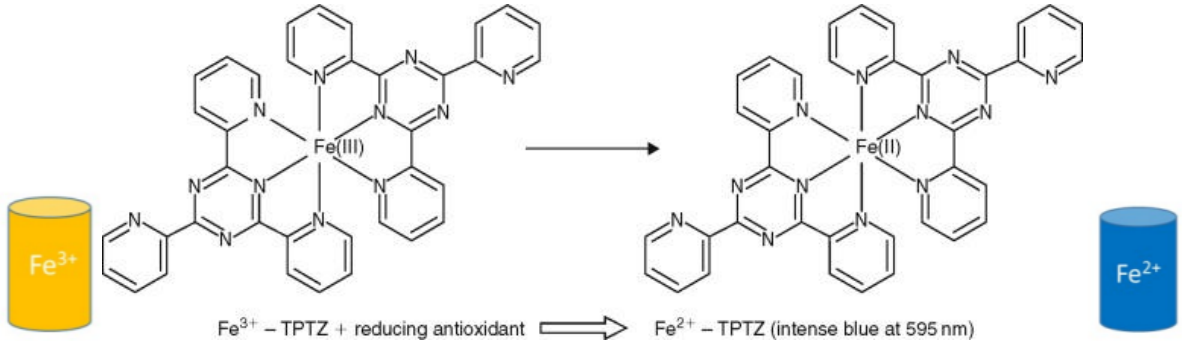

Figure 6. Principle of FRAP assay

Superoxide radical scavenging activity: As shown in figure 7, the $O_2^{\cdot -}$ produced in the coupling reaction of PMS–NADH. $O_2^{\cdot -}$ reduces NBT to formazan (purple color). Antioxidants scavenge the $O_2^{\cdot}$ and decrease of absorbance at 560 nm [29].

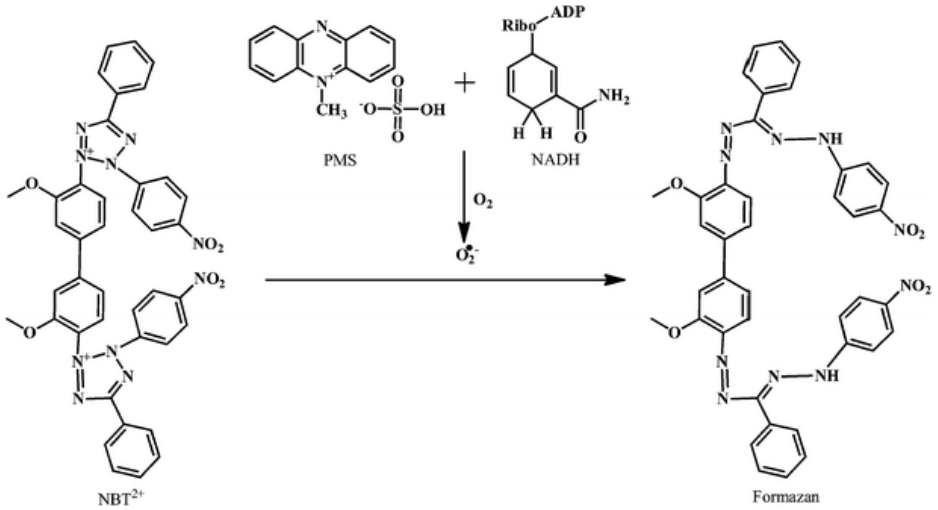

Figure 7. Principle of Superoxide radical scavenging activity assay

Hydroxyl radical scavenging activity assay: As shown in figure 8

A/ the Fe^{2+} reacts with 1,10-phenanthroline form tri-phenanthroline complex (orange color).

B/ the hydroxyl radicals were produced by the Fenton reaction between H_2O_2 and Fe^{2+}, the Fe^{2+} was oxidized to Fe^{3+}, no tri-phenanthroline complex can form, resulting in a decrease in absorbance at 536 nm.

C. In the presence of antioxidants, they scavenge the hydroxyl radicals and prevent the Fenton reation. The degree absorbance at the 536 nm reflected the hydroxyl radical scavenging activity of the samples [30], [31].

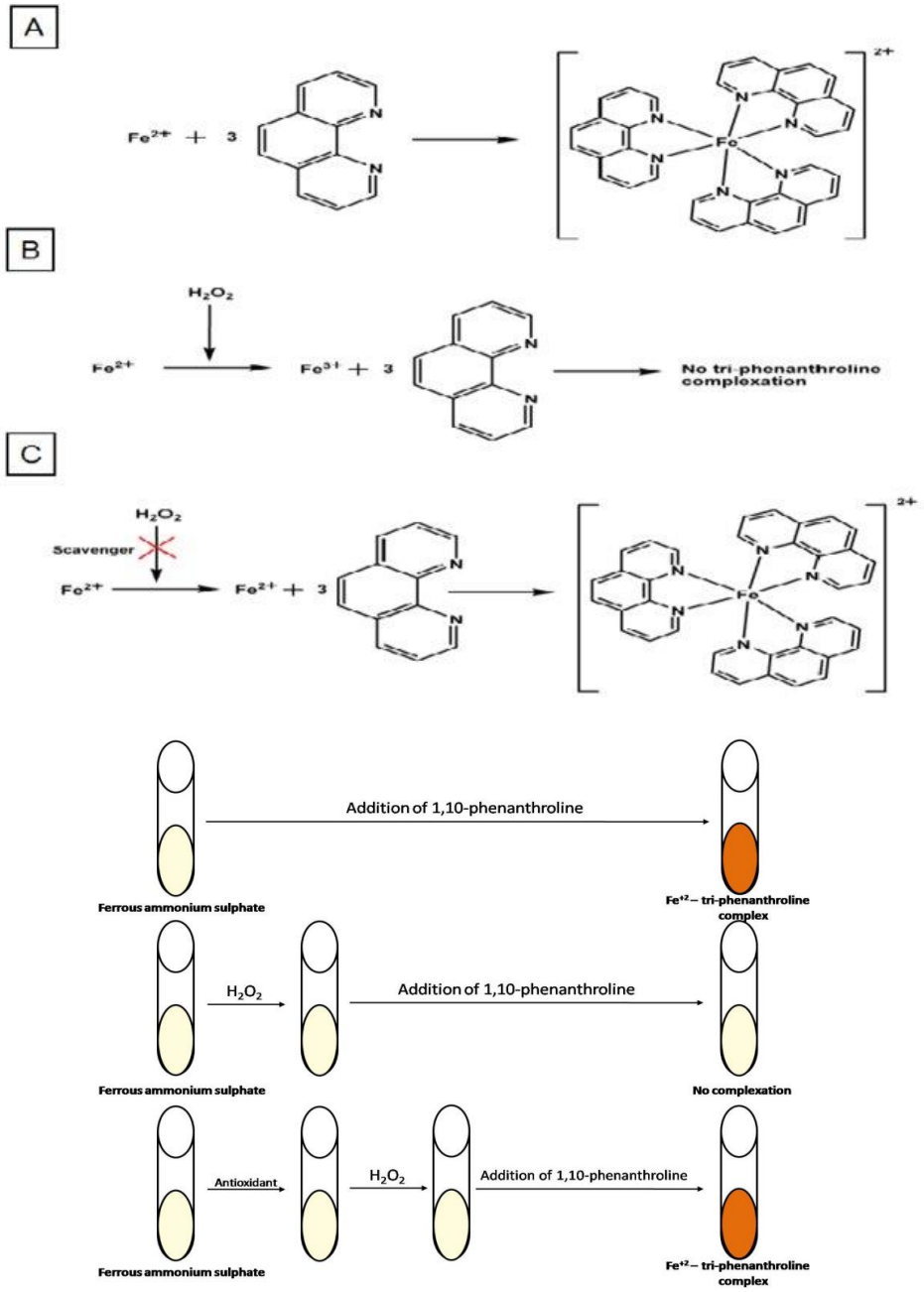

Figure 8. Principle of Hydroxyl radical scavenging activity assay

Metal chelating activity assay: In a slightly acidic medium (pH= 6), Fe^{2+} reacts with ferrozine, forming a blue-colored complex that can be monitored spectrophotometrically – Figure 9. However, in the presence of antioxidant compounds, there is disruption of the formation of the metallic complex (due to the binding of Fe^{2+} with antioxidants) which leads to decrease blue color or decrease in the absorbance at λ = 562 nm [32]

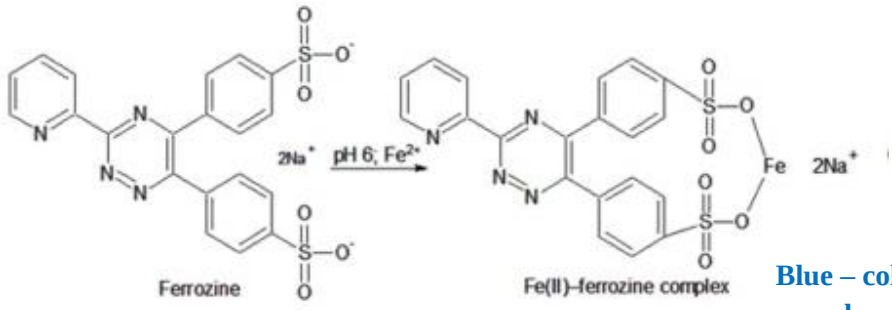

Figure 9. Principle of Metal chelating activity assay

Table 2 Test- tube models in evaluation anti-oxidant activity [33]

	Name of methods
1	DPPH scavenging activity
2	Hydrogen peroxide scavenging (H_2O_2) assay
3	Nitric oxide scavenging activity
4	Peroxynitrite radical scavenging activity
5	Trolox equivalent antioxidant capacity (TEAC) method/ ABTS radical cation decolorization assay
6	Total radical-trapping antioxidant parameter (TRAP) method
7	Ferric reducing-antioxidant power (FRAP) assay
8	Superoxide radical scavenging activity
9	Hydroxyl radical scavenging activity
10	Hydroxyl radical averting capacity (HORAC) method
11	Oxygen radical absorbance capacity (ORAC) Method
12	Reducing power method (RP)
13	Phosphomolybdenum method
14	Ferric thiocyanate (FTC) method
15	Thiobarbituric acid (TBA) method
16	DMPD (N, N-dimethyl-p-phenylene diamine dihydrochloride) method
17	β-carotene linoleic acid method/conjugated diene assay
18	Xanthine oxidase method
19	Cupric ion reducing antioxidant capacity (CUPRAC) method
20	Metal chelating activity

***In vitro* cell-based assays**

Recently, live-cell assays have been developed and provided information about the antioxidant mechanism at the subcellular level. HepG2 is by far the most common human cell line used for antioxidant analysis, CaCo2 (resembling enterocytes from the small intestine), HaCaT (keratinocytes), and SH-SY5Y (neuron-like) cells are other very common cell systems used in antioxidant studies. Up to date, there are only four assays that achieve standardization: (1) the catalase-like assay (2) the cell antioxidant assay (CAA), (3) the AOP1 assay, (4) the Nrf2/ARE gene reporter assay. Among them, the Nrf2/ARE gene reporter displays the most standardized assay with high-throughput applications. AOP1 is the most promising tool for the assessment ability to scavenging ROS at the cellular level [26].

5.3 In vivo models

5.3.1 *In vivo* models for the evaluation of anti-inflammatory activity

Over the past century, *in vivo* models have been used to test new medicines, assure their efficacy and safety before moving into the next phase for testing them on humans. Each model displays both advantages and limitations, making them useful in certain phases of drug discovery. There are many kinds of animals used in drug discovery such as *Drosophila, Caenorhabditis elegans*, mice, rats, rabbits, dogs, cats, fish. Among them, rodents and fish hold over 95% of all animals used in preclinical research[34]. Figure 10 describes the number of papers using animals for evaluating the anti-inflammatory activity.

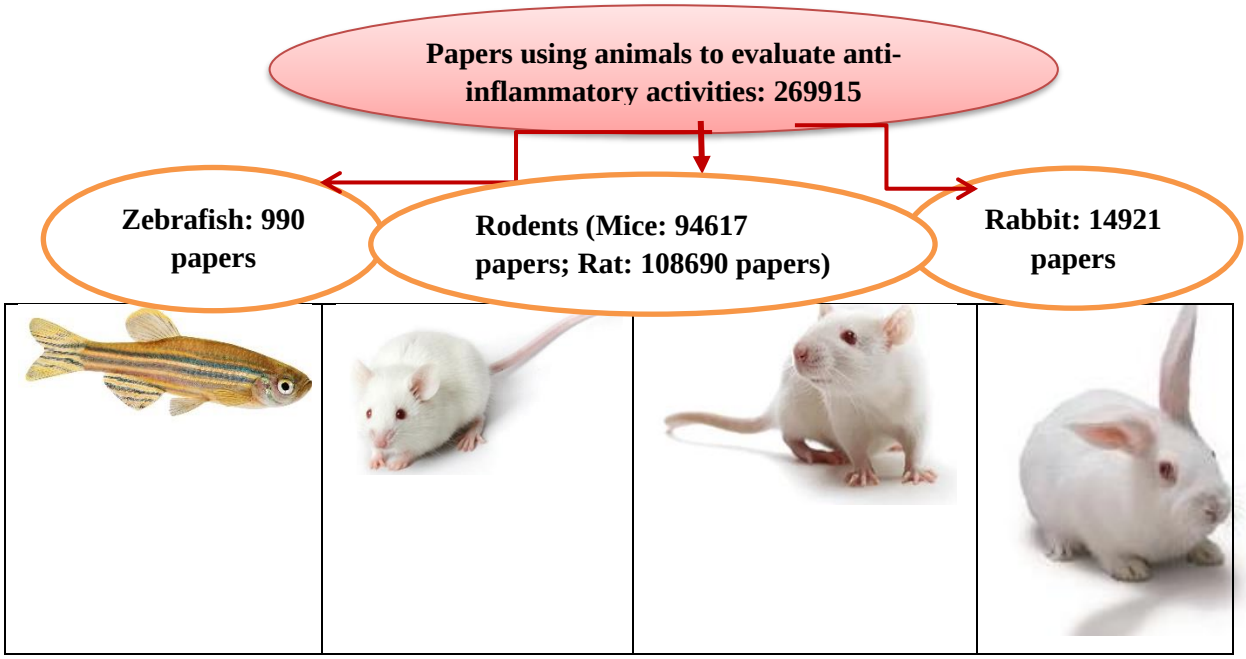

Figure 10. The number of publications based on a search of Pubmed with the settings "Zebrafish/rat/mouse/rabbit" and "inflammatory"

Zebrafish model

The use of zebrafish as an animal model to investigate the inflammatory activities started in the 2000s, with a considerable increase in the number of publications from 2011 till now (Figure 11). Zebrafish can be used for the evaluation of anti-inflammatory activity of novel synthetic compounds or plant extracts as well as phytochemicals isolated from the plant extracts because of the advantages described below.

Advantages: The assay on zebrafish provides a whole animal model advantage over the *in vitro* model of cell lines [35]. Zebrafish provides a complementary model between *in vitro* cell-based assays and rodent models[36]. Because of its small size, a large number of animals can be kept in small space, the regular care and maintenance of zebrafish are simple with low maintenance costs, relatively rapid life cycle, and ease of breeding. Zebrafish genomes are approximately 75% similar to humans, extensive genetic data is available for zebrafish [36], [37]. Pathological features of inflammation in zebrafish were similar to those of humans. Additionally, the zebrafish larvae below 120 hours old are not required animal test authorization according to European legislation (EU Directive, 2010/63/EU). Zebrafish has been considered as an alternative model in drug discovery that satisfies for 3R concept: the reduce, refine, replace to eliminate and/or minimize animal testing [38]. The transparency of the zebrafish larvae allows real-time visualization of the inflammatory cell migration[39]. Furthermore, many transgenic zebrafish types have been established, which makes it convenient to observe the inflammatory cells such as neutrophils and macrophages. Because of these reasons, zebrafish has been established as an good model for evaluating anti-inflammatory activity and high-throughput *in vivo* screening of active compounds [38].

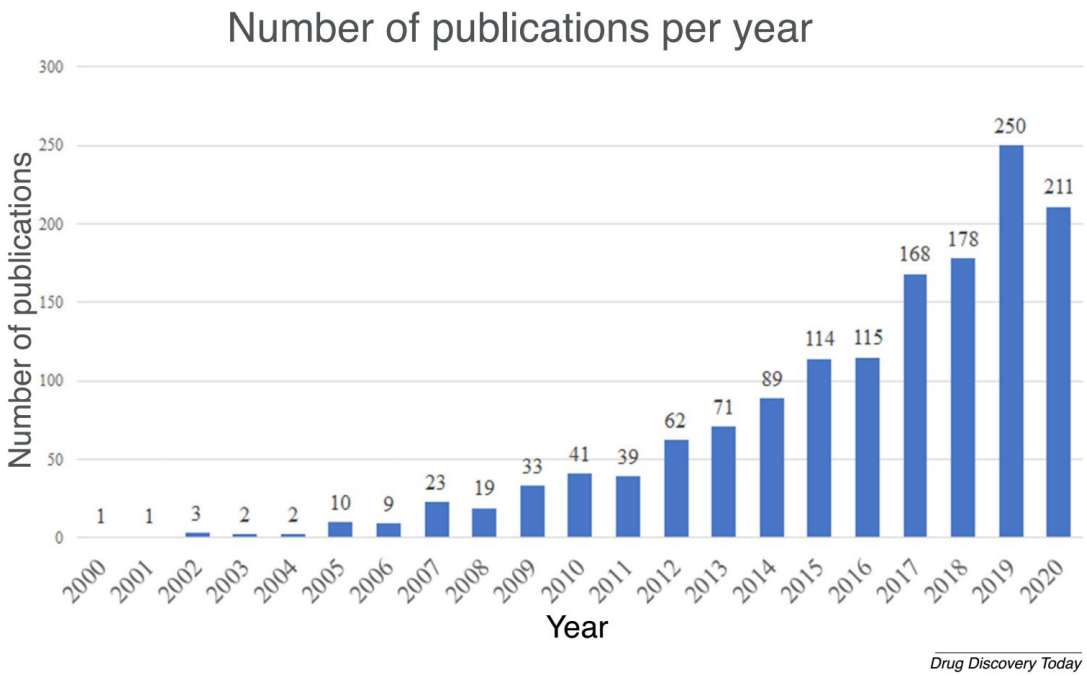

Figure 11. Number of publications per year (1999–2019) based on a search of Scopus with the settings: TITLE-ABS (zebrafish OR 'zebrafish' OR 'danio rerio' AND inflammation OR inflammatory) [40]

Limitations:

The first consideration is pharmacokinetics. Rats and other mammals are generally exposed to test compounds through gavage or injection. In zebrafish, by contrast, the most popular method for dosing is through immersion in a medium with drugs. This poses two issues; one is that it is not suitable for chemicals with poor solubility; the second is that in-water dosing may yield unique exposures compared to other animal routes[41]. It also is difficult to extrapolate the dose from human to fish. Because of its small size, technical skills are required for administration and get samples (blood/tissue) in adult zebrafish. Multi-endpoint assays or pharmacokinetics, pharmacodynamics (PK/PD) analysis in a single animal are difficult to conduct/evaluate because of the limited amount of biological samples (blood, tissues). Some tissues/organs of zebrafish are different from those of mammals [42]. Because of these limitations, the number of research using zebrafish for investigating anti-inflammatory activity is still low compared to the rodent model, with only 211 publications in 2020. Figure 12 describes the position of zebrafish in the drug discovery pipeline, it is a bioassay for the screening phase and a bridge between *in vitro* models and animal models.

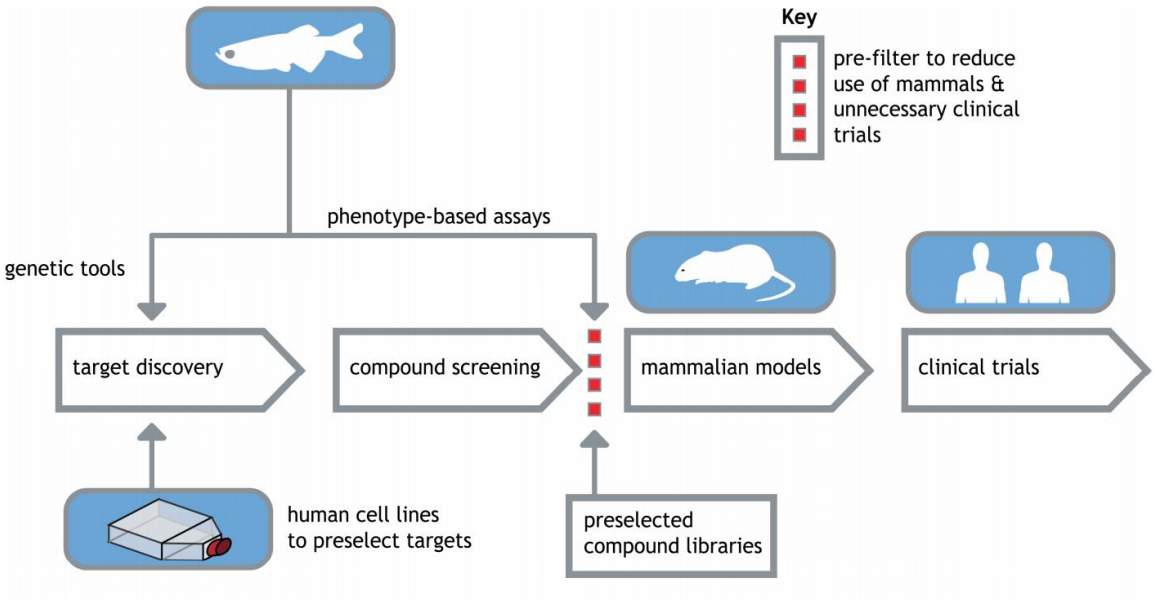

Figure 12. Drug discovery pipeline involving novel zebrafish models [40]

Zebrafish inflammation modeling

Several inflammation models using zebrafish have been developed over the past few years. Similar to rodents, zebrafish inflammation can be triggered by physical, chemical, or biological stimuli.

Inflammatory model induced by physical injury

In this model, inflammation was induced by tail transaction in zebrafish larvae or adults. Inflammatory was semi-quantitatively scored through observing the accumulation of macrophages and neutrophils near the wounded area, fin regeneration and the expression of inflammatory markers [40]. Migrating zebrafish leukocytes were detected using the staining method or transgenic fish [43], [38]. Besides direct transection, the injury can also be triggered by laser irradiation of the epidermis on flank skin[44], the yolk sac and skeletal muscle tissue[45]. This assay has provided the basis for genetic and pharmacological screens for new anti-inflammatory drugs. A lot of natural compounds, well-characterized drugs have been evaluated and screened in this model, for example, meisoindigo was proved to have anti-inflammatory through the inhibition of leukocyte migration to the inflammatory site [46], isopimpinellin displayed anti-inflammatory activity through inhibiting the recruitment of leukocytes and promoting the resolution phase [47]. However, the tail transaction model is time-consuming and requires high technical skill, unsuitable for high-throughput screens.

Inflammatory model induced by chemicals

Exposure to $CuSO_4$ was reported to induce oxidative damage and inflammatory in zebrafish. Upon $CuSO_4$ treatment, neutrophils and immune-related processes are activated. The accumulation of neutrophils in the neuromasts is observed in this model as one of the indicators for assessing anti-inflammatory drugs [48],[49]. This model is non- invasive, allows rapid screening of thousands of compounds and genetic screens aimed at identifying key molecular components of the innate immune response. The model is considered the ideal model for inflammatory research. The number of publications that use this model increased, from 1 publication in 2010 to 7 publications in 2020 (Pubmed data).

LPS-induced inflammation

LPS is an endotoxin of Gram-negative bacteria. It induces inflammatory response upon recognition through TLRs, and leads to the production of proinflammatory cytokines, proteases, eicosanoids, and reactive oxygen and nitrogen species [50]. In the model, an inflammatory reaction is induced by injecting LPS into the yolk of zebrafish larvae or by immersing LPS in the embryo medium [38]. Inflammation can be assayed through observation of the migration of macrophages and neutrophils and assessed by measuring the expression of key cytokines IL-1β, IL-6, and TNF-α using RT-PCR. A lot of compounds and extracts from traditional plants have been evaluated using this model[38], [51]. However, the TLR4 identified in zebrafish does not recognize LPS. Moreover LPS tolerance of zebrafish to LPS can happen in some cases[50]. This affects the stability of the model and requires control carefully in the experiment process. Recently, some studies used the combination of LPS treatment and tail wounding to enhance leukocyte accumulation near the wound [52], [43].

Chemical-induced intestinal inflammation:

Intestinal inflammation can be induced in zebrafish using various chemicals. Intraperitoneal injection of carrageenan caused abdominal edema in zebrafish [53]. Inflammatory bowel disease (IBD) can be induced using dextran sodium sulfate (DSS) [54]. Exposure to TNBS increased the number of neutrophils in the inflamed intestine and increased expression of *il1b, tnf-a, mmp9, ccl20,* and *il8* [55]. Similarly, the rectal administration of oxazolone can induce a model of enterocolitis, which is characterized by the infiltration of granulocytes, epithelial damage, and upregulated expression of cytokines [56]. These models are useful for the investigation of the cellular and molecular mechanisms underlying the inflammatory response, and the identification of potential drug targets.

Rodent models

Different types of animals can be used for the evaluation of anti-inflammatory: rat, mouse, rabbit, zebrafish, goat… Amongst these models, rodents are considered as the ideal model for inflammatory research since they display similarities to humans in terms of genetics, anatomy, and physiology, can be genetically modified to resemble different human disease models. In addition, rodent models are often selected because of their unlimited supply and ease of manipulation.

Acute inflammatory models: In these models, paw edema is induced by injection carrageenan/ histamin/serotonin, bradykinin, dextran or LPS in the right hind paw of rats. The paw volume is measured at different times after the injection. These chemicals can induce inflammation through various mechanisms: carrageenan initiates the early phase of inflammation through stimulation of phospholipase A2 while histamine and 5-HT increase the vascular permeability, acting with prostaglandins. The bradykinin enhances the prostaglandins (PGs) and the stimulation of phospholipase activity. Dextran increases vascular permeability, activates kinins, the release of histamine and serotonin. LPS increases the TNF-α, IL-1β expression and myeloperoxidase activity [57]. Among the different models, carrageenan-induced hind paw edema is the most common animal model to evaluate the acute anti-inflammatory potential of pharmacological substances. It is highly sensitive and reproducible.

Chronic inflammatory models: Cotton pellet-induced granuloma is the most popular model for evaluating chronic anti-inflammatory of new compounds. It is based on the release of granuloma induced by implanting cotton pellets in the groins of the animals subcutaneously. The wet weight of the pellets is associated with transudate, while the dry weight has a direct relationship with the size of granulomatous tissues[20].

Table 3 described the advantages and limitations of each model in the evaluation of anti-inflammatory drugs. The selection of the model should be based on the purpose of the research and the target mechanism of each compound.

Table 3. Rodent models in evaluation anti-inflammatory effect of drugs [57]

	The name of the model	**Advantages**	**Limitations**
Acute inflammation	Carrageenan-Induced Paw Edema	Involvement of multiple mechanisms, sensitive to drugs relates to the cyclooxygenase inhibition of prostaglandin synthesis, highly reproducible.	involves animal stress; difficulty with edema measurement, the investigator should be trained.

	Histamine/5-HT-Induced Paw Edema	Suitable for the assessment of drugs that act through the histamine and/or 5-HT inhibition	Not readily available; produce minimal edema
	Bradykinin-Induced Paw Edema	Drugs inhibiting prostaglandins are effective in this model	only mild and transient edema
	Dextran-Induced Paw Edema	It is suitable to assess anti-histaminic or anti-serotonin drugs; the development of edema is rapid	not suitable for lipid mediators
	LPS-Induced Paw Edema	Suitable for the recognition of anti-inflammatory agents that acts through cytokine modulation.	
	Pleurisy tests	Suitable to evaluate phenomena like fluid extravasation, leukocyte migration and biochemical parameters in the exudate.	Induce severe pain and accompanied with systemic infections
	Arachidonic Acid-Induced Ear Edema	Suitable for topical inflammation; requires mice, suitable for drug relate to eicosanoids inhibition	Animals are sacrificed; measurement is relative.
	Croton oil/TPA-Induced Ear Edema	Suitable for topical inflammation; requires mice	
	Oxazolone-Induced Ear Edema	Well recognized model of delayed-type hypersensitivity, topical inflammation; requires mice	
	Acetic Acid/Compound 48/80-Induced Vascular Permeability	Suitable to assess mast cell stabilization or anti-histaminic activity of a drug	
Sub-acute inflammation	Granuloma Pouch Model Administration [20]	Good for sub-acute inflammation; direct contact of test drugs with the target cells; monitors immune system response	Severe animal stress; requires anesthesia; involves loss of many animals; animals are sacrificed
Chronic inflammation	Cotton Pellet-Induced Granuloma[57], [20]	Widely used model of chronic inflammation. Ideal for chronic inflammation; gives information about the proliferative changes	needs anesthetics and surgical skills, sepsis may occur; need of sacrifice of the animals
	Complete Freund's Adjuvant (CFA)-Induced arthritis[20]	Ideal for chronic inflammation and arthritic alterations	Sophisticated; tedious; severe animal pain and stress; animals are sacrificed

	Formalin-Induced Paw Edema	This is the chronic inflammation model that similar to human arthritis.	Formalin is a severely irritating agent and can cause severe pain when exposes to the experimental animals

5.3.2 I*n vivo* models currently used in the evaluation of anti-oxidant activity

In all *in vivo* methods, the plant extracts are administered to the testing animals (mice, rats, etc.). After a period of time, the animals are killed and blood or tissues are used for the assay. Enzymatic antioxidants superoxide dismutase (SOD), catalases, glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GPX) from blood or tissue are measured using various methods. *In vivo* models used for anti-oxidant activity are shown in table 4, the frequency for using these models is shown in figure 13. The LPO method is the most frequently used among a total of 74 papers reviewed by Md. Nur Alam (2013) [27]. It is followed by CAT, SOD and GPx.

Table 4. *In vivo* models used in the evaluation of anti-oxidant effect of drugs

	Name of methods
1	Ferric reducing ability of plasma
2	Reduced glutathione (GSH) estimation
3	Glutathione peroxidase (GPx) estimation
4	Glutathione-S-transferase (GSt)
5	Superoxide dismutase (SOD) method
6	Catalase (CAT)
7	c-Glutamyl transpeptidase activity (GGT) assay
8	Glutathione reductase (GR) assay
9	Lipid peroxidation (LPO) assay
10	LDL assay

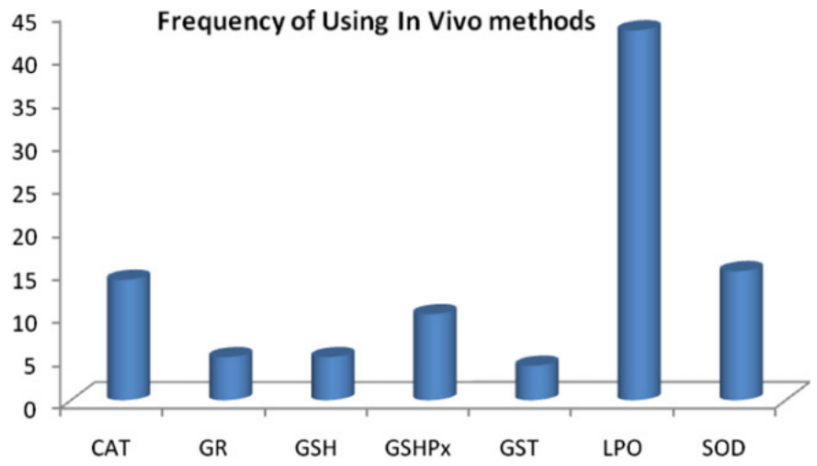

Figure 13. Frequency of using invivo methods for evaluation anti-oxidant activity[27]

6. Ethnomedicinal, phytochemical and pharmacological profile of *Clerodendrum cyrtophyllum* Turz.

Science name: *Clerodendrum cyrtophyllum* Turz. Lamiaceae (Verbenaceae).

Synonym name: *Clerodendrum amplius* Hance, *Clerodendrum formosanum* Maxim

Botanical description

In Viet Nam, there are 30 species of *Clerodendrum* found. *Clerodendrum cyrtophyllum* Turz is the species with the most popular distribution. The plant often distributes in delta provinces and in the mountain with an altitude lower than 1000 m, especially in midland provinces: Thai Nguyen, Cao Bang, Lang Son, Bac Giang, Vinh Phuc, Phu Tho, Ha Noi. *C. cyrtophyllum* often grows in a cluster in the hill, along roadsides and abandoned fields. It is a shrub or small tree, 1 m high. Leaves are oval or long lanceolate, 5-13 cm x 3-7 cm, leave petiole 1 – 6 cm long. Flowers are white, inflorescences grow at the tips of branches, the main axis of the inflorescences is short, many branches carry flowers. Figure 14 describes the leaves, branches, flowers, fruit, and the seed of *C. cyrtophyllum.*

Traditional medicinal uses and ethnopharmacology

All parts of the plant can be used in folk medicine for different purposes. Young leaves are boiled or steam with digestive benefits. They are prescribed for mothers, after having given birth, in order to restore their appetite[58]. Leaves boiled in water to treat scabies[59]. Roots for the treatment of rheumatism, asthma, febrifuge, incephalagia, ophthalmia and other inflammatory diseases. The decoction of whole plant is used to treat colds, high fever, inflammation of the throat, epidemic encephalitis, furuncles, rheumatic arthritis, carbuncles, and snakebites [60].

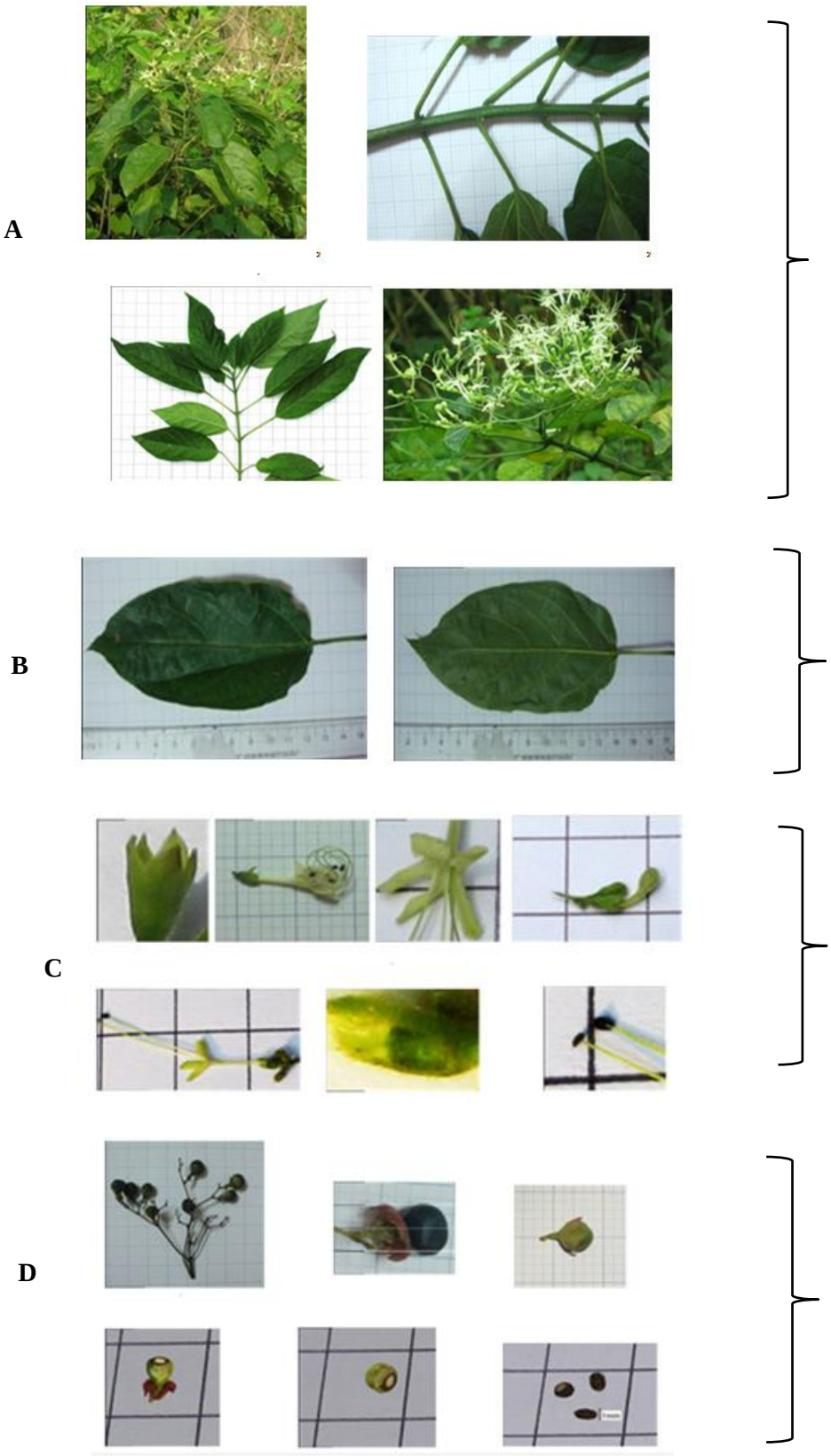

Figure 14. Photos of *Clerodendrum cyrtophyllum* plant

A. Branch, **B**. Leave, **C**. Different parts of a flower, **D**. Different parts of fruit

Phytochemical constituents

Phenolic acids, polyketides, diterpenes, triterpenes, glucosides, proteins, and sterols have been isolated from various plant parts, and phenols are the major constituents of the plant [60]. Clerodendiod A and B, together with seven known glycosidic were isolated from the branches of *C. cyrtophyllum* by Peng Wang et al (2012)[61]. Jing Zhou (2020) isolated 12 compounds from leaves of *C. cyrtophyllum*: acteoside, jionoside C: jionoside D, martynoside, luteolin, cirsilineol, cirsimartin, cirsilineol-40-O-β-D-glucoside, cirsimarin, jaceosidin 7-O-β-D-glucoside, (1-p-Hydorxy-cis-cinnamoyl) cinnamic acid, esculetin.

Pharmacology

Despite the wide application of this plant in folk medicine, there has not been much pharmacology research for its bioactivity. Some *in vitro* studies indicated that the crude extract and the fraction from leaves of this plant possess anti-oxidant activity through scavenging DPPH, ABTS and superoxide anion radicals [62]. The methanol extract from the plant also demonstrated anti-inflammatory through the inhibition of NO release and the downregulation of the expression of COX-2 and iNOS in RAW 264.7 cells[63]. Some pure compounds were isolated from *C. cyrtophyllum* by Jing Zhou (2020), among them acteoside was found to be a major component and potential for antioxidant, IC50 values for scavenging DPPH and ABTS were 79.65±3.4 and 23.00±1.5 μg/ml, respectively. These studies have provided some first evidence for using the plant in folk medicine. However, it is still far from the purpose to develop the plant to the drug. We need more pharmacology studies, phytochemical studies and clinical studies to confirm the efficacy, safety and deep investigation for the molecular mechanism of the crude extract as well as lead compounds. Drug discovery and development are complex processes, very expensive, time-consuming, and with many failures but it is necessary for pursuing an “evidence-based herbal medicine,” make people’s health care better and safer.

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Chapter 2

Objectives, outline of the thesis and hypotheses

1. Objectives

General objective: This thesis aimed to investigate the anti-inflammatory and anti-oxidant efficacy, the protective activity against copper toxicity, phytochemistry and toxicity as well as the underlying molecular mechanisms of action of the extracts from *Clerodendrum cyrtophyllum* Turz leaves.

Specific objectives:

- To investigate the anti-inflammatory activity and the mechanisms of action of the extracts from *Clerodendrum cyrtophyllum* leaves.
- To investigate the anti-oxidant activity and the mechanisms of action of the extracts from *Clerodendrum cyrtophyllum* leaves.
- To investigate the protective activity against copper toxicity and the mechanisms of action of the extracts from *Clerodendrum cyrtophyllum* leaves.
- To assess the embryotoxicity and toxicity mechanisms of the extract from *Clerodendrum cyrtophyllum* leaves.
- To identify the phytochemicals from the extracts of *Clerodendrum cyrtophyllum* leaves.

2. Context of thesis

The thesis is a part of the research project "Exploring the medical, (Eco-toxicological and socio-economic potential of natural extracts in North Vietnam". This project aims to explore medicinal plants in Northern Vietnam. For this purpose, experts in both Vietnam and Belgium set up and validated a standard extract procedure using ethanol and solvents with increasing polarity (n-hexane, dichloromethane, ethyl acetate, methanol) for the extraction.

Sample collection: different parts of *C. cyrtophyllum* such as leaves, roots, or the whole plant are used in traditional medicine. However, the extract from leaves of this plant has been proved to have the potential for anti-oxidant and anti-inflammatory activities in *in vitro* tests in recent studies. In addition, leaf collection is easier than roots or the whole plant. Leaf collection is also a way for the conservation and sustainable use of medicinal plants. Therefore, we selected the leaves of the plant as material for extraction and for evaluating their bioactivity.

Increasing concentrations of the extract from *Clerodendrum cyrtophyllum* leaves used in *in vitro* tests based on a preliminary study. From the result of the prelimnary study, the main study was implemented with at least 5 concentrations to determine a dose-response curve. For *in vivo* tests, doses were selected from the toxicity screening test. Only concentrations inducing no lethal and low immunotoxicity were then selected.

3. Outline of the thesis

Chapter 1: General introduction

Chapter 2: Objectives, hypothesis, and outline of the thesis

Chapter 3: *In vitro* and *in vivo* anti-inflammatory properties of the ethanol extract from *Clerodendrum cyrtophyllum* Turcz based on murine cells and zebrafish models

Chapter 4: Anti–inflammatory and antioxidant properties of the ethanol extract of *Clerodendrum cyrtophyllum* Turcz in copper sulfate-induced inflammation in zebrafish

Chapter 5: Ethanolic extract of *Clerodendrum cyrtophyllum* Turcz leaves exerts antioxidant activity against $CuSO_4$-induced toxicity, involving the Nrf2/keap1/ARE signalling pathway

Chapter 6: Effect of the ethanol extract from *Clerodendrum cyrtophyllum* leaves on zebrafish larvae proteins in response to $CuSO_4$

Chapter 7: Developmental Toxicity of *Clerodendrum cyrtophyllum* Turcz ethanol extract in zebrafish embryo

Chapter 8: Anti-inflammatory potential of the extracts from *Clerodendrum cyrtophyllum* Turcz and comparison to the ethanol extract through *in vitro* and *in vivo* assays

Chapter 9: General discussion

Chapter 10: Conclusions and perspectives

4. Hypotheses

Research hypothesis 1 (chapter 3):

Medicinal plants have provided an endless source of new drugs. Recent studies showed that phytochemicals (flavonoids, triterpenoids, polyphenolic compounds, limonoids…) present in medicinal plants are potential anti-inflammatory compounds. Leaves of *Clerodendrum cyrtophyllum* Turcz (Lamiaceae), a popular medicinal plant in Vietnam and in many countries, have been used widely for the treatment of inflammatory diseases: colds, high fever, inflammation of the throat, rheumatic arthritis. So our research hypothesis in chapter 3 is that "*C.cyrtophyllum* leaves has anti-inflammatory activity". To prove this hypothesis, some research questions are suggested:

1/ What is the best solvent for the extraction *C. cyrtophyllum* leaves?

2/ Which *in vitro* or *in vivo* tests should be used?

3/ What are the suitable doses for *in vitro* and *in vivo* tests?

After reviewing the literature carefully and thoroughly we chose the ethanol solvent for the extract of *C. cyrtophyllum* leaves, because it was identified as the most effective solvent for the extraction, resulting in the highest extraction yield and less toxic to human and animal cells. To prove the anti-inflammatory activity of the ethanol extract from *C. cyrtophyllum* leaves (EE), we used some *in vitro* tests. We found that blood cell membrane stabilization and NO assay were simple, rapid and cheap tests. The ethanol extract thus was screened for anti-inflammatory effects using blood cell membrane stabilization and NO assay at various concentrations. To confirm anti-inflammatory activity as well as the underlying molecular mechanism of action of the extract from *C. cyrtophyllum* leaves, we used the cut-tail zebrafish model. Zebrafish has been established as an ideal model for evaluating anti-inflammatory activity and high-throughput in vivo screening of active compounds. The cut-tail zebrafish model is an inflammatory model specific for physical injury. The dose of EE for this test was built based on immunotoxicity tests and only concentrations with no or low immunotoxicity were selected. The expression of inflammatory genes was evaluated in the cut-tail zebrafish model using q-PCR analysis.

Research hypothesis 2 (chapter 4):

Inflammation can be induced by many reagents. To prove that EE also has anti-inflammatory activity in the inflammatory model induced by chemicals, we evaluated the effects of EE on the copper sulfate-induced zebrafish inflammation model. However, recent studies reported that $CuSO_4$ induces an inflammatory response in zebrafish larvae through the oxidative stress reaction. Some studies on *C. cyrtophyllum* leaves found that its extracts had *in vitro* antioxidant activity. Thus, the hypothesis for this chapter was “EE has both anti-oxidant and anti-inflammatory activity in the copper sulfate-induced zebrafish inflammation model”. To prove this research hypothesis, we evaluated the capacity of EE to scavenging DPPH radicals, chelate Cu^{2+} and the effect of EE on the reactive oxygen species (ROS) formation induced by $CuSO_4$ using fluorescence probes. We also evaluated the effects of EE on the expression of inflammatory and oxidative stress genes on the copper sulfate-induced zebrafish inflammation model.

Research hypothesis 3 (chapter 5):

Oxidative stress in the cell can be prevented in two ways: direct and/or indirect. Exogenous antioxidants can directly scavenge free radicals, RNS, ROS or indirectly increase endogenous antioxidant enzymes activity. In the oxidative stress signaling pathways, the Nrf2/Keap1/ARE plays a central role which initiates cellular defense by regulating the expression of ARE-driven genes including antioxidant and phase II detoxifying enzymes. Our research hypothesis in this chapter was that "EE can be involved directly and indirectly as antioxidant, involving Nrf2/keap1/ARE pathway". To prove this hypothesis, we used test-tube assays to evaluate the direct anti-oxidant activity of EE and the copper sulfate-induced zebrafish inflammation model to evaluate indirect anti-oxidant activity. Activity of endogenous antioxidants (SOD, CAT, GSH, GST) were measured after 24 h exposure copper. We also investigated the effects of EE on the expression of genes of Nrf2/keap1/ARE pathway to prove the involvement of it and this pathway.

Research hypothesis 4 (chapter 6)

EE's protective activity relates to its effects on proteins involved in different pathways in response to $CuSO_4$ and on the expression of copper metabolism genes. To prove this hypothesis, we used proteomics as a tool to evaluate EE's effects on protein alterations, biological processes, and protein-protein interaction networks in response to $CuSO_4$. We also used qPCR to investigate EE's effects on the expression of copper metabolism genes. Proteomic results combined with results on copper metabolism genes could provide a comprehensive understanding of the action mechanisms of EE against copper toxicity.

Research hypothesis 5 (chapter 7)

Pharmacology effects of EE were proved in previous chapters. However, it needs to assure safety before moving into the next phase for testing this plant extract on humans. *C. cyrtophyllum* leaves have been used widely for the treatment of human diseases. The research hypothesis in this chapter is that EE is safety to zebrafish embryo. To prove this hypothesis, we treated zebrafish embryos with different concentrations of EE and observed different teratogenicity indicators. We also evaluated the effects of EE on the expression of genes related to fish embryo's development such as heart development (*arntl2, cry2b, nr1d1, nkx 2.5*), oxidative stress (*cat, sod1, gpx4, gstp2*), wnt pathway (β-*catenin, wnt3a, wnt5, wnt8a,* and *wnt11*), cell apoptosis (*pt53, bax, bcl2, casp3, casp8, casp9, apaf-1, gadd45bb)* to elucidate the possible toxicity mechanisms involved.

Research hypothesis 6 (chapter 8)

The extracts with solvents with increasing polarity from *Clerodendrum cyrtophyllum* also have anti-inflammatory activity and more potential than the ethanol extract. To prove this hypothesis, we extracted *C. cyrtophyllum* leaves with 4 solvents with increasing polarity: n-hexane, dichloromethane, ethyl acetate and methanol. *In vitro* assays were used for screening anti-inflammatory activity of these extracts. The best extract was then used for phytochemical analysis and compared to the ethanol extract for anti-inflammatory activity in the *in vivo* model.

SECTION II

Foreword: Medicinal plants have provided an endless source of new drugs. Recent studies showed that phytochemicals (flavonoids, triterpenoids, polyphenolic compounds, limonoids…) are potential anti-inflammatory compounds. Therefore, medicinal plants are important sources for the development of anti-inflammatory drugs. Leafs of *Clerodendrum cyrtophyllum* Turcz (Lamiaceae), a popular medicinal plant in Vietnam and many other countries, has been used widely for the treatment of inflammatory diseases. This chapter aimed to evaluate the anti-inflammatory activity of the ethanol extract from *C. cyrtophyllum* leaves.

Chapter 3

In vitro and in vivo anti-inflammatory properties of the ethanol extract from *Clerodendrum cyrtophyllum* Turcz based on murine cells and zebrafish models

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Abstract

Ethnopharmacological relevance: *Clerodendrum cyrtophyllum* Turcz, a plant belonging to the Verbenaceae family, has been used in traditional medicine for the treatment of various inflammatory diseases in many Asian countries.

Aim of the study: The study aimed to evaluate anti-inflammatory properties of the ethanol extract from *Clerodendrum cyrthophyllum* Turcz leaves (EE) through *in vitro* and *in vivo* models.

Material and Methods: Total phenolic and flavonoid contents in the extract were determined using colorimetric methods and HPTLC. In red blood cell membrane stabilization model, rat erythrocyte suspension was treated with crude ethanol extract at different concentrations, the hemoglobin content of the supernatant solution released by red blood hemolysis was estimated. We also evaluated the effects of the ethanol extract from this plant on the production of nitric oxide (NO), tumor necrosis factor alpha (TNF-α) in stimulated RAW 264.7 cells. In order to elucidate its anti-inflammatory molecular mechanisms, we further evaluated the effects of the EE on the expression of the inflammatory genes in inflammation-induced zebrafish model by tail-cutting using qPCR analysis.

Results: Colorimetric methods and HPTLC revealed high phenolic and flavonoid contents in the extract. In the red blood cell membrane stabilization model, the amount of hemoglobin released by red blood hemolysis significantly decreased in the presence of EE, demonstrating a strong membrane stabilizing activity. EE did not cause any toxic effect on cell viability but strongly inhibited NO and TNF-α release due to LPS induction. The association with EE significantly reduced the expression of *cox-2, pla2, c3a, il-1(il1fma), il-8* (*cxcl8b.1*), *tnf-α,* and *nf-ƙb*, while increased the expression of the anti-inflammatory cytokine *il-10* gene in cut-tail induced inflammation of zebrafish model.

Conclusions: Taken together, the results suggest that the raw ethanol extract from C. *cyrtophyllum* Turcz leaves presents potent anti-inflammatory activities and may be useful for the treatment of various inflammatory diseases.

Abbreviations: EE, the ethanol extract from *Clerodendrum cyrthophyllum* Turcz leaves; NO, nitric oxide; TNF-α, tumor necrosis factor alpha; LPS, lipopolysaccharide; HPTLC, High performance thin layer chromatography; QE, quercetin; GAE, gallic acid equivalent; quantitative polymerase chain reaction (qPCR); dpf, day post fertilization; RBC, red blood cell.

Keywords: RAW 264.7; zebrafish; RBC membrane stabilization; Nitric oxid; Anti-inflammatory; *Clerodendrum cyrtophyllum*.

1. Introduction

Inflammation is the protective response of the body against infectious agents and injury, allergic or chemical irritation by removing injurious stimuli and initiating the healing process. Two important components taking part of the inflammation process include cells of the immune system and inflammatory mediators that come from these cells or plasma proteins. Cells of the immune system are mast cells, neutrophils and monocytes/macrophages, T cells and B cells. Inflammatory mediators include: nitric oxide, prostaglandins, leukotrienes, vasoactive amines

(histamine, serotonin), and cytokines, proteases, chemokines. In response to injurious stimuli, the body initiates a chemical signaling cascade that stimulates responses to repair the damage. Inflammatory pathways are activated. These signals activate the immune cell chemotaxis from the general circulation to the sites of injury. These activated cells produce chemical mediators that bind to specific target receptors on the cells and induce the characteristic signs of inflammation (swell, heat, red, pain and loss of function)(Chen et al., 2018). Usually, cellular and molecular events in acute inflammatory responses can lead to recovery from infection and to healing. However, if the acute inflammation is not controlled or resolved, inflammation cascades can lead to the development of a variety of chronic inflammatory diseases such as asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis, diseases of the cardiovascular, metabolic.

Lysosomal enzymes released during inflammation produce a variety of disorders leading to tissue injury, by macromolecules damaging and membranes peroxidation. Stabilization of the lysosomal membrane thus has an important role in inhibiting the release of lysosomal constituents from activated neutrophils and limit the inflammatory response (Seema Chaitanya Chippada et al., 2011).

Macrophages are the main pro-inflammatory cells, playing critical roles in immune reactions, allergy, inflammation, and protecting the organism from external intruders through phagocytosis. Upon activation, macrophages become potent secretory cells that release large amounts of pro-inflammatory mediators including NO, prostaglandins and the cytokines TNF-α, IL-1β, and IL-6 (Shao et al., 2013). Production of NO and prostaglandins are regulated by iNOS and COX-2. Overproduction of NO can trigger some inflammatory diseases such as heart attacks, septic shocks, rheumatoid arthritis (Jung et al., 2014). NF-κB is a transcription factor that plays an important role in the transcription of genes, which are involved in immune and inflammatory responses. Upon stimulation, NF-κB is liberated from a complex with I-κB, now becoming enabled to migrate to the nucleus where it binds to the cis-acting κB enhancer element of target genes and induces transcription of its target genes such as *COX-2*, *iNOS*, *TNF-α*, *IL-1β*, and *IL-6*, chemokines and adhesion molecules (Karin and Ben-neriah, 2000). Inhibition of the production and/or release of many of these mediators are well-established targets for the discovery of anti-inflammatory drugs, with nonsteroidal anti-inflammatory drugs (NSAIDs) as COX-inhibitors being the most prominent drug class. However, due to the various side effects associated with many NSAIDs, especially gastrointestinal irritation and increased risk of

myocardial infarction and stroke, there is still an immense need for anti-inflammatory agents with a better benefit-risk profile (Pompermaier et al., 2018). For this purpose, ethnobotanical studies represent an increasingly attractive approach for applying indigenous knowledge of plant use to modern societies, with the final aim of developing new remedies.

Clerodendrum cyrtophyllum Turcz, a plant belonging to the Verbenaceae family, is widely distributed in Vietnam, China, India, Japan, Korea and Thailand (Kar et al., 2014). In Vietnam, the plant is called ‘‘Bọ mẩy’’. In Vietnam and China, it has been used for the treatment of some diseases such as inflammation of the throat, rheumatic arthritis, colds, high fever, migraines (Zhou et al., 2013). In several studies, phenolic acids, polyketides, diterpenes, triterpenes, glycosides, proteins, sterols and flavonoids have been isolated, and (poly)phenols are the major constituents of the plant (Zhou et al., 2013). Among the phytochemical compounds isolated from this plant, the glycosidated coumaramide compound clerodendiod B was shown to have potent activity in inhibiting the NF-κB pathway (Wang et al., 2012). Total phenols and flavonoids displayed high antioxidant activity and potential anti-inflammatory activity in several experimental models (Liu et al., 2011). However, underlying mechanisms remain uncertain.

In this study, we investigated the anti-inflammatory effects and the underlying molecular mechanisms of the ethanol extract from *Clerodendrum cyrtophyllum* Turcz on different biological models. For simple, fast and inexpensive testing, we selected the rat red blood cell membrane stabilization model by measuring hemoglobin release, as well as activated RAW264.7 macrophage cells to measure survival, NO production, and TNF-α secretion. Among animal models, zebrafish has the advantage to display pathological features of inflammation similar to those of humans. This fish model has been established as an ideal model for studying the pathophysiology of human inflammatory-related diseases (Yang et al., 2014). To complement the preliminary *in vitro* cellular tests, we chose the zebrafish larva as an alternative to animal testing (Saeidnia et al., 2015) to confirm the anti-inflammatory effects of the ethanol extract as well as to elucidate the molecular mechanisms of its effects.

2. Materials and methods

2.1.Chemicals

RPMI 1640, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Hyclone (Thermo Scientific, Waltham, MA, USA). Mouse TNF-α ELISA kits were purchased from Abcam (ab208348, Cambridge, CB20AX, UK). LPS (*Escherichia coli* 0111.B4), 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulfanilamide (S9251-100G), phosphoric acid and naphthyl ethylenediamine dihydrochloride (N9125-10G) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest commercially available grade.

2.2. Cell Culture

RAW 264.7 cells, a murine macrophage cell line, were obtained from American Type Cell Culture (ATCC; Rockville, MD) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37^{o}C in an incubator containing 5% CO_2.

2.3. Preparation of the ethanol extract of *Clerodendrum cyrtophyllum* (EE)

Plant collection*:* Leaves of *Clerodendrum cyrtophyllum* Turcz were collected from Northern provinces in Vietnam during June, 2018 by Dr Nguyen Kim Thanh, from Vietnam National University **(VNU-BIOL).** An official herbarium number (HNU 024106) was deposited at Botanical Museum of Hanoi, University of Science. Leaves were cleaned, dried for 72 h to a constant weight using a hot air oven at 40^{o}C and ground in a blender to fine powder (size smaller than 0.5 mm).

Preparation of total extract: Given that polar (aqueous) extracts are used in traditional practice, we selected ethanol to extract polar secondary metabolites, minimizing the extraction of mineral salts and polysaccharides. Hundred g dried leaves powder were extracted with 1000 ml 95% ethanol, at 60^{o}C in a water bath with 360 rpm agitation for 4 h. The extract was then filtered and collected. The residue was extracted for another two times using the same procedure. The collected ethanol extracts were then combined, concentrated on a rotary evaporator (40^{o}C) under reduced pressure and lyophilized to obtain the crude extract. The yield of extract was 11.3 % relative to the dried leaves powder. The crude extract was stored at -20^{o}C until use.

Determination of total phenolics and flavonoids content

The extract total phenolic content was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method of Dorman et al., 2003 (Dorman et al., 2003). The total phenolics content was expressed as mg gallic acid equivalents (GAE mg/g) using the following equation based on the calibration curve: $y = 0.0013x - 0.0062$ ($R^2 = 0.995$), where y was the absorbance and x the gallic acid concentration (µg/mL). Data presented are the average of three independent measurements, expressed in dry weight of leaves.

The extract flavonoids content was determined spectrophotometrically using a method based on the formation of a complex flavonoid-aluminum (Brahmi et al., 2012)(Fatiha et al., 2012). The total flavonoids content was calculated as quercetin (QE mg/g) equivalents using the following equation based on the calibration curve: $y = 0.042x + 0.1188$ ($R^2 = 0.990$) where y was the absorbance and x the quercetin concentration (µg/mL). Data presented are the average of three independent measurements, expressed in dry weight of leaves.

High performance thin layer chromatography (HPTLC) profiling

HPTLC was performed according to the procedure of the European Pharmacopeia 10 (Europe, 2019) using Camag Automatic TLC Sampler (ATS 4), Automatic Developing Chamber 2 (ADC 2), Derivatizer and TLC Visualizer 2, systems operated under the software VisionCATS version 2.5. The HPTLC profiling of phenolic compounds/flavonoids was performed on silica gel 60 F254 HPTLC plates (Merck, Germany), using ethyl acetate – methylethylketone – formic acid – water (5:3:1:1, v/v/v/v) as a solvent system; after being heated at 100°C for 3 min, the plates were sprayed with a 1 % MeOH solution of aminoethanol diphenyl borate and 5 % MeOH solution of macrogol 400 and visualized under UV 365nm and visible light.

The HPTLC profiling of iridoids was performed on silica gel 60 F254 HPTLC plates (Merck, Germany). Ethyl acetate – methanol – water (77:25:8 v/v/v) was used as a solvent system; plates were sprayed with a solution of vanillin (1 %) and sulfuric acid (2 %) in EtOH, heated for 5 min at 100°C and visualized under UV 365nm and visible light.

HPLC-DAD-ESI-MS analysis

High performance liquid chromatography analysis with diode array and MS detection (HPLC-DAD-ESI-MS) was performed on the extracts. LC-DAD analysis was carried out on an Agilent 1260 Infinty II system connected to an Advion expression L mass spectrometer via an electrospray ionization (ESI) interface. Chromatographic separations were achieved at a constant flow rate of 1.2 mL/min on a Agilent InfinityLab Poroshell 120 EC-C18 column (4.6 x 100 mm; 4 µm) maintained at 32°C. A gradient elution was performed with mobile phases A (Acetonitrile) and B (Water - 0.1% formic acid) as follows: 0 min, 5 % A; 2 min, 5 % A; 30 min, 30 % A; 60 min, 80 % A; 75 min, 100 % A. Using a post-column passive splitter, a 0.2 mL/min flow rate was delivered into the mass spectrometer ionization source. DAD detection wavelengths were set at 254, 270 and 330 with peak scanning between 200 and 700 nm. Full scan mass spectra were registered in negative and positive modes between 100 and 1000 m/z. Chromatographic and mass spectrometry data were acquired and processed using the instrument built-in software.

Fish and experimental conditions

The adult wild – type AB zebrafish (*Danio rerio*) were maintained in a recirculating ZebTec housing system (Techniplast) at 28°C with a 12:12 h (light/dark) photoperiod. Conductivity was maintained at approximately 500 µS/cm, pH at 7.2. Fish were fed *ad libitum* three times daily. The day before mating, males and females (2:2) were placed in spawning tanks. Naturally spawned embryos were obtained within 30 min after the lights were switched on in the morning. The embryos were selected visually using a binocular microscope and only fertilized and normally developed eggs were selected. Selected eggs were placed in embryo medium (clean water from housing system) at 28^{o}C. Embryo medium was renewed every 24h. Hatched larvae (at 4dpf) were used for subsequent experiments. The use of zebrafish was in accordance with the animal welfare act. Since zebrafish larvae below 120 h old are not considered animals (Lackmann et al., 2018) and hence no animal test authorization is required according to European legislation (EU Directive, 2010/63/EU). All experiments were terminated at 120 hpf. The term "larvae" refers to hatched embryos up to 120 hpf that are using up the yolk-sac reserves and still do not feed externally (also called yolk-sac larva, early larva, pre-larva or eleuthero embryo) (Lackmann et al., 2018).

2.4. Experimental design

Preparation of test samples

Dry ethanol extract was dissolved in DMSO to make a stock solution of 50 mg/mL. Depending on the experiment type, the stock solution was diluted to different concentrations using NaCl 0.9% with heat-induced hemolysis experiments, RPMI medium for experiments using RAW 264.7 cells, or in fresh larvae medium to the final concentration in each experiment well to be 5, 20, 25, 40, 50, 80, 100, 200, 500 µg/ml.

2.4.1. *In vitro* experiments

Heat-induced hemolysis

The heat-induced hemolysis of erythrocytes was carried out as described by Ranasinghe et al. (2012) (Ranasinghe et al., 2012) with some modifications. Fresh rat blood was collected and transferred to heparinized centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min, and the resultant erythrocyte pellet washed three times with equal volume of NaCl 0.9%. Final erythrocyte pellet was suspended in NaCl 0.9 % to make a 10 % suspension containing 10 % v/v of RBCs in NaCl 0.9 %.

Various concentrations of ethanol extract from *Clerodendrum cyrtophyllum* Turcz (EE at 500, 200, 100, 50 and 25 µl/ml) were prepared. The test mixture consisted of 20 µL of the sample, 60 µL NaCl 0.9 %, 20 µL 10 mM sodium phosphate buffer pH 7.4, 100 µL of the 10 % RBC suspension in test tubes. Sodium diclofenac (600 µg/ml) was used as positive control (Seema Chaitanya Chippada et al., 2011)(Begum et al., 2016). DMSO was used as control. The concentration of DMSO in control group was the same as in all other groups. The reaction mixture was incubated at 55°C for 20 min. After heating, the reaction mixtures were centrifuged at 5400 rpm for 5 min. 100 µl of supernatant were collected. Hemoglobin content of the supernatant solution was measured indirectly through its absorbance (O.D.) at 540 nm using a microplate reader (Epoch biotek plate reader). The blank was prepared as above, except that the reaction mixture was incubated at 4°C. The percent inhibition of hemolysis or percent of protection from hemolysis was calculated using the following formula:

$$\% \text{ protection} = \frac{(Control\ OD - Blank\ OD) - (Sample\ OD - Blank\ OD)}{Control\ OD - Blank\ OD}\ x100$$

Cell Culture Preparation

The RAW 264.7 cells were seeded in 96-well culture plates with 1×10^5 cells/well and incubated for 24 h at 37°C in an incubator containing 5% CO_2. The cells were pretreated with various concentrations (12.5, 25, 50, 100 and 200 µg/mL) of EE or of indomethacin 100 µg/ml (positive control) for 1 h (Lee and Park, 2016) (Sudsai and Wattanapiromsakul, 2013). DMSO was used as control. The concentration of DMSO in the control group was the same as in all other groups. The cells were stimulated with or without LPS (1 µg/mL) at 37°C for 24 h. After a 24h incubation at 37°C, 100 µl of supernatant were collected for the nitric oxide (NO) assay and 50 µl for the TNF-α assay. The remaining cells were used for assessing cell viability using the MTT assay.

Nitric oxide (NO) Assay

The amount of nitric oxide (NO) in the culture medium was measured by the Griess reaction (Fabiola et al., 2016). Briefly, 100 μL of cell culture medium were mixed with 100 μL of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid, and 0.1% (w/v) naphthyl ethylenediamine dihydrochloride). The reaction mix was incubated at room temperature for 10 min, the absorbance at 540 nm was measured using a microplate reader (Epoch biotek plate reader). The concentration of NO in the samples was calculated according to a standard curve of sodium nitrite. Fresh culture medium was used as blank in all experiments.

Measurement of cytokines (TNF-α): The levels of TNF-α in the culture medium (supernatant) were measured with enzyme-linked immunosorbent assay (ELISA) kit (Abcam) in accordance with the manufacturer's protocols. The absorbance was measured at 540 nm using a microplate reader. The concentrations of TNF-α were calculated according to the standard curve using each of the recombinant cytokines in the ELISA kits.

MTT assay

Cell viability was assessed by MTT assay. After collecting the supernatant for NO and TNF-α assay, the remaining cells in each well were incubated with 100 µL medium containing MTT (500 µg/mL) for 4 h at 37°C. Viable cells with active metabolism convert MTT into a purple colored formazan product. The medium was discarded, and the formazan was dissolved with 100 µl DMSO by shaking for 5 min. The absorbance of each well at 540 nm, measured using a microplate reader (Epoch biotek plate reader), was considered directly proportional to the number of viable cells.

2.4.2. *In vivo* experiments

The toxicity screening test

Four-days post fertilization (dpf) wild-type zebrafish larvae were treated with EE diluted in fresh embryo medium at different final concentrations (0, 5, 20, 40, and 80 µg/mL) for 2, 8, 14, 24 h. Treatments were performed in triplicate (20 larvae per group per dose). After incubation, larvae were observed for mortality. At non-lethal EE concentrations, larvae were collected, washed and analysed using qPCR to evaluate the effects of EE on immune gene expression. Only EE concentrations having no or low immunotoxicity were selected for further experiments.

In vivo anti-inflammatory test in cut tail zebrafish larval model

Four-days post fertilization (dpf) wild-type zebrafish larvae were anesthetized with ice slush (ice and water admixture) (Chen et al., 2013). After anesthesia, to trigger tail-cutting-induced inflammation, the tail of the zebrafish larvae was cut, around 0.5 mm (±0.2 mm, tolerated range) from the tip of each larval tail (Lee et al., 2013)(Cordero-maldonado et al., 2013). To make wounds as consistently sized as possible, tail-cutting was performed using a blade (*Swann – Morton Stainless steel surgical blade BS2982 ISO7740)* under a microscope. As soon as tail-cutting was completed, the zebrafish larvae were transferred into fresh larvae medium. Zebrafish larvae were randomly divided into groups, 20 larvae/well, 3 wells for a treatment. The zebrafish larvae were treated with dexamethasone 5 µg/ml, or EE diluted in fresh embryo medium at different final concentrations (selected from the toxicity screening test) for 24 h.

DMSO was used as control. The concentration of DMSO in the control group was the same as in all other groups. After 24 h, zebrafish larvae were collected, frozen and stored in -80^{o}C for qPCR analysis.

Quantitative PCR

Total RNA extraction and reverse transcription

Total RNA was extracted from these larvae using Trizol Reagent solution (Ambion, Thermofisher Scientific) following the manufacture's instructions. The pellet containing RNA was dried and resuspended in 100 µl of RNase-free water. The concentration of total RNA was determined spectrophotometrically at A_{260}/A_{280} and A_{260}/A_{230} nm using a NanoDrop^{TM} 1000 (Thermo Scientific). RNA integrity was checked by gel electrophoresis (1.2 % agarose). The extracted RNA samples were then subjected to DNase treatment (DNA-*free*™ DNA Removal Kit- Invitrogen) to avoid genomic DNA contamination. 1 µg total RNA was reverse-transcribed into double-stranded cDNA using the RevertAid RT kit (Thermo Scientific) according to the manufacturer's instructions.

The resulting cDNA was used to measure the expression of 12 immune system genes in a real-time quantitative polymerase chain reaction (qPCR). The mean expression of two housekeeping genes (ß-actin, elongation factor 1α EF1α) were used as the reference. The expression of genes encoding antibacterial protein (*lysozyme*), pro-inflammatory cytokines *(il-1(il1fma*), *il-8* (*cxcl8b.1*), *tnf-α),* neutrophil (*mpo*), complement 3a (*c3a*), phospholipase A2 (*pla2*), *cox-2,* nitric oxide synthase (*inos*), transcription factor NF-***κ***B *(nf-ƙb),* vascular cell adhesion molecule (*vcam)* and an anti-inflammatory response cytokine (*il-10*) was evaluated. The list of specific primers used is given in table 1. Primers were designed on Primer3 software. Amplifix software was used to check the quality of the primers. The efficiency of each primer was confirmed by RT-PCR.

Table 1. Primers pairs used in this study

Gene name	**Product length**	**GenBank Accession No.**	**Forward and reverse primer sequences (5' -3')**
ß-actin	193	AF057040	*Fwd:* CCCCATTGAGCACGGTATTG *Rev*: ATACATGGCAGGGGTGTTGA
efl1-α	150	L23807.1	*Fwd:* CCAAGGAAGTCAGCGCATAC *Rev*: CCTCCTTGCGCTCAATCTTC
Interleukin-1 *il-1 (il1fma*)	185	NM_001290418.1	*Fwd:* AGGAGCGAACAGTCATGAGT *Rev:* ACAACCTCGAGCTACAACCA

Interleukin -8 *il-8* (*cxcl8b.1*)	234	NM_001327985.1	*Fwd:* GCCTTCATGCTTCTGATCTGC *Rev*: AATCACCCACGTCTCGGTAGGA
Cyclooxygenase-2 (*ptgs2a* or *cox2*)	240	NM_153657.1	*Fwd:* ACAGATGCGCTACCAGTCTT *Rev*: CCCATGAGGCCTTTGAGAGA
inos (nos2a)	206	NM_001104937.1	*Fwd:* GCATTTGGAGACGGAGGTTC *Rev*: TGACCTTGCATCTCCGAAGT
Phospholipase A2 *pla2* (*pla2g4aa*)	177	NM_131295.2	*Fwd:* TCATGTCTCCTGGGCTGTTT *Rev*: CCAGCTCCTCCTCCATAGTG
Tumor necrosis factor *(tnfa)*	227	AB183467	*Fwd:* CACAAAGGCTGCCATTCACT *Rev*: GATTGATGGTGTGGCTCAGGT
nf-ƙb (nkap)	210	NM_001003414.1	*Fwd:*GGTCGGACAGAGATCACGGATT *Rev*: TGCTGTTCTTCACGTCCTCT
Interleukin-10 (*il-10*)	100	AY887900.1	*Fwd:* AGTCATCCTTTCTGCTCTGCT *Rev*: AAAGCCCTCCACAAATGAGC
c3a (c3a.1)	235	NM_131242.1	*Fwd:* GTACGAGGCGAACAACTGGA *Rev*: CATCATACGCCGCAGCTTTC
lysozyme	236	BC162644.1	*Fwd:* TCAGCTGATACGGGGAAGGA *Rev*: CCTTTTACCCAGCGGGACAT
vcam *(vcam1b)*	153	NM_001083835.1	Fwd: GCTAAGCAGGGTTGTGGATG Rev: TCCAGACTGTCGCTGTGATT
mpo	152	AF349034.1	Fwd: GTGGTCGTGTCGGTTCTCTT Rev: GCAGATTATGCGGGCCATTG

RT-PCR was performed using an ABI Step One Plus Real Time PCR system (Applied Biosystems). Five (5) µl of 25-fold diluted cDNA template was mixed with 5 µl of each primer (5 µM) and 10 µl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 20 µl. The standard cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. All reactions were performed as technical triplicates. For analysis, a standard curve of a pool of the cDNA of all samples was constructed following the absolute quantification method (Bio-Rad) (Bio-Rad, 2006). The equation for the linear regression line CT = m(log quantity) + b. Where b is the y-intercept and m is the slope of the linear regression. Based on the equation for the linear regression, we can determine the quantity of an unknown sample:

$$\text{Quantity} = 10^{(CT-b)/m}$$

The ratios of the quantity of candidate gene / average quantity of 2 housekeeping genes were subsequently calculated for each candidate gene and used to assess the differences in expression levels between experimental groups.

2.5. Data presentation and statistical analyses

Data analyses were performed using SPSS software version 16.0. Results are presented as the means ± S.E.M of at least three independent experiments. Datas followed a normal distribution. Therefore in this case, statistical significance was determined by one-way analysis of variance ANOVA followed by LSD's multiple comparison test to determine treatment differences. Differences were considered statistically significant when p values were < 0.05. For *in vitro* experiments, concentration providing 50 % inhibition (IC50) was calculated by non-linear regression using GraphPad Prism Software (San Diego, CA, USA).

3. Results

3.1.Total phenolic and flavonoid contents

The ethanolic extract of *C. cyrtophyllum* leaves (EE) presented a concentration of total phenolic compounds of 23.3 ± 1.5 GAE mg/g expressed in dry weight of leaves material. Flavonoids content was evaluated at 2.97 ± 0.01 QE mg/g expressed in dry weight of leaves material.

3.2 HPTLC profiling

The phenolic compounds/flavonoids (Figure 1A) and iridoids (Figure 1B) of EE were profiled by HPTLC and visualized under UV 365 nm and visible light after derivatization. Such characteristic profiles will allow to control the identification of further plant samples; the formal identification of major bands however requires comparison with reference compounds described in the literature; to our best knowledge, these are not commercially available. Further work in HPLC-DAD-MS yielded 3 major peaks but no characteristic m/z could be identified (Figure 2).

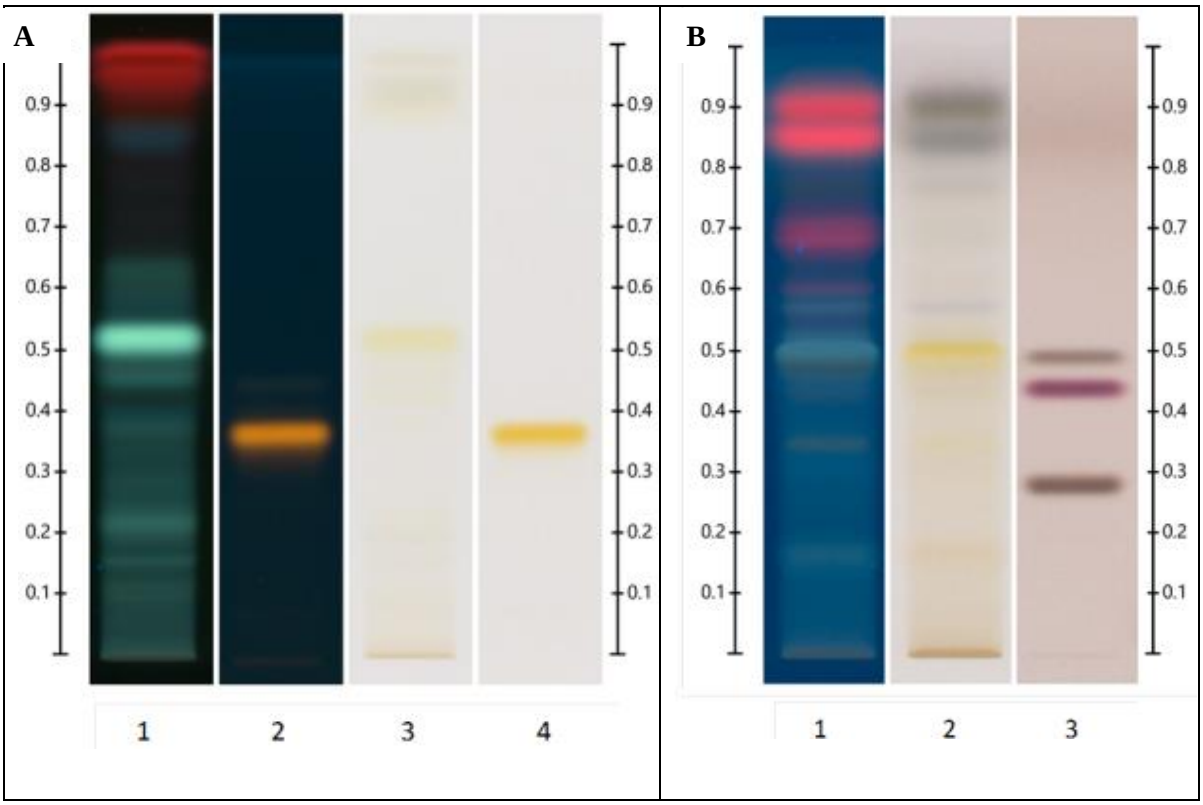


Figure. 1A: HPTLC profile of ethanolic extract (1 and 3) of *Clerodendrum cyrtophyllum* leaves (EE) and rutin (2 and 4). Analysis was performed on silica gel 60 F254 HPTLC plates (Merck, Germany), using ethyl acetate – methylethylketone – formic acid – water (5:3:1:1, v/v/v/v) as a solvent system; plates were sprayed with a 1 % MeOH solution of aminoethanol diphenylborate and 5 % MeOH solution of macrogol 400 after being heated at 100°C for 3 minutes and visualized under UV 365 nm (1 and 2) and visible light (3 and 4).

Figure. 1B: HPTLC profile of *Clerodendrum cyrtophyllum* leaves ethanolic extract (EE, 1 and 2) and iridoids standard mix (from top to bottom: agnuside, geniposide, aucubin). Analysis was performed on silica gel 60 F254 HPTLC plates (Merck, Germany), using ethyl acetate – methanol – water (77:25:8, v/v/v) as a solvent system; plates were sprayed with a solution of vanillin (1 %) and sulfuric acid (2 %) in EtOH, heated for 5 min at 100°C and visualized under UV 365 nm (1) and visible light (2 and 3).

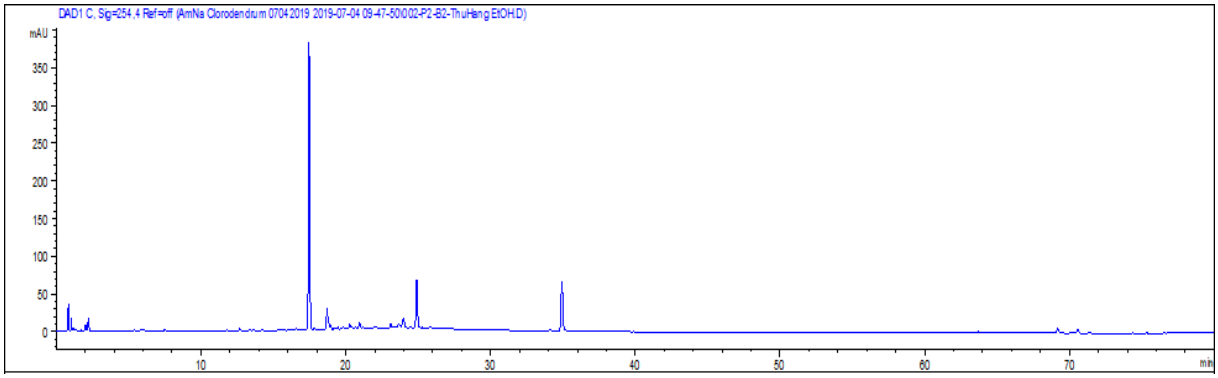


Figure. 2: Typical HPLC chromatogram of the *C. cyrtophyllum* ethanolic extract (EE) recorded at 254 nm.

3.3. *In vitro* experiments

3.3.1. Effect of the ethanol extract from the leaves of *C. cyrtophyllum* (EE) on hemolysis of red blood cells induced by heat

Heating the assay units at 55^{o}C induced 100 % hemolysis in the control samples. The NaCl 0.9% control caused a very weak protection 0.5%, while the samples treated with diclofenac experienced were protected to 78% (Figure 3). Addition of the ethanol extract from *C. cyrtophyllum* caused a concentration-dependent stabilization of the red blood cell membranes. At the highest concentration of 500 µg/ml, EE inhibited 93 % of hemolysis. At the concentrations of 200, 100, 50, 25 µg/ml, the % inhibition of hemolysis was, respectively 81; 56; 36 and 22% (Figure 3). No significant differences were observed between EE 200 and 500 µg/ml, between EE 50 and 100 µg/ml in inhibition of hemolysis. However, EE 200 µg/ml was more potent than EE 50 and 100 µg/ml. The IC50 of EE on hemolysis was found to be 75 µg/ml, compared with diclofenac 294 µg/ml (Table 2). Thus, the effect of EE on red blood cell membrane stabilization was even higher than the positive control diclofenac.

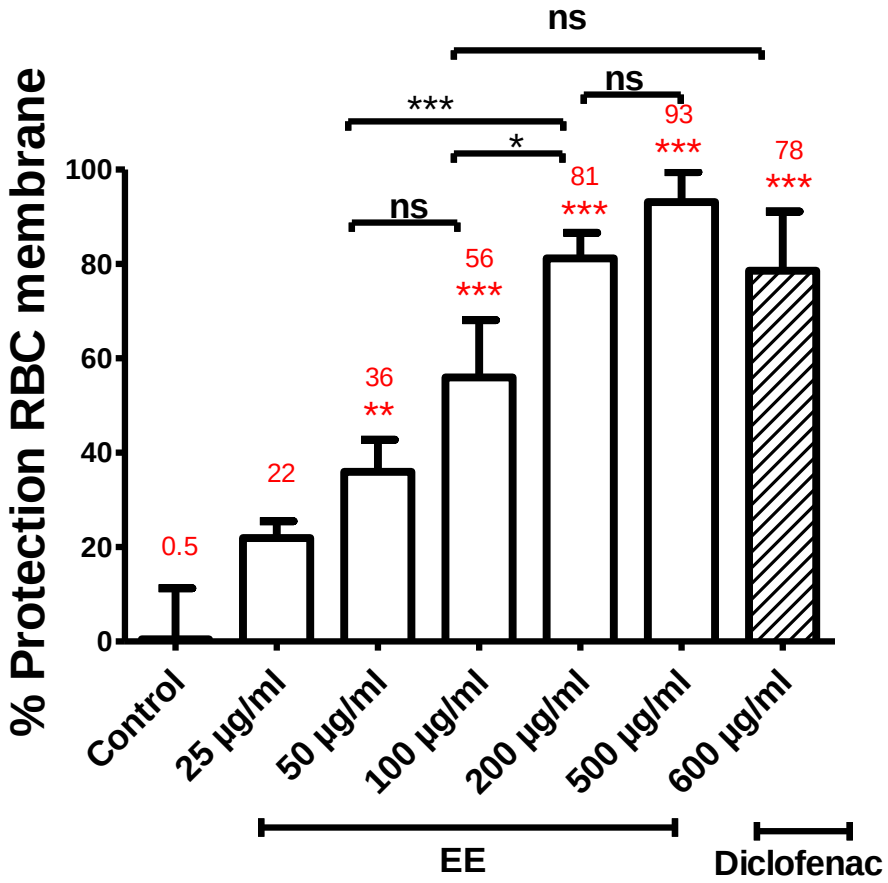


Figure 3. Effect of the ethanol extract from *C. cyrtophyllum* leaves (EE) on the hemolysis of red blood cells induced by heat. Each value is expressed as mean ± SD (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

Table 2. IC50 of EE on hemolysis of red blood cells induced by heat.

	EE	**Diclofenac**
IC50 (µg/ml)	75	294
Std. Error	1.05	1.10
95% Confidence Intervals	62.8 - 90.4	213.4 - 404.6
R^2	0.9919	0.9596
Number of points analyzed	5	5

3.3.2. Effect of the ethanol extract from leaves of *C. cyrtophyllum* (EE) on cell viability and NO production of LPS-induced RAW 264.7 macrophages

As shown in Figure 4A, RAW 264.7 cells treated with LPS alone dramatically increased NO release as compared with the non-treated cells, while indomethacin inhibited this increase. EE

inhibited NO production in a dose-dependent manner. No significant differences were observed between EE 12.5 and 25 µg/ml, between EE 50 and 100 µg/ml in inhibition of NO production. However, EE 50 µg/ml was more potent than EE 12.5 and 25 µg/ml and EE 200 µg/ml was more potent than EE 50 and 100 µg/ml at reducing NO production. The IC50 value of EE on NO release was found to be 48 µg/ml, compared with indomethacin 47 µg/ml (Table 3).

The effect of EE on the viability of activated RAW 264.7 cells was studied using MTT assay. No toxic effects were observed in presence of EE at concentrations up to 200 µg/ml after 24h LPS treatment. Interestingly, it seems that the extract protects from LPS-induced cell death. (Figure 4B).

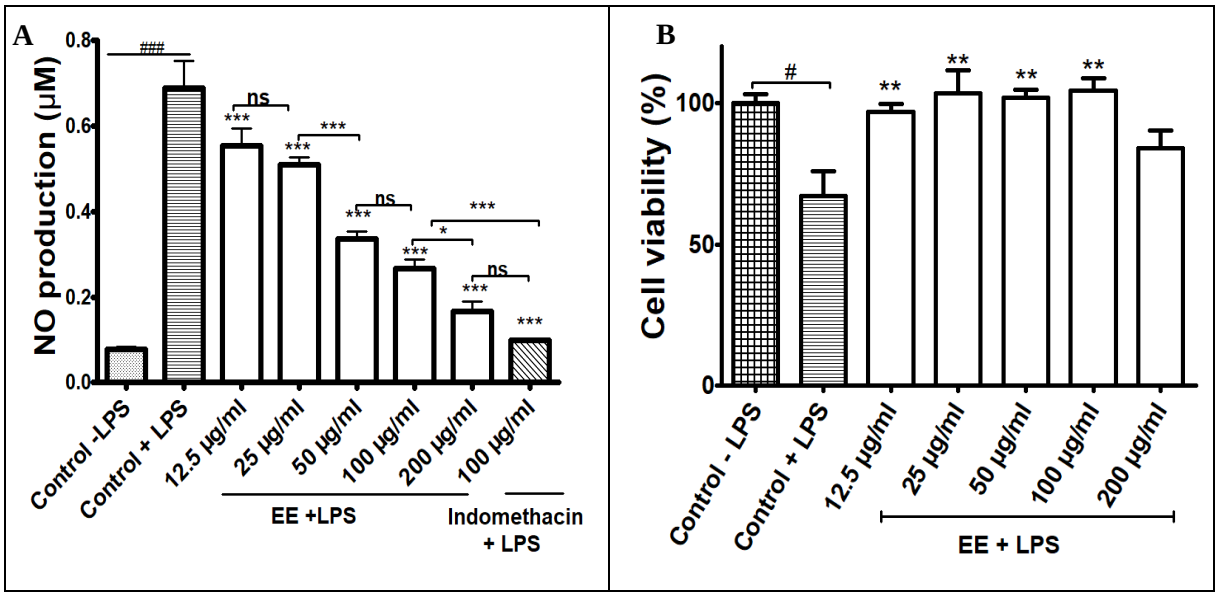


Figure 4. A. Effect of ethanol extract from leaves of *C. cyrtophyllum* (EE) on NO production B. Effect of ethanol extract from leaves of *C. cyrtophyllum* (EE) on cell viability *of RAW 264.7* macrophages. The data are presented as mean ± SD for three different experiments performed in triplicate, $^{###}$ $p < 0.001$ compared to control-LPS, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control + LPS.

Table 3. IC50 of EE on NO production.

	EE	**Indomethacin**
IC50 (µg/ml)	48	47
Std. Error	1.1	1.2
95% Confidence intervals	36.4 - 64.4	28.3 - 79.2
R^2	0.9787	0.9298
Number of points analyzed	5	5

3.3.3. Effect of EE on the production of TNF-α in LPS induced RAW 264.7 Cells

To further investigate the anti-inflammatory effect of EE on LPS-stimulated macrophages, the production of pro-inflammatory cytokines was evaluated by ELISA. As shown in Figure 5, the cells treated with LPS alone showed a marked increase in TNF-α compared to untreated control; this stimulation was significantly inhibited by treatment with EE ($p < 0.01$) at concentrations of 50, 100, and 200 µg/ml. EE 100 µg/ml was more potent than EE 50 µg/ml. No significant differences were observed between EE 100 and 200 µg/ml in inhibition of TNF-α production. No differences were found in TNF-α production at the lower EE concentrations of 12.5 and 25 µg/ml compare to control group (the cells treated with LPS alone). These data suggest that EE has the ability to inhibit LPS-induced TNF-α production in mouse macrophages.

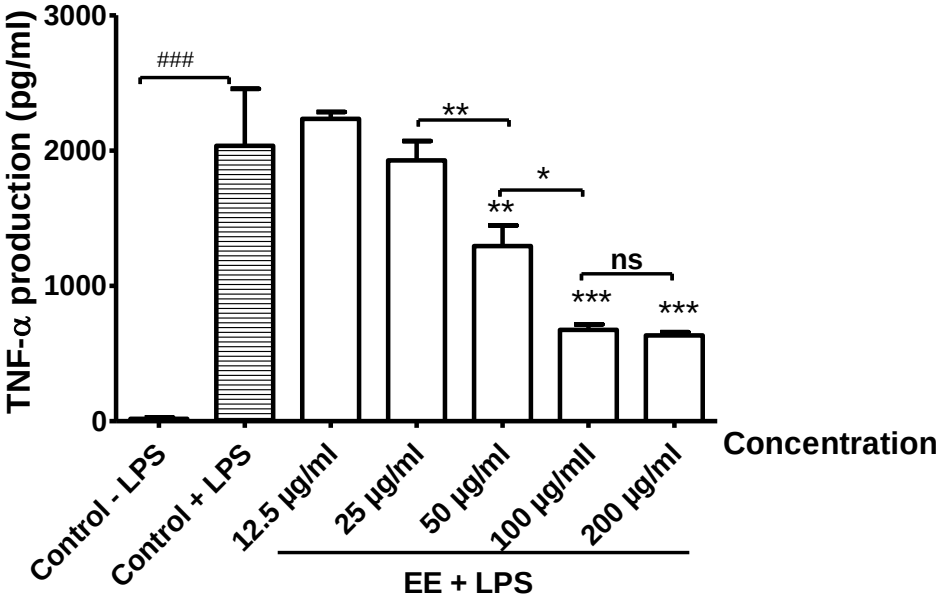


Figure 5. Effects of EE on TNF-α in LPS-induced RAW264.7 cells.

The data are presented as mean ± SD for three different experiments performed in triplicate,* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control + LPS.

3.4 *In vivo* experiment

3.4.1 The toxicity screening test

Effect of EE on survival rate of zebrafish larvae

To determine the optimal stage for treatment during the *in vivo* anti-inflammatory experiments, identical doses of EE were exposed to 4 dpf larvae. Our preliminary experiment revealed that the administration of EE at 80 µg/ml induced changes in survival rate (%) of animals at 24 h

compared with control (Figure 6A). Continuous EE exposure at dose 80 µg/ml can induce higher mortality in zebrafish larvae. The time 24 h was therefore preferable. With the lower EE doses (5, 20, 40 µg/ml) all the animals were physically active and no deaths were observed.

Effect of EE on expression of the immune genes in normal larvae

In order to determine the optimal dose for treatment, we continued to evaluate the effects of EE at doses 5, 20, 40 and 80 µg/ml on immune gene expression. Results are shown in Figure 6 B, C.

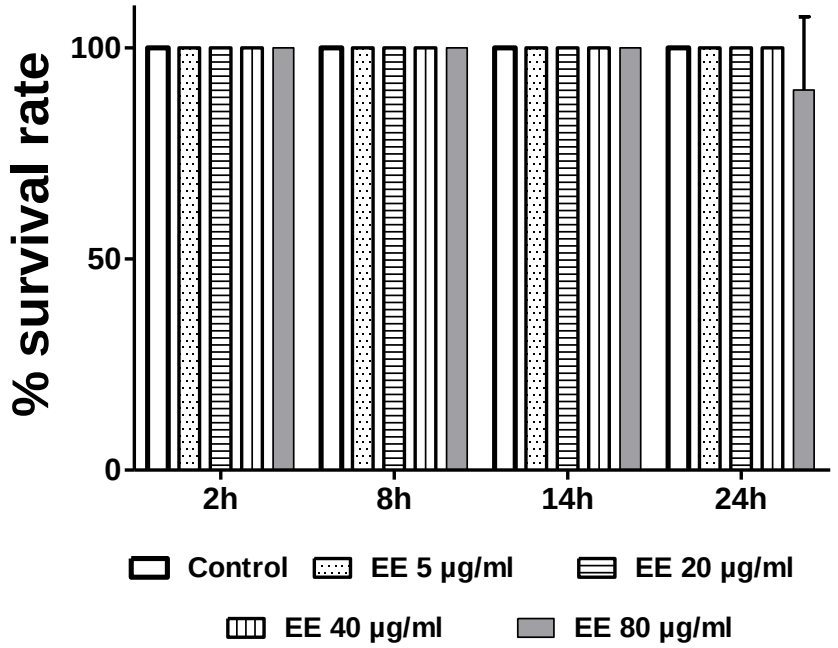
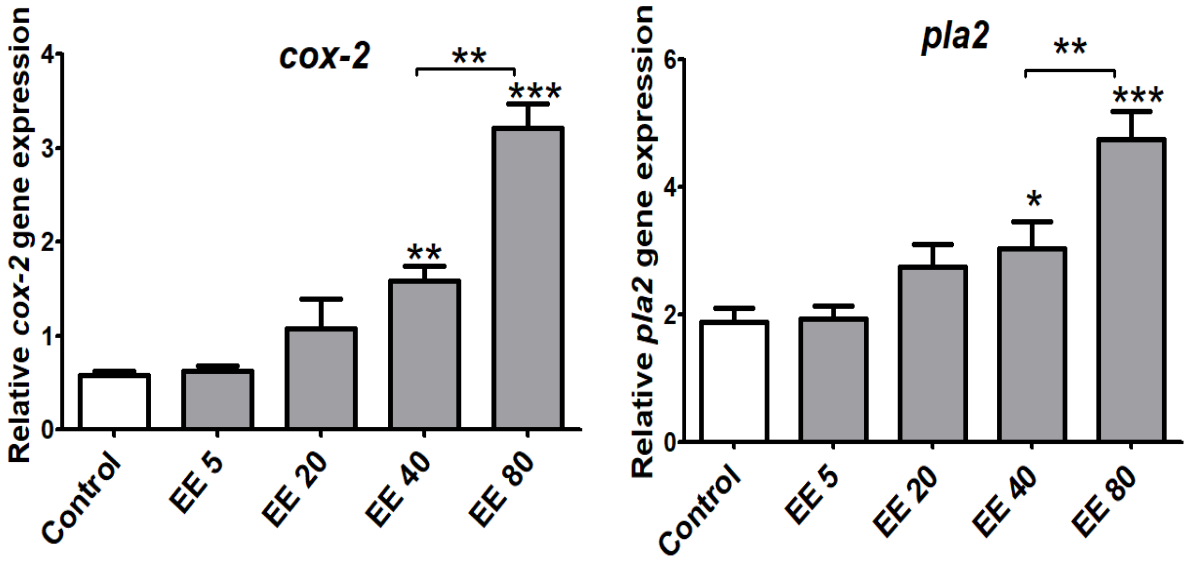


Figure 6A. Effect of EE on survival rate of zebrafish larvae



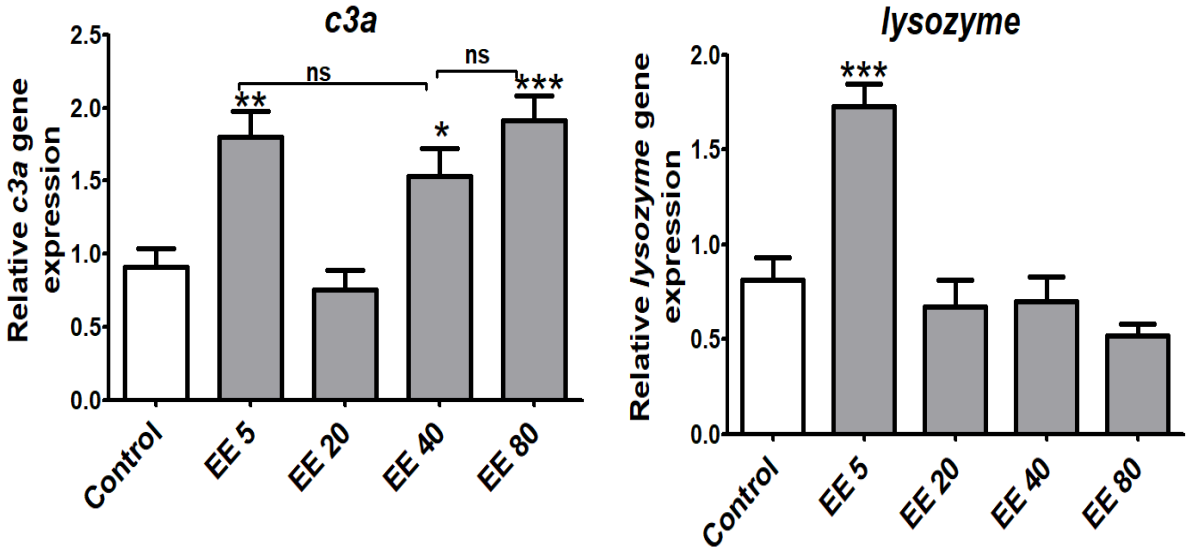
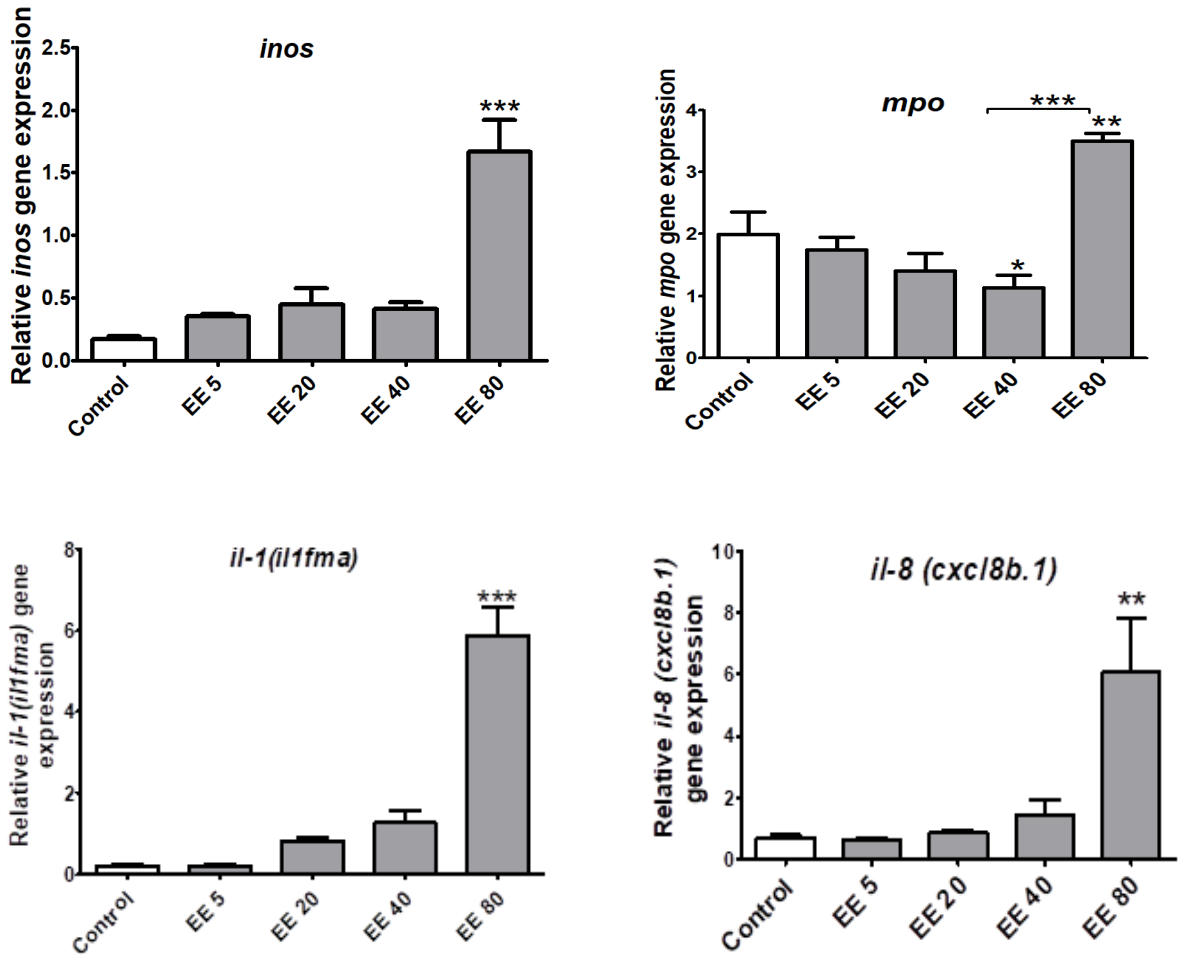


Figure 6B. Transcription of genes related to eicosanoid pathway (*pla2, cox-2*), *c3a, lysozyme* in zebrafish after exposure to various concentrations of EE for 24 h.



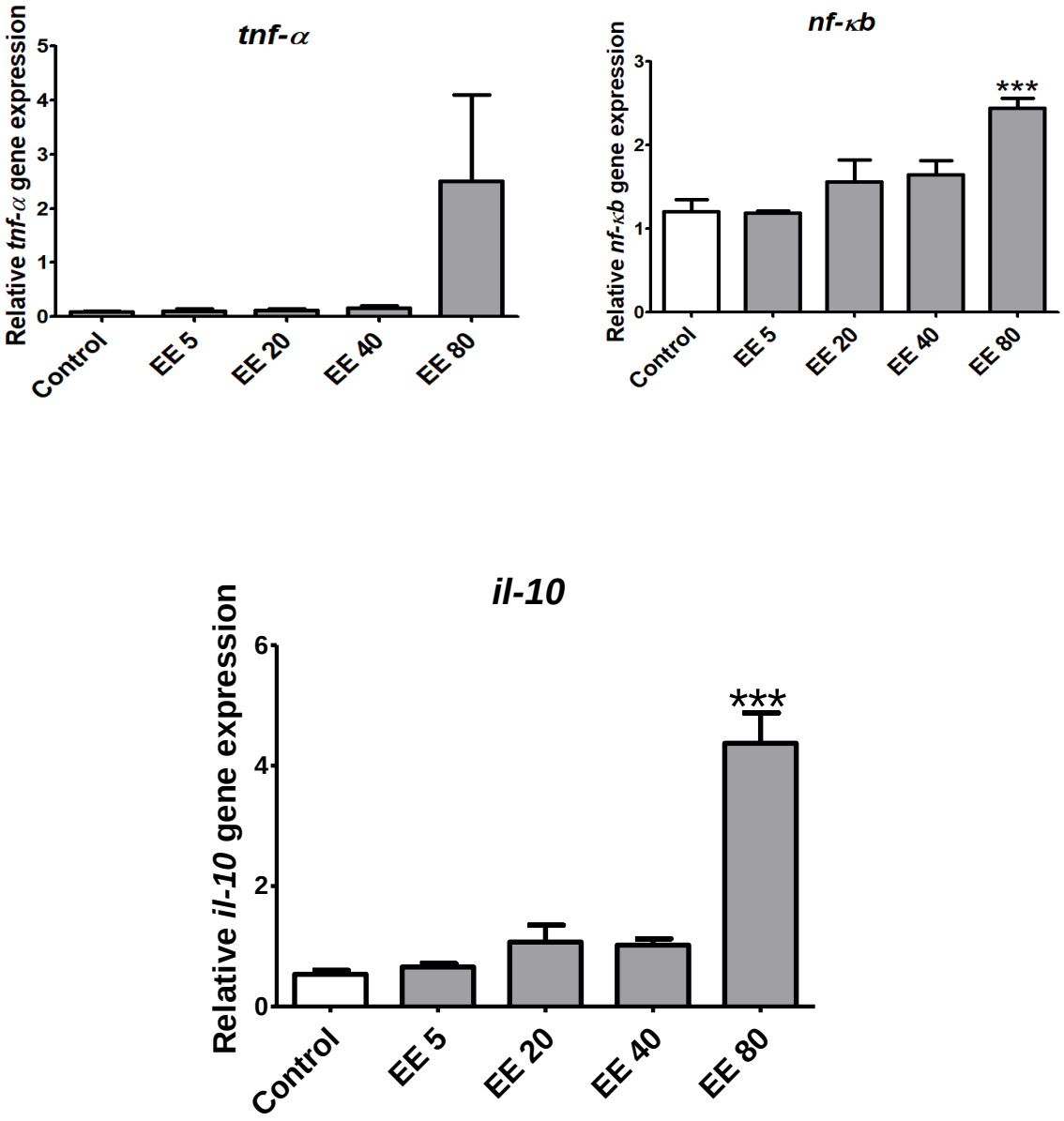


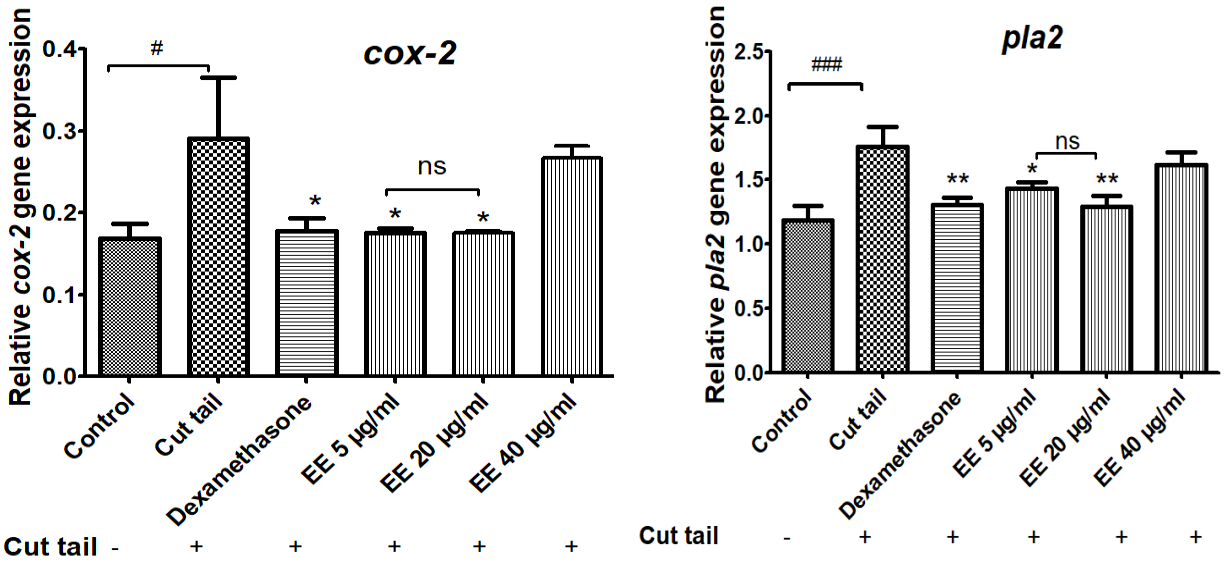
Figure 6C. Transcription of genes related to cytokines, *inos, mpo* in zebrafish after exposure to various concentrations of EE for 24 h. Data shown are mean values ± SEM of 3 independent wells, 20 larvae/well, * $p < 0.05$; ** $p < 0.01$;*** $p < 0.001$ compared to control group.

EE at high dose (80 µg/ml) had immunotoxicity through up-regulation in almost all immune genes tested when compared with the control group ($p < 0.05$). At a lower dose of 40 µg/ml, EE increased the expression of *cox-2, c3a, pla2.* However, no significant differences in immune gene expression were observed at the doses of 5 and 20 µg/ml. These doses of 5, 20 and 40 µg/ml were considered no or less immunotoxic and were used for further experiments.

3.4.2 Effect of the ethanol extract from leaves of *C. cyrtophyllum* on the expression of immune genes in cut tail zebrafish larvae

The EE inhibited the transcription of cox-2, c3a, pla2 in cut tail zebrafish larvae

To further investigate the mechanism of action, we determined the effects of EE on the expression of two genes involved in the eicosanoid pathway (*cox-2, pla2*), and of the complement gene *c3a* and the *lysozyme* gene. As shown in Figure 7, cut tail increased the expression of *cox-2, c3a, pla2* genes significantly when compared with the control group (no cut tail). Treatment with dexamethasone reduced the inflammatory reaction, as revealed by down-regulating expressions of *cox-2* and *pla2* ($p<0.05$). Similarly to dexamethasone, EE at the dose of 20 µg/ml and 5 µg/ml prominently suppressed the cut tail-induced mRNA levels of *cox-2, pla2 and c3a*. In general, these results are consistent with the anti-inflammatory activity of the extract. At the highest dose of 40 µg/ml, EE only inhibited expression of *c3a* gene (Figure 7 C). No differences were observed on *lysozyme* expression in all treatment groups when compared with the cut tail and no treatment group (Figure 7D).



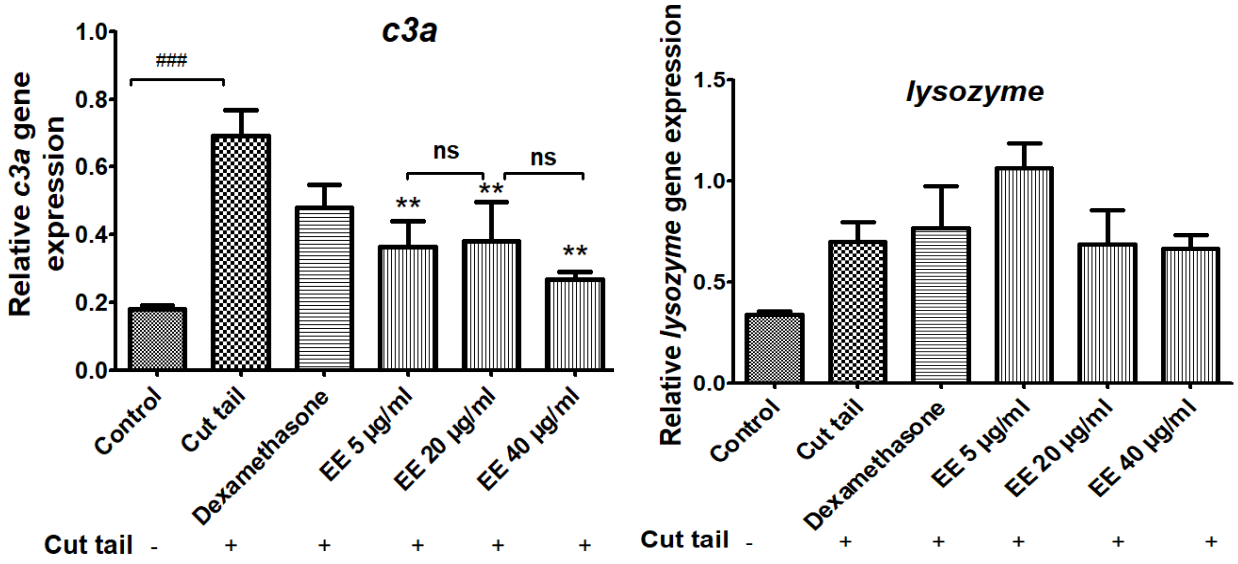


Figure 7. Effect of ethanol extract from leaves of *C. cyrtophyllum* (EE) on the expression of genes in eicosanoid pathway (pla2, cox-2) and c3a, lysozyme.

Data shown are mean values ± SEM of 3 independent wells, 20 larvae/well. $^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$ compared to control group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to cut tail group.

EE inhibited the transcription of tail cut-induced pro-inflammatory cytokines in zebrafish larvae

In response to cut tail, macrophages in zebrafish larvae could release pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6 and IL-8. The aberrant release of these cytokines contributes to the pathogenesis of many chronic inflammatory diseases such as rheumatoid arthritis (RA) and inflammatory bowel diseases. We thus examined whether the expression of these genes is increased by the tail cut, and subsequently whether EE inhibits tail cut-induced expression of *tnf-α, il-1(il1fma*), and *il-8* (*cxcl8b.1*) in the concentration range of 5, 20 and 40 µg/ml. Real-time RT-PCR analysis revealed that tail cut activated inflammation by increasing the expression of *il-1(il1fma*), *il-8* (*cxcl8b.1*) and *tnf-α* compared to the control group (no cut tail). Dexamethasone 5 µg/ml exerted its anti-inflammatory activity by reverting the increase in expression of *il-1(il1fma*) and *il-8* (*cxcl8b.1*) genes ($p < 0.05$), but not of *tnf-α*. Exposing the zebrafish larvae to EE resulted in a similar decrease in the mRNA levels of *il-8* (*cxcl8b.1*) and *il-1(il1fma*), and also in *tnf- α* at 20 µg/ml ($p < 0.05$). EE down-regulated *il-1(il1fma*) expression even at both 5 µg/ml and 40 µg/ml (Figures 8 A, B, C). These results suggest that EE in the

concentration range of 5 - 40 µg/ml inhibits the increase in expression of pro-inflammatory cytokines that are induced by cutting the tail in zebrafish larvae.

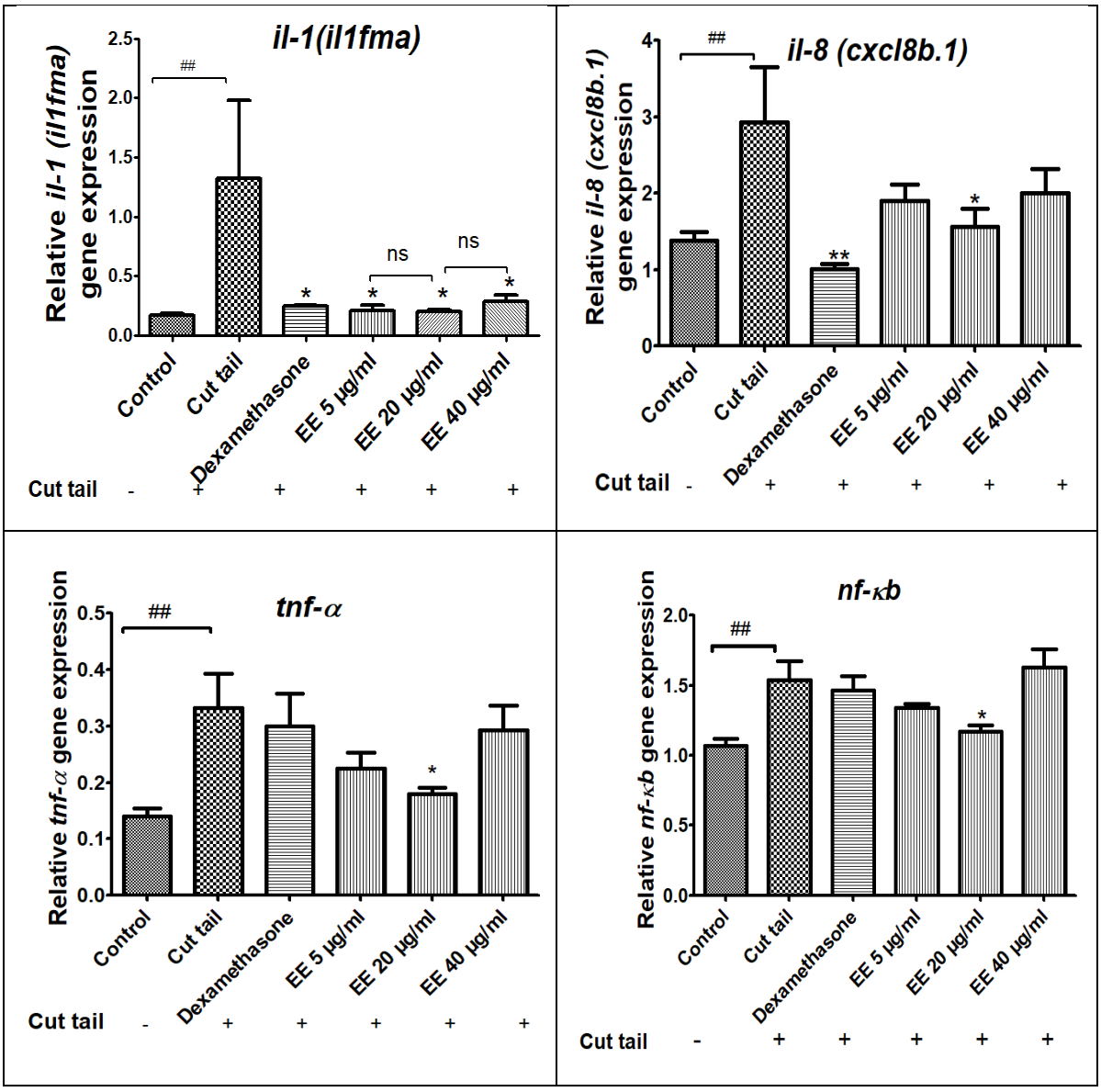


Figure 8. Relative expression of genes involved in immune responses (*il-1(il1fma)*,*il-8* (*cxcl8b.1*), *tnf-α, nf-ƙb*) of zebrafish larvae after tail cut and treatment with the ethanol extract (EE) from *C. cyrtophyllum.* Data shown are mean values ± SEM of 3 independent wells, 20 larvae/well. ## $p < 0.01$; ### $p < 0.001$ compared to control group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to cut tail group.

EE reduced cut tail-induced expression of nf-ƙb

NF-κB is a major transcription factor that modulates the expression of many pro-inflammatory cytokines (*tnf-α, il-1β, and il-6).* As shown in Fig 8D, the tail cut caused a significant increase

in *nf-ƙb* expression, which was inhibited by EE at 20 µg/ml ($p < 0.05$), suggesting that its ability to inhibit *nf-ƙb* expression may underlie its anti-inflammatory effects.

EE up-regulated expression of anti-inflammatory cytokine il-10

Interleukin 10 (IL-10) is a cytokine with potent anti-inflammatory properties that plays a central role in limiting the host's immune response to pathogens, thereby preventing damage to the host and maintaining normal tissue homeostasis (Iyer and Cheng, 2012). As shown in Figure 9, EE at 40 µg/ml up-regulated *il-10* expression ($p < 0.05$).

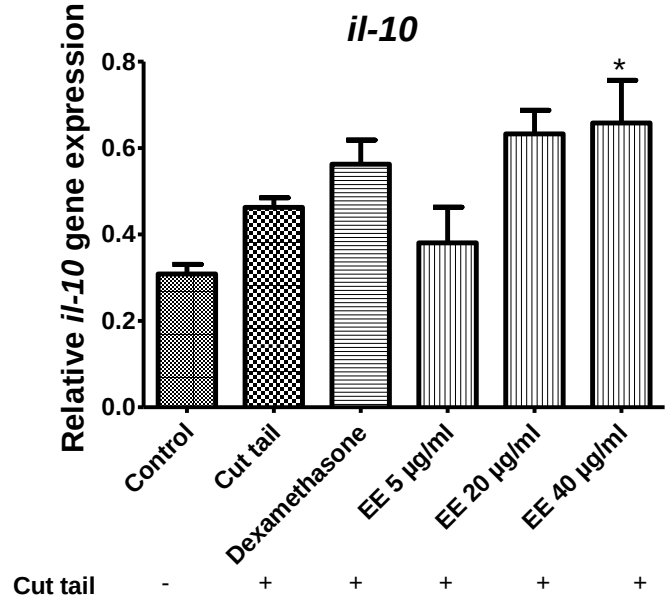


Figure 9. Effects of EE on the expression of the anti-inflammatory gene *il-10* in inflammation-induced zebrafish model.

Data shown are mean values ± SEM of 3 independent wells, 20 larvae/well. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to tail cut group.

Effect of the ethanol extract from leaves of *C. cyrtophyllum* Turcz on expression of the *vcam, inos, mpo* genes

Cutting the tail of 4dpf zebrafish larvae decreased the expression of *inos* and increased the expression of *mpo* after 24h, while the levels of *vcam* were not affected. No differences were observed on *inos, vcam* expression in all treatment groups when compared with the cut tail group (Figure 10).

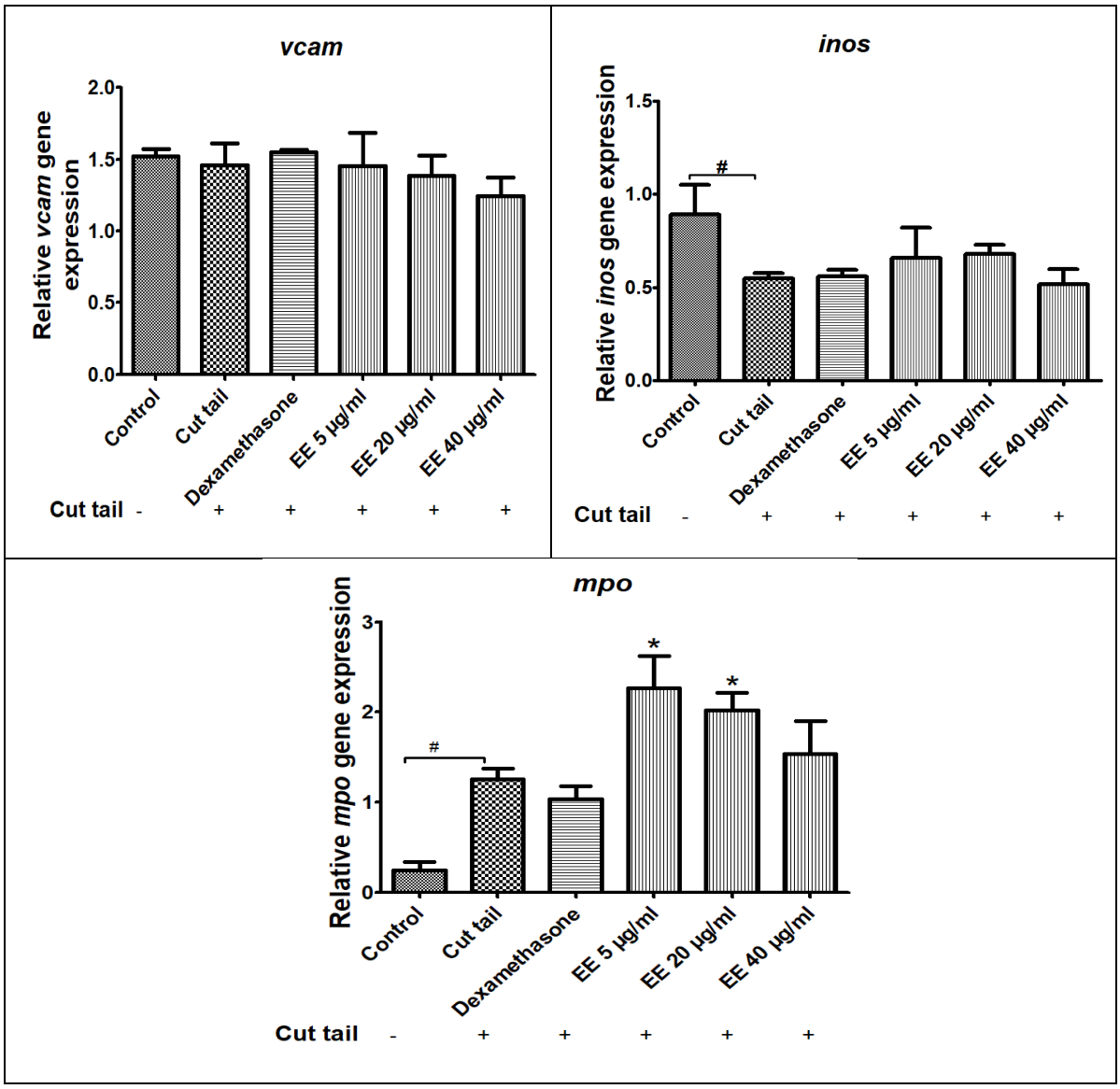


Figure 10. Effect of the ethanol extract (EE) from leaves of *C. cyrtophyllum* Turcz on the expression of the vcam, inos, mpo genes

Data shown are mean values ± SEM of 3 independent wells, 20 larvae/well. $^{\#}$ $p < 0.05$ compared to control group; * $p < 0.05$ compared to cut tail group.

4. Discussion

4.1 Total phenolic and flavonoid contents

Phenolic compounds play an important role in a variety of medicinal applications. The ethanolic extract of the *C. cyrtophyllum* leaves analysed in this study presented a concentration of total phenolic compounds of 23.3 ± 1.5 GAE mg/g expressed in dry weight of leave material. This concentration is higher than the total phenolic content reported by Li et al (2008) (11.9 ± 0.3 GAE mg/g DW) for methanolic extract of *C. cyrtophyllum* obtained by maceration at 40°C and

by Zhou et al (2013) (16.8 ± 0.2 GAE mg/g DW) for an ethanolic ultrasonic-assisted extraction at 63.3 °C of *C. cyrtophyllum* leaves. Flavonoids content was evaluated at 2.97 ± 0.01 QE mg/g expressed in dry weight of leaves. This is higher than the flavonoids content reported by Liu et al (2011) (0.50 ± 0.47 QE mg/g DW) for methanolic extract of *C. cyrtophyllum*.

4.2. Influence of the ethanol extract *from C. cyrtophyllum* Turcz on inhibition of hemolysis

The erythrocyte membrane is similar to the lysosomal membrane (Kumar et al., 2011). The stabilization of the lysosomal membrane prevents the release of lysosomal constituents and has an important role in limiting the inflammatory response (Parvin et al., 2015). In this study, the ethanol extract from *C. cyrtophyllum* leaves exhibited erythrocyte membrane stabilization by inhibiting heat-induced hemolysis. This indicates that the ethanol extract of *C. cyrtophyllum* leaves possesses biological membrane stabilization properties, preventing or delaying stress-induced destruction of the plasma membrane.

4.3. Influence of the ethanol extract from *C. cyrtophyllum* on NO production

Macrophages are an important component in the immune defense mechanism. During the progress of inflammation, macrophages actively participate in the inflammatory responses by releasing proinflammatory cytokines and mediators such as free radicals and NO as well as cytokines (Bak et al., 2013). An overproduction of NO has been implicated in the pathogenesis of septic shock, inflammation, and carcinogenesis. Thus, agents that decrease NO production in macrophages may have a potential therapeutic effect on the treatment of inflammatory and infectious diseases. Our results demonstrate that the ethanol extract from *C. cyrtophyllum* leaves strongly inhibited LPS-induced NO production without notable cytotoxicity in RAW264.7 macrophages. This result is in line with a previously paper by Liu et al. (2011) in which a *C. cyrtophyllum* methanolic extract significantly inhibited NO production through down-regulation of iNOS expression in LPS-stimulated RAW264.7 cells. In contrast to the induction of iNOS expression in LPS-stimulated RAW264.7 macrophages, the zebrafish larvae model showed that tail cut down-regulated the *inos* gene expression, while EE had no effect on *inos* expression. The difference most probably results from differences in complexity between cell cultures and whole organisms. In vitro models reflect pathway and gene expression modulations resulting from direct action of compounds on the cells, while more complex interactions between different systems take place in in vivo systems. This is a disadvantage of the in vitro tests and it remains one of the bottlenecks in drug development (Otava et al., 2015).

Depending on the concentration of NO released into the tissue microenvironment and the type and stage of inflammation, NO seems to exhibit either anti- or pro-inflammatory effects (Hyun et al., 2004). It has been well documented that excessive production of NO via iNOS is involved in the pathogenesis of several inflammatory disorders. Studies with iNOS-deficient mice however indicate that the production of NO might be needed as a protective factor in various models of acute or chronic inflammation (Kenyon et al., 2002). In a model of contact hypersensitivity, an inhibition of NO synthesis caused the release of inflammatory mediators such as histamine and platelet-activating factor (PAF) from mast cells. Impairment of NO production resulted in a pattern of leukocyte adhesion and emigration that is characteristic of acute inflammation (Kubes et al., 1991). Further studies are raising the possibility that the use of NO donors may be a reasonable therapeutic approach to reducing mast cell-dependent inflammation. Anti-inflammatory drugs that have been modified to include a NO-releasing moiety have been recently developed and have shown enhanced anti-inflammatory activities together with reduced side-effects (Hyun et al., 2004). In line with these observations, in the tail cut zebrafish model, our results show that cutting the tail significantly inhibited *inos* expression, suggesting that inflammation is associated with decreased NO production. However, EE didn't protect larvae from NO deficiency.

4.4. Influence of the ethanol extract from *C. cyrtophyllum* on *vcam* and *mpo* expression in tail cut-induced inflammation in zebrafish larvae

VCAM was identified as a cytokine-inducible adhesion molecule present on endothelial cells and mediating their binding to leukocytes. IL-1 and TNF-α signaling regulate the response of cells through upregulating VCAM expression, with maximal activity reached after 6–12 h (Imhof and Dunon, 1995). In our study, no significant changes in *vcam* expression were observed between experimental groups, suggesting that the expression of *vcam* was unaffected by those treatments or that the timing (24 h) was not correct.

Neutrophils are the first leukocyte to migrate to the site of the infection. There, they phagocyte pathogens and subsequently kill the invading microbes (Mayadas et al., 2014). MPO, the major protein in neutrophil granules, is one of the key players in the neutrophil functions described above (Odobasic et al., 2016). Based on the principle of generating a mechanical injury to attract leukocytes to damaged zones, tail transection results in a robust recruitment of leukocytes to the zone of injury as illustrated by *in situ* myeloperoxidase (MPO) staining (Cordero-maldonado et al., 2013). Unexpectedly, exposure to EE led to an even higher *mpo* expression;

possibly due to tail cutting, EE could be directly absorbed by contact with the zone of injury. The body recognizes EE as *"foreign"* and activates phagocyting neutrophils to tail transection position. Such an exposure would lead to an even greater migratory response of neutrophils to the injury site and higher expression of *mpo* expression. Beside, the compounds contained in EE also can be directly immunogenic. These compounds can penetrate intact larval skin or absorpt through digestive tract (intestinal absorption) and express immunogenic activity. In larvae with intact skin, only the high dose of EE at 80 µg/ml expressed immunogenic activity, increased *mpo* expression (Figure 6C). At lower doses (5, 20, 40 µg/ml), EE did not show any immunogenic activity in intact larvae. However, in cut tail larvae, EE can absorpt through open injury. This increased extent of absorption of of EE. So at low doses (5, 20, 40 µg/ml), EE can express immunogenic activity and may induce expression of *mpo*.

4.5. Influence of the ethanol extract from *C. cyrtophyllum* on enzyme expression in eicosanoid pathway.

In mammalian cells, eicosanoid biosynthesis is usually initiated by the activation of phospholipase A2 and the release of arachidonic acid (AA) from membrane phospholipids. The AA is subsequently transformed by cyclooxygenase (COX) and lipoxygenase (LO) pathways to prostaglandins, thromboxane and leukotrienes, collectively termed *"eicosanoids"*. Both COX-2 and PLA2 are important enzymes in the eicosanoid pathway. Inhibiting the expression of these 2 enzymes resulted in decreased production of mediators such as prostaglandins, arachidonic acid, thromboxane and leukotrienes which is beneficial for treating pain and inflammation. The inhibition of this pathway is the major target for conventional drugs to treat pain and inflammation in clinical test (Khanapure et al., 2007). EE at the dose of 5 and 20 µg/ml prominently suppressed the tail cut -induced mRNA levels of *cox-2* and *pla2*. These results suggest that EE could be a promising option for the prevention and treatment of inflammation through inhibition of inflammatory mediators formation.

4.6. Influence of the ethanol extract from *C. cyrtophyllum* on the expression of cytokines

Proinflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-2, TNF-α, IL-6, IL-8, IFN-γ are involved in the process of pathological inflammation. Because of this, the cytokine system constitutes a very interesting target for the development of clinically relevant anti-inflammatory drugs. In this

study, EE down-regulated the expression of *tnf-α, il-8* (*cxcl8b.1*) at 20 µg/ml and *il-1(il1fma*) at 5, 20 and 40 µg/ml.

The regulation of inflammatory gene transcription is controlled by specific signaling pathways and transcription factors, such as NF-κB and AP-1. In response to stimulation, NF-κB is liberated from a complex with I-κB and induces transcription of inflammatory genes such as iNOS, COX-2, TNF-α, IL-1, and IL-6 chemokines and adhesion molecules that cause acute inflammation and a systemic inflammatory response syndrome (Liu and Malik, 2006). Therefore, blocking the NF-κB transcriptional activity can suppress the expression of iNOS, COX-2, and proinflammatory cytokines. In this study, the ethanol extract from C. cyrtophyllum inhibited the expression of *nf-ƙb* at the dose of 20 µg/ml. That explains the decrease of TNF-α and NO production in LPS induced RAW264.7 cells.

4.7 Influence of the ethanol extract from *C. cyrtophyllum* on the expression of anti-inflammatory cytokine- il-10

The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response. Major anti-inflammatory cytokines include IL-1 receptor antagonist, IL-4, IL-10, IL-11, and IL-13. Among all the anti-inflammatory cytokines, IL-10 is a cytokine with potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1 by activated macrophages. In addition, IL-10 can up-regulate endogenous anti-cytokines and down-regulate pro-inflammatory cytokine receptors. Thus, it can counter-regulate the production and function of pro-inflammatory cytokines at multiple levels (Jun-Ming Zhang and Jianxiong An, 2007). In our study, EE upregulated *il-10* expression. That promises in the treatment of diseases related to an inflammatory process.

4.8 The relation between dose and response

At low dose 5 µg/ml, EE induced an anti-inflammatory effect through the down regulation of *cox-2, pla2, c3a, il-1(il1fma*). This effect increased with increasing of doses; at the dose of 20 µg/ml, EE generally down-regulated almost tested genes. However, at a dose of 40 µg/ml, EE sometimes showed lower or no effects. A likely explanation for the lack of a dose–response curve for EE might be related to immunotoxicity. At a dose of 40 µg/ml, EE alone expressed immunotoxicity through up-regulated expression of *cox-2, pla2, c3a.* This probably explains a decreasing anti-inflammatory effect compared to the dose of 20 µg/ml.

4.9 The relation between the chemical constituent(s) of the plant and anti-inflammatory effects

The exact mechanism for the anti-inflammatory effects induced by *C. cyrtophyllum* extract and the chemical constituent(s) responsible for this effect are not yet known. The present study indicates a high level of phenols and flavonoids in EE.

Phenolic compounds play an important role in a large variety of medicinal applications. The production of ROS, such as lipid peroxides and superoxides, is reported to be accountable for cell membrane destabilization. Phenolic compounds are reported to act as antioxidant agents through inhibition of ROS formation and/or upregulation of antioxidant defenses (Kumar and Pandey, 2013). Thus, it is not unreasonable to postulate that phenolic compounds in *C .cyrtophyllum* Turcz leaf extracts could be responsible for the membrane-stabilizing effect observed in this study.

Flavonoids are one of the major groups present in *Clerodendrum* genus possessing promising biological activities (Shrivastava and Patel, 2007). "Several studies have shown that flavonoids exhibit analgesic and anti-inflammatory effects as a result of their membrane- stabilizing ability in various experimental models "(Ranasinghe et al., 2012). It has been reported that flavonoids can inhibit expression of iNOS, COX, LOX, which are responsible for the production of a great number of mediators of the inflammatory process such as NO, prostaglandin, leukotriene as well as adhesion molecules (Kumar and Pandey, 2013). It has been found that several flavonoids can decrease the expression of different pro-inflammatory cytokines/chemokines, among which TNF-α, IL-1β, IL-6, IL-8, MCP-1, in many cell types (Santangelo et al., 2007). These studies strongly support the idea that flavonoids can modulate the immune response and have potent anti-inflammatory activity.

Other phytochemicals could however be important for the investigated activities. In previous studies, the glycosidated coumaramide clerodendiod B, isolated from *C. cyrtophyllum*, has been reported to exhibit anti-inflammatory effects through the inhibition of the NF-κB pathway in NF-κB reporter Luciferase Assay (Wang et al., 2012). Iridoid glycosides described in other *Clerodendron* species (Erukainure et al., 2014, Kanchanapoom et al., 2005, Kanchanapoom et al., 2001) could also account for anti-inflammatory activities and their presence should be investigated in *C. cyrtophyllum*.

5. Conclusions

In conclusion, the ethanol extract from *C. cyrtophyllum* Turcz displayed significant *in vitro* anti-inflammatory activity through lysosome membrane stabilization and decrease of proinflammatory mediator production (TNF-α and NO) in LPS-induced macrophages. Furthermore, *in vivo* results in zebrafish confirm that the ethanol extract from *C. cyrtophyllum* inhibits the inflammation via the downregulation of inflammatory genes expression *(cox-2, pla2, c3a, il-1(il1fma*), *il-8* (*cxcl8b.1*), *tnf-α, nf-ƙb)* and the upregulation of the anti-inflammatory gene *il-10*. These *in vivo* results contribute to understanding the molecular mechanisms of this action. Taken together, these findings provide a pharmacological validation to the traditional use of *C. cyrtophyllum* Turcz leaves in the treatment of inflammatory disorders.

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Foreword: Inflammation can be induced by many reagents: physical factors, infections, chemicals, ... In the previous chapter, the ethanol extract from *Clerodendrum cyrtophyllum* leaves (EE) was proved to display anti-inflammatory activity in cut tail zebrafish model. The copper sulfate-induced inflammation zebrafish model has been used and presents several advantages in comparison to other methods. The copper induced inflammation responses mainly through the oxidative stress reaction in zebrafish model. This chapter aimed to study anti-oxidant and anti-inflammatory effects of the ethanol extract from *Clerodendrum cyrtophyllum* leaves in this model to further understand its bioactivity.

Chapter 4

Anti – inflammatory and antioxidant properties of the ethanol extract from *Clerodendrum cyrtophyllum* Turz in copper sulfate induced inflammation in zebrafish

antioxidants

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(This article belongs to the Special Issue **Antioxidants in the Prevention and Treatment of Chronic Diseases**)

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Abstract: Oxidative stress and inflammation are commonly present in many chronic diseases. These responses are closely related to pathophysiological processes. The inflammatory process can induce oxidative stress and *vice-versa* through the activation of multiple pathways. Therefore, agents with antioxidant and/or anti-inflammatory activities are very useful in the treatment of many pathologies. *Clerodendrum cyrthophyllum* Turcz, a plant belonging to the Verbenaceae family, is used in Vietnamese traditional medicine for treating migraine, hypertension, inflammation of the throat, and rheumatic arthritis. Despite its usefulness,

studies on its biological properties are still scarce. In this study, ethanol extract (EE) of leaves of *C. cyrtophyllum* showed protective activity against $CuSO_4$ toxicity. The protective activity was proven to relate to antioxidant and anti-inflammatory properties. EE exhibited relatively high antioxidant activity (IC_{50} of 16.45 µg/ml) as measured by DPPH assay. In an *in vivo* anti-antioxidant test, 3 day-post fertilisation (dpf) zebrafish larvae were treated with different concentrations of EE for 1 h and then exposed to 10 µM $CuSO_4$ for 20 min to induce oxidative stress. Fluorescent probes were used to detect and quantify oxidative stress by measuring the fluorescent intensity (FI) in larvae. FI significantly decreased in the presence of EE at 5 and 20µg/ml, demonstrating EE's profound antioxidant effects, reducing or preventing oxidative stress from $CuSO_4$. Moreover, the co-administration of EE also protected zebrafish larvae against oxidative damage from $CuSO_4$ through down-regulation of *hsp70* and *gadd45bb* expression and upregulation of *sod*. Due to copper accumulation in zebrafish tissues, the damage and oxidative stress were exacerbated overtime, resulting in the upregulation of genes related to inflammatory processes such as *cox-2*, *pla2*, *c3a*, *mpo*, pro- and anti- inflammatory cytokines (*il-1ß*, *il-8*, *tnf-α*, and *il-10*, respectively). However, the association of $CuSO_4$ with EE significantly decreased the expression of *cox-2*, *pla2*, *c3a*, *mpo*, *il-8*, and *il-1ß*. Taken together, the results suggest that EE has potent antioxidant and anti-inflammatory activities and may be useful in the treatment of various inflammatory diseases.

Keywords: Anti-inflammation, oxidative stress, antioxidant, $CuSO_4$, zebrafish larvae, *Clerodendrum cyrtophyllum*

1. Introduction

Oxidative stress and inflammation contribute to the pathogenesis of many chronic diseases such as diabetes, cardiovascular diseases, neurodegenerative diseases, cancer, and aging [1]. They are tightly linked with one another. If inflammation is the primary event, the activated macrophages and neutrophils liberate large amounts of reactive species at the site of inflammation [2]. As a consequence, oxidative stress will develop and further increase inflammation. Conversely, if oxidative stress appears, the overproduced reactive oxygen/nitrogen species can induce inflammation through upregulation of pro-inflammatory gene expression [1,3]. Inflammation will eventually further accentuate oxidative stress. In clinical practice, identification and treatment of the primary abnormality are very important. However, the relationship between inflammation and oxidative stress is interdependent; treating

only the primary abnormality may not always be successful [1]. Therefore, finding agents that inhibit both ROS production and inflammatory mediators has been considered a promising strategy for preventing or treating diseases associated with chronic inflammatory conditions.

Zebrafish (*Danio rerio*) have been widely used in developmental biology and drug discovery. Several characteristics make zebrafish a convincing tool in drug discovery, such as low maintenance costs, rapid embryogenesis, transparency, high similarity between the human and zebrafish genomes, etc [4]. More specifically related to this research, oxidative and inflammatory responses can be robustly and easily induced and visualised in zebrafish, particularly in the early stages [5]. Copper is an essential micronutrient but misregulation of intracellular levels can become toxic to many cell types. Under inflammatory conditions, serum copper levels increase and trigger oxidative stress responses that activate inflammatory responses [5]. The zebrafish copper-induced inflammation model has been used previously and presents several advantages as it is a non-invasive and sterile method, in comparison to methods involving physical damage and the use of infectious agents, besides its gentle manipulation of the larvae [6]. In this study, we used $CuSO_4$ as an agent to induce an inflammatory response through the oxidative stress reaction to study the antioxidant and anti-inflammatory effects of ethanol extracts of *Clerodendrum cyrthophyllum* Turcz.

C. cyrthophyllum, a plant belonging to the Verbenaceae family, is widely distributed in tropical countries such as Vietnam, China, India, Japan, Korea, and Thailand [7]. In Vietnam it is used for treating migraine, hypertension, inflammation of the throat, and rheumatic arthritis. In several studies, phenolic acids and flavonoids have been isolated from various plant parts [8]. Total phenols and flavonoids displayed good antioxidant activity and potential anti-inflammatory activity in several experimental models [9]. Investigations on this plant suggest that it has potential as an alternative treatment for inflammatory diseases. However, further investigation is needed to identify the exact mechanism of action and pathways that are modulated by this plant. In this study we investigated the antioxidant and anti-inflammatory effects of the ethanol extract of *C. cyrthophyllum* using the zebrafish copper-induced inflammation model to elucidate the molecular mechanisms to explain its effects, and provide scientific evidence for using it in traditional medicine.

2. Materials and Methods

2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), quercetin, 2′,7′-dichlorodihydrofluorescein diacetate

(H2DCFDA), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), pyrocatechol violet (PV) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of the highest commercially available grade.

2.2. Preparation of the ethanol extract of *Clerodendrum cyrtophyllum* Turcz (EE)

Plant collection: Leaves of *Clerodendrum cyrtophyllum* Turcz Verbenaceae were collected from northern provinces of Vietnam in June, 2018, by Dr Nguyen Kim Thanh from Vietnam National University **(VNU-BIOL).** A voucher specimen (HNU 024106) was deposited at the Botanical Museum of Hanoi, University of Science. Leaves were cleaned and dried for 72 h to a constant weight using a hot air oven at 40°C before extraction. Dried leaves were then ground in a blender to fine powder (size smaller than 0.5 mm).

Preparation of total extract: Given that polar (aqueous) extracts are used in traditional practice, we selected ethanol to extract polar secondary metabolites, minimising the extraction of mineral salts and polysaccharides. For this, 100 g of dried leaf powder was extracted with 1000 ml 95% ethanol at 60°C in a water bath with 360 rpm agitation for 4 h. The extract was then filtered and collected. The residue was extracted another two times using the same procedure. The collected ethanol extracts were then combined, concentrated on a rotary evaporator (40°C) under reduced pressure and lyophilised to obtain the crude extract. The yield of extract was 11.3% relative to the dried leaf powder. The crude extract was stored at –20°C until use. After the extraction, the EE was analysed with various analytical techniques including colorometric methods, HPTLC (high performance thin layer chromatography) and mass spectrometry analyses. The extract presents a concentration of total phenolic compounds of 23.3 ± 1.5 GAE mg/g and flavonoid of 2.97 ± 0.01 QE mg/g expressed in dry weight of leaves material.

2.3 Fish and experimental conditions

The adult wild–type AB zebrafish (*Danio rerio*) were maintained in a recirculating ZebTec housing system (Techniplast) at 28°C with a 12:12 h (light/dark) photoperiod. Conductivity was maintained at approximately 500µS/cm, pH 7.2. Fish were fed *ad libitum* three times daily. The day before mating, males and females (2:2) were placed in spawning tanks. Naturally spawned embryos were obtained within 30 min after the lights were switched on in the morning. The embryos were selected visually using a binocular microscope and only fertilised and normally developed eggs were selected. Selected eggs were placed in embryo medium at 28°C. Embryo medium was renewed every 24 h. Hatched larvae at 3 days post fertilization (dpf) were

used for subsequent experiments. Since zebrafish larvae below 120 h old are not considered animals [10] no animal test authorisation is required according to European legislation (EU Directive, 2010/63/EU). All experiments were terminated at 120 h post fertilization (hpf). The term "larvae" refers to hatched embryos up to 120 hpf that are using up the yolk-sac reserves and do not yet feed externally (also called yolk-sac larva, early larva, pre-larva, or eleuthero embryos) [10].

2.4. Experimental design

Preparation of test samples

The dry ethanol extract of *Clerodendrum cyrtophyllum* Turcz ***(EE)*** was dissolved in DMSO to make a stock solution of 50 mg/mL. The stock solution was diluted to different concentrations using fresh larvae medium (dechlorinated water with conductivity approximately 500µS/cm, pH 7.2) so that the final concentration in each experimental well was 5, 20, and 40 µg/mL.

The experiments

2.4.1. Protective effect of EE against $CuSO_4$ toxicity in zebrafish larvae

Mortality was used as an initial endpoint to characterise and evaluate the protective effect of the EE against $CuSO_4$ toxicity in zebrafish larvae. Zebrafish larvae 3 dpf at a density of twenty larvae per well were incubated with EE at different doses (5, 20, 40 µg/mL) for 1 h then stimulated with a lethal dose (20 µM) of $CuSO_4$ for 24 h. All treatments were performed in 6-well culture plates. The parameter assessed was mortality rate at 24 h.

2.4.2. Anti-oxidant effect of EE

In vitro antioxidant tests:

Measurement of DPPH radical scavenging capacity

The ability of the prepared extract to scavenge $DPPH^{\bullet}$ radicals was determined by the method described by Cheng-zhong Wang et al. (2013) [11] with slight modifications. Briefly, a 20 µL solution of EE in different concentrations (5, 10, 25, 50, 100, 250, 500 µg/mL) was added to 180 µL methanol solution of $DPPH^{\bullet}$ (0.1 mM). Quercetin (2, 4, 8, 20, 40, 80, 200 µg/mL) was used as a positive control and methanol was used as a blank. All the reaction mixtures were shaken and incubated in the dark for 30 min at room temperature. The absorbance was then measured at 517 nm using a UV-Vis spectrophotometer. All samples were analysed in

triplicate. The DPPH scavenging ability of the plant extracts was calculated using the following equation:

%Scavenging Activity = (Abs control –Abs sample) x 100/ (Abs control)

Where Abs control is the absorbance of the blank, and Abs sample is the absorbance of the sample.

The antioxidant activity was reported in terms of IC_{50} (concentration of extract necessary to decrease the initial concentration of DPPH by 50%).

Cu^{2+} chelation ability

The ability of EE to chelate Cu^{2+} was assessed using the method described by Santos et al. (2017) that employs pyrocatechol violet (PV) as the chromogen agent [12]. Briefly, in each well, 30 µL solution of EE in different concentrations (5, 10, 25, 50, 100, 250, 500 µg/mL) or water (control) were mixed with 200 µL of sodium acetate buffer (50 mmol/L, pH 6.0). Then, 30 µL of a 100 mg/L $CuSO_4.5H_2O$ solution were added in each well and let react for 2 min. After 2 min, 8.5 µL of solution pyrocatechol violet 2mmol/L were added to initiate the reaction. All the reaction mixtures were shaken and incubated for 10 min at 25°C. The absorbance was then measured at 632 nm using a UV-Vis spectrophotometer. Cu^{2+} chelating ability of EE was calculated as:

Cu^{2+} chelating ability of EE (%) = [(Abs control - Abs sample)/Abs control] ×100.

In vivo antioxidant test

Generation of reactive oxygen species was determined using the membrane-permeable fluorescent dye 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCFDA). H2DCFDA is hydrolysed inside cells to the non-fluorescent compound 2′,7′-dichlorodihydrofluorescein, which emits fluorescence when oxidised to 2′,7′-dichlorofluorescein (DCF). Thus the fluorescence emitted by DCF directly reflects the overall oxidative status of a cell [13,14]. Zebrafish larvae (3 dpf) were transferred into 12-well plates containing 940 µL embryo media, 6 larvae per well. Groups of 6 hatched larvae were treated with EE at different doses of 5, 20, and 40µg/mL or 100 mM quercetin for 1 h and then exposed to 10 µM $CuSO_4$ for 20 min to induce oxidative stress. After 20 min of exposure, larvae were washed twice with fresh larvae medium and incubated with H2DCFDA solution (20µg/ml) for 1 h in the dark at 28 ± 1°C. After incubation, zebrafish larvae were washed 3 times in fresh larvae medium, anesthetised with 0.0003% MS-222 (tricaine methane-sulfonate) and transferred to flat 24-well plates (one

larva per well) before observation and photography under a fluorescence microscope. Each larva was placed on the lateral side with the head preferably on the left side with respect to the observer. Fluorescence intensity of an individual larva was quantified using Autovision software and a BD pathway 855 system (BD Biosciences). Larvae exposed to $CuSO_4$ but not treated with EE were used as controls.

The stock solutions of H2DCFDA were prepared in DMSO. The solutions were protected from light and stored at –20°C until used for the analysis. Working solutions were prepared on the day of the experiment.

Quantitative Real–Time PCR

Three dpf zebrafish larvae were transferred into 12-well plates containing 940 µl embryo media, 20 larvae per well. A group of 20 hatched larvae were first treated with EE at different doses of 5, 20, and 40 µg/mL for 1 h and then exposed to 10 µM $CuSO_4$ (a concentration capable of inducing oxidative stress and inflammation) for 4 h. After 4 h, larvae were collected and stored at –80ºC for qPCR analysis to evaluate the effects of EE on the expression of genes related to oxidative stress, such as *sod*, *gpx4*, *hsp70,* and *gadd45bb.*

Total RNA extraction, DNase treatment and reverse transcription

Total RNA was extracted from these larvae using Trizol Reagent solution (Ambion, Thermo Fisher Scientific) following the manufacturer's instructions. The pellet containing RNA was dried and resuspended in 100 µl of RNase-free water. The concentration of total RNA was determined spectrophotometrically at A_{260}/A_{280} and A_{260}/A_{230} nm using a NanoDrop™ 1000 (Thermo Scientific). RNA integrity was checked by denaturating gel electrophoresis (1.2% agarose). The extracted RNA samples were then subjected to DNase treatment (DNA-*free*™ DNA Removal Kit, Invitrogen) to avoid DNA contamination. One µg of total RNA was reverse-transcribed into double-stranded cDNA using the RevertAid RT kit (Thermo Scientific) according to the manufacturer's instructions.

The resulting cDNA was used to measure the expression of 5 oxidant genes in a real-time quantitative polymerase chain reaction (qPCR). Two housekeeping genes (ß-actin and elongation factor 1α (*efl1-α*)) were used as references. The expression of genes encoding *sod*, *cat*, *gpx4*, *hsp70,* and *gadd45bb* was evaluated. The list of specific primers used is given in Table 1. Primers were designed on Primer3 software. Amplifix software was used to check the quality of the primers. The efficiency of each primer was confirmed by RT-PCR.

RT-PCR was performed using an ABI StepOnePlus Real-Time PCR system (Applied Biosystems). Five µL of 25-fold diluted cDNA template was mixed with 5 µL of each primer (5 µM) and 10 µL of SYBR green PCR master mix (Applied Biosystems) to a final volume of 20 µL. The standard cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. All reactions were performed as technical triplicates. For analysis, a standard curve of a pool of the cDNA of all samples was constructed following the absolute quantification method (Bio-Rad)[15]. The equation for the linear regression line

CT = m(log quantity) + b.

Where b is the y-intercept and m is the slope of the linear regression. Based on the equation for the linear regression, we can determine the quantity of an unknown sample:

Quantity $=10^{(CT-b)/m}$

The ratios of the quantity of candidate gene/average quantity of housekeeping genes were subsequently calculated for each candidate gene and used to assess the differences in expression levels between experimental groups.

2.4.3. Anti-inflammatory effect of EE

To evaluate the anti-inflammatory properties of EE a similar experiment was designed using 10 µM $CuSO_4$ to stimulate inflammation. After 4 and 24 h of $CuSO_4$ exposure, the expression of genes related to inflammatory processes were analysed in zebrafish larvae using qPCR.

The resulting cDNA was used to measure the expression of 9 immune system genes in a real-time quantitative polymerase chain reaction (qPCR). Two housekeeping genes (*ß-actin, efl1-α*) were used as the reference. The expression of genes encoding pro-inflammatory cytokines (*il-1ß*, *il-8*, *tnf-α*), neutrophil (*mpo*), complement 3a (*c3a*), phospholipase A2 (*pla2*), *cox-2*, transcription factor NF-**κ**B (*nf-ƙb*), and an anti-inflammatory response cytokine (*il-10*) were evaluated. The list of specific primers used is given in Table 1. Primers were designed on Primer3 software. Amplifix software was used to check the quality of the primers. The efficiency of each primer was confirmed by RT-PCR.

Table 1. Primer pairs used in this study

Gene name	Function	GenBank Accession No.	Forward and reverse primer sequences (5'-3')
ß-actin	Housekeeping gene	AF057040	*Fwd:* CCCCATTGAGCACGGTATTG *Rev*: ATACATGGCAGGGGTGTTGA
Elongation factor 1 alpha (*efl1-α*)	Housekeeping gene	L23807.1	*Fwd:* CCAAGGAAGTCAGCGCATAC *Rev*: CCTCCTTGCGCTCAATCTTC
Interleukin-1 *(il-1ß*)	Pro-inflammatory cytokine	NM_212844.2	*Fwd:* AAAGTGCGCTTCAGCATGTC *Rev:* GCTGGTCGTATCCGTTTGGA
Interleukin -8 *il-8* (*cxcl8b.1*)	cytokine	NM_001327985.1	*Fwd:* GCCTTCATGCTTCTGATCTGC *Rev*: AATCACCCACGTCTCGGTAGGA
Cyclooxygenase -2 (*ptgs2a* or *cox2*)	Catalyse the formation of prostaglandin, thromboxane	NM_153657.1	*Fwd:* ACAGATGCGCTACCAGTCTT *Rev*: CCCATGAGGCCTTTGAGAGA
Phospholipase A2 *pla2* (*pla2g4aa*)	Provide precursors for generation of eicosanoids	NM_131295.2	*Fwd:* TCATGTCTCCTGGGCTGTTT *Rev*: CCAGCTCCTCCTCCATAGTG
Tumor necrosis factor *(tnf-α)*	Pro-inflammatory cytokine	AB183467	*Fwd:* CACAAAGGCTGCCATTCACT *Rev*: GATTGATGGTGTGGCTCAGGT
nf-ƙb (nkap)	A ubiquitous transcription factor	NM_001003414.1	*Fwd:*GGTCGGACAGAGATCACGGATT *Rev*: TGCTGTTCTTCACGTCCTCT
Interleukin -10 (*il-10*)	Anti-inflammatory cytokine	AY887900.1	*Fwd:* AGTCATCCTTTCTGCTCTGCT *Rev*: AAAGCCCTCCACAAATGAGC
c3a (c3a.1)	Complement 3a	NM_131242.1	*Fwd: GTACGAGGCGAACAACTGGA* *Rev: CATCATACGCCGCAGCTTTC*
mpo	Myeloperoxidase	AF349034.1	Fwd: GTGGTCGTGTCGGTTCTCTT Rev: GCAGATTATGCGGGCCATTG
sod (sod1)	Superoxide dismutases	NM_131294.1	*Fwd*: ATGGTGAACAAGGCCGTTTG *Rev*: AAAGCATGGACGTGGAAACC
gpx4b	glutathione peroxidase	BC095133.1	*Fwd:*TGAGAAGGGTTTACGCATCCTG *Rev*: TGTTGTTCCCCAGTGTTCCT
hsp70	Protect cell from oxidative stress	AF210640.1	*Fwd:* CAACGTGCTGATCTTTGACC *Rev*: TCCTCTTGGCTCGTTCACAT
gadd45bb	Growth arrest and DNA-damage-inducible	NM_001012386.2	*Fwd:* CGCTTCAGATCCACTTCACG *Rev*: TCCCACTTCCTTCAGCTTGA

2.5. Data presentation and statistical analyses

Data analyses were performed using SPSS 16.0 (SPSS Inc, Chicago). In the case of data with a normal distribution, data are shown as mean ± SEM, a *t*-test and one-way analysis of variance with LSD post hoc test were used for statistical comparisons to determine treatment differences. A probability level of $p<0.05$ was considered significant.

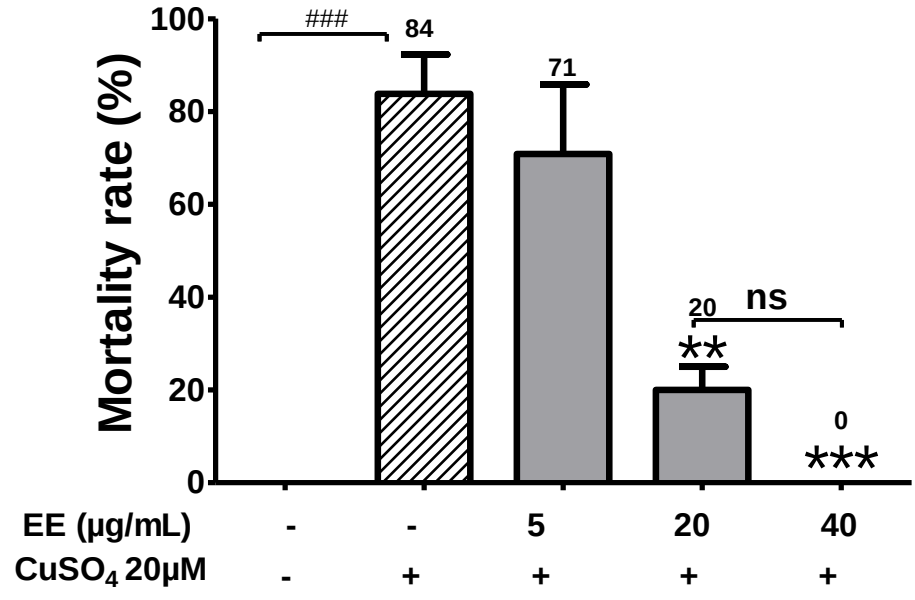
In the case the data were not normally distributed. The non-parametric Kruskal-Wallis one–way analysis of variance by ranks was performed, followed by a Mann-Whitney test to determine significant differences between the experimental groups. When non-parametric statistics were used, the data were presented using Turkey's boxplot (bottom and top of the box are 25th and 75th percentile, the horizontal line in the box is the median and the ends of the whiskers represent 1.5 times of the interquartile distance; the individual dots are values that fall outside the whiskers.

3. Results

3.1. Protective activity of EE against $CuSO_4$ toxicity in zebrafish larvae

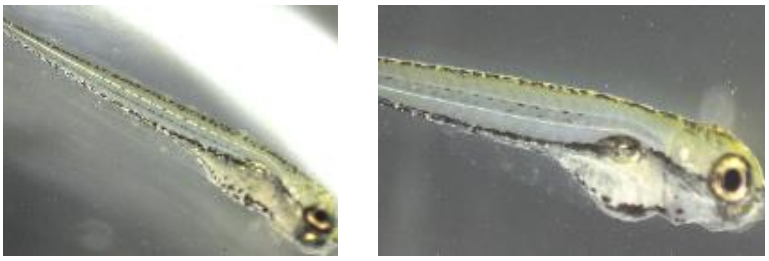
In the present study, we showed that the exposure of $CuSO_4$ at high dose of 20 µM induced mortality in zebrafish larvae (Figure 1A), and EE at the doses of 20 and 40 µg/ml effectively inhibited the toxicity of $CuSO_4$, improving fish survival. The mortality of larvae which were treated with $CuSO_4$ alone was 84%, while the mortality rate was 20% for co-administration with EE at the 20 µg/mL dose. At the high EE dose 40 µg/mL, no dead larvae were observed after 24 h.

A



B

Control-water $CuSO_4$ $CuSO_4$ + EE 5 µg/mL



$CuSO_4$ + EE 20 µg/mL $CuSO_4$ + EE 40 µg/mL

Figure 1 Protective effect of the ethanol extract of *C. cyrtophyllum* leaves (EE) against $CuSO_4$ toxicity **A**. Mortality rates of zebrafish larvae after treatment with 20 uM $CuSO_4$ and ethanol extracts of C. cyrtophyllum (EE) at different doses **B.** Representative photographs of zebrafish larvae treated with 20 uM $CuSO_4$ and ethanol extracts of *C. cyrtophyllum* (EE) at different doses.

*The data are presented as mean ± S.E. for three different experiments performed in triplicate. ### p<0.001, **p<0.01, and ***p<0.001 compared to the $CuSO_4$ alone group.*

The protective effect of EE against $CuSO_4$ was further confirmed by representative photographs of zebrafish larvae treated with 20 uM $CuSO_4$ and EE at different doses. Photographs of zebrafish showed that treatment only with $CuSO_4$ induced toxicity in larvae. Several malformations were observed include trunk curvature, shorter body length, loss of moving ability. Interestingly, the groups that were treated with copper plus EE, extent of morphological abnormality decreased. At high dose, EE 40 µg/mL protected zebrafish larvae against toxicity of $CuSO_4$, so no morphological abnormality was observed (Figure 1B). These results provided evidence that EE protected larvae from $CuSO_4$ toxicity in a dose-dependent manner. To elucidate the protective mechanisms of EE, we further evaluated its antioxidant and anti-inflammatory activities.

3.2. Antioxidant effect

3.2.1. *In vitro* antioxidant activity of EE

Effect of the ethanol extract of C. cyrtophyllum leaves (EE) on *DPPH radical scavenging*

Table 2. DPPH radical scavenging activity of EE

	EE	**Quercetin**
Emax (%)	94.24	93.82
IC_{50} (µg/ml)	16.45 ± 1.11	3.93 ± 1.09
95% Confidence interval	11.63-23.27	2.98-5.17
R^2	0.9958	0.98995
Number of points analysed	7	7

The free-radical scavenging ability of EE is shown in Table 2. The positive control, quercetin, has a strong DPPH scavenging property with a maximum achievable response (Emax) of 93.82%. The concentration that gave the half-maximal effect (IC_{50}) was 3.93 ± 1.09 µg/mL. Similarly to quercetin, EE showed a strong antioxidant activity with an IC_{50} value of 16.45 ± 1.11 µg/ mL and an Emax of 94.24%. The results indicate that EE may act as a DPPH radical scavenger.

Effect of the ethanol extract of C. cyrtophyllum leaves (EE) on Cu^{2+} chelation ability

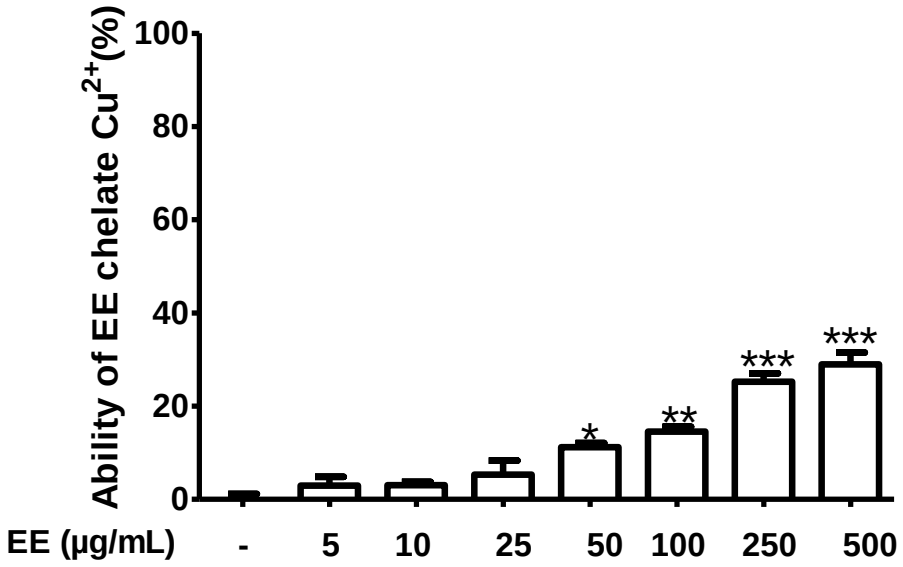
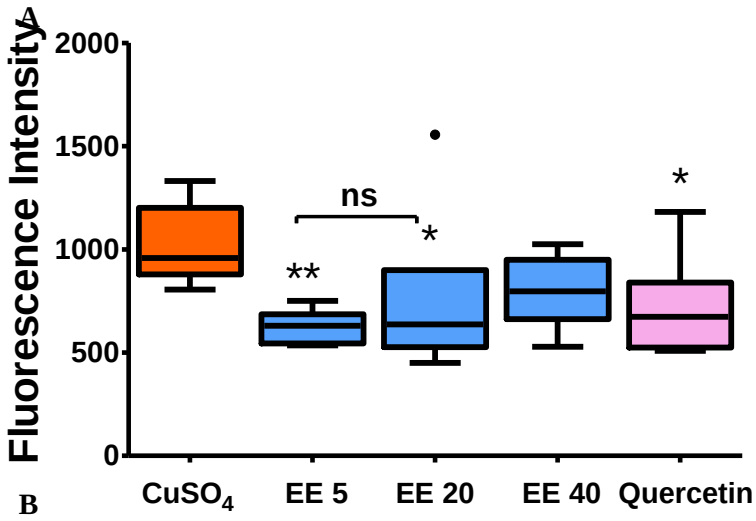


Figure 2. Effect of the ethanol extract of *C. cyrtophyllum* leaves (EE) on Cu^{2+} chelation ability

*Each bar represents the mean ± S.E. for three different experiments performed in triplicate. *p<0.05, ** and ***p<0.001 compared to the control group.*

Ability of EE to chelate Cu^{2+} is shown in Figure 2. Compounds in EE bind Cu^{2+} with the formation of a complex EE-Cu^{2+}. EE showed a weak copper chelating activity. At the highest concentration of 500µg/ml, Cu^{2+} chelating ability of EE reached 29% only.

3.2.2. Effect of the ethanol extract of *C. cyrtophyllum* leaves (EE) on the generation of reactive oxygen species



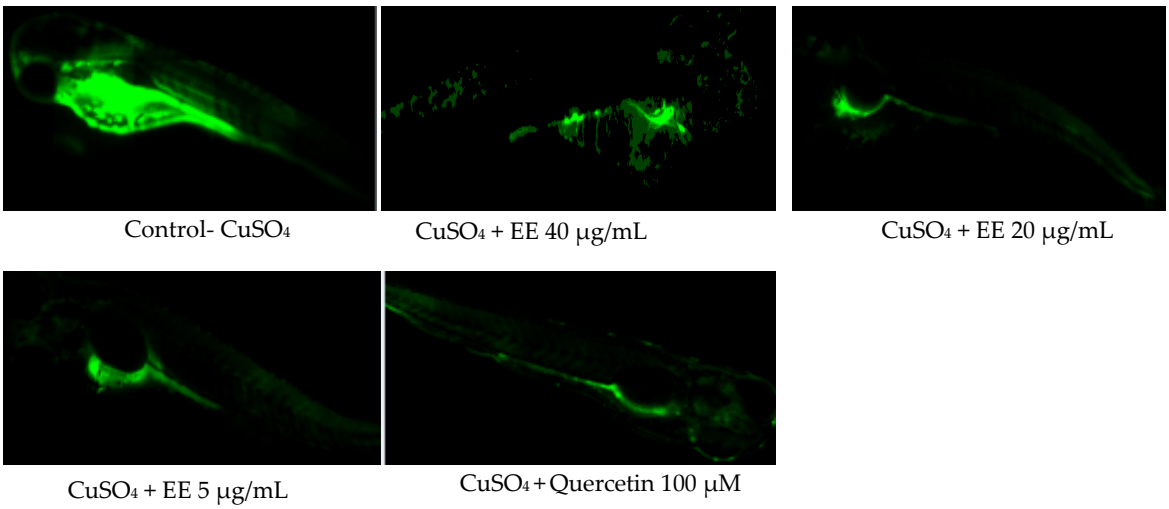


Figure 3. Inhibitory effect of ethanol extract (EE) from leaves of *C. cyrtophyllum* Turcz on $CuSO_4$-stimulated ROS production in zebrafish larvae. The zebrafish were exposed to different concentrations of EE (5, 20 and 40 µg/mL) or 100 µM quercetin for 1 h and then exposed to 10 µM $CuSO_4$ for 20 min to induce oxidative stress. After 20 min, larvae were incubated with H2DCFDA (20 µg/ml) for 1 h. Fluorescent probes were used to detect and quantify oxidative

stress by measuring the fluorescent intensity (FI) in larvae using Autovision software and a BD pathway 855 system (BD Biosciences). **A**. Fluorescence intensity obtained from individual zebrafish larvae. The data are presented as medians for 6 different larvae, *p<0.05, **p<0.01 compared to the $CuSO_4$ alone group **B**. Representative fluorescence micrographs of ROS production

As shown in Figure 3, quercetin 100 µM inhibited ROS formation, so fluorescence intensity significantly decreased compared to the $CuSO_4$ group. Similarly to quercetin, co-administration with EE at 5 and 20 µg/mL significantly decreased fluorescence intensity. These results suggest that administration of EE can limit the generation of reactive oxygen species generated in response to $CuSO_4$.

3.2.3. Effect of the ethanol extract of *C. cyrtophyllum* leaves (EE) on antioxidant gene expression

To further investigate the mechanism of antioxidant action, we measured the effects of EE on the expression of two genes involved in antioxidant systems (*sod, gpx4*). As shown in Figure 4, exposure to copper sulfate resulted in a significant reduction in *sod* compared to the control group. EE at 20 and 40 µg/mL significantly increased *sod*, whereas expression of *gp4x* was not affected by any treatment.

We also designed experiments to examine whether copper sulfate is capable of inducing stress response or growth arrest and DNA damage through evaluating the expression levels of the growth arrest and DNA-damage gene (*gadd45bb*) and stress-related gene (*hsp70).* As shown in Figure 4, $CuSO_4$ induced a stress response; the mRNA expression levels of *hsp70* and *gadd45bb* were significantly upregulated. The co-administration of EE at the doses of 5, 20, and 40 µg/ml protected zebrafish larvae against oxidative damage due to $CuSO_4$ and therefore the expression of *hsp70* and *gadd45bb* were down-regulated.

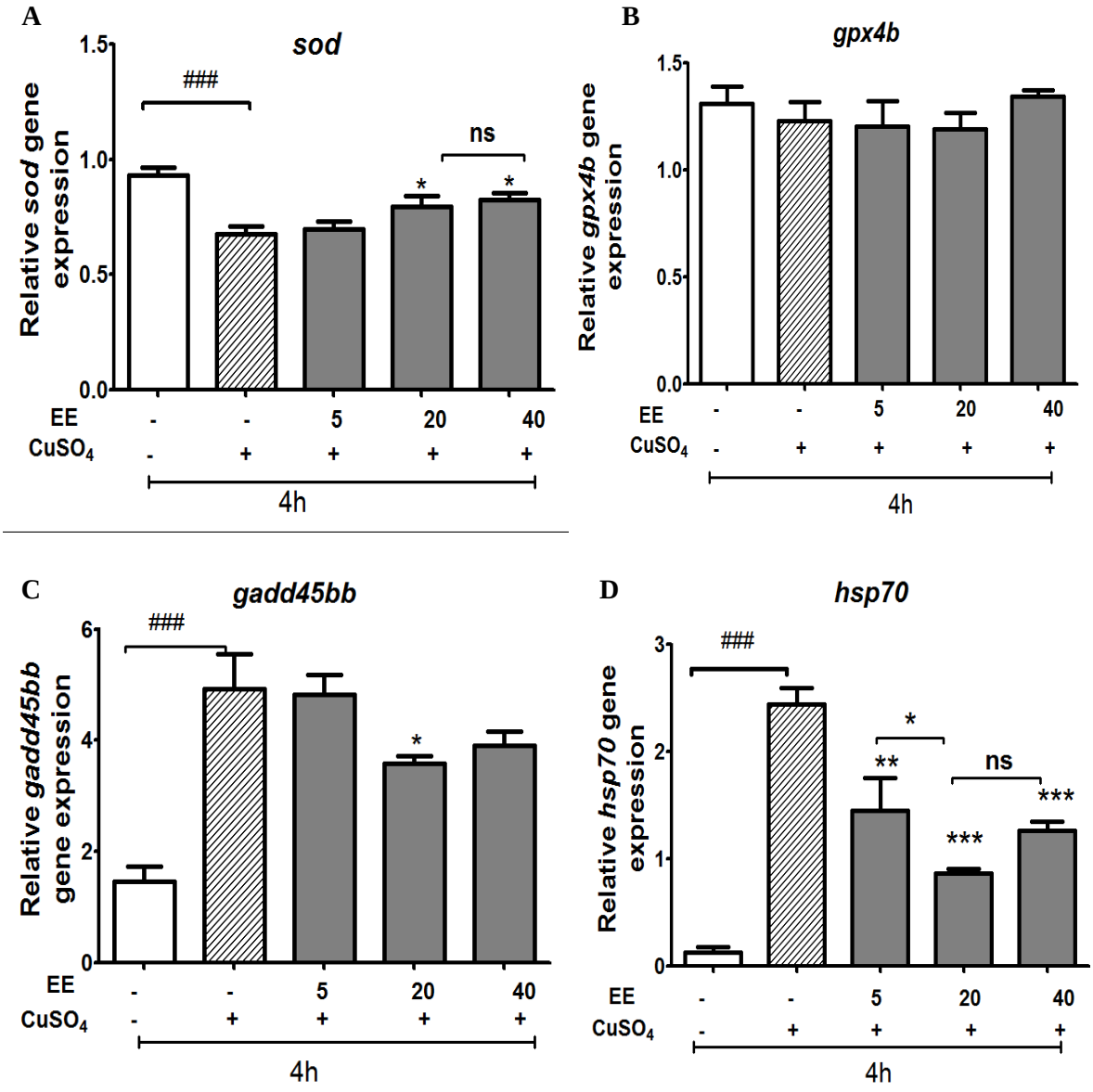
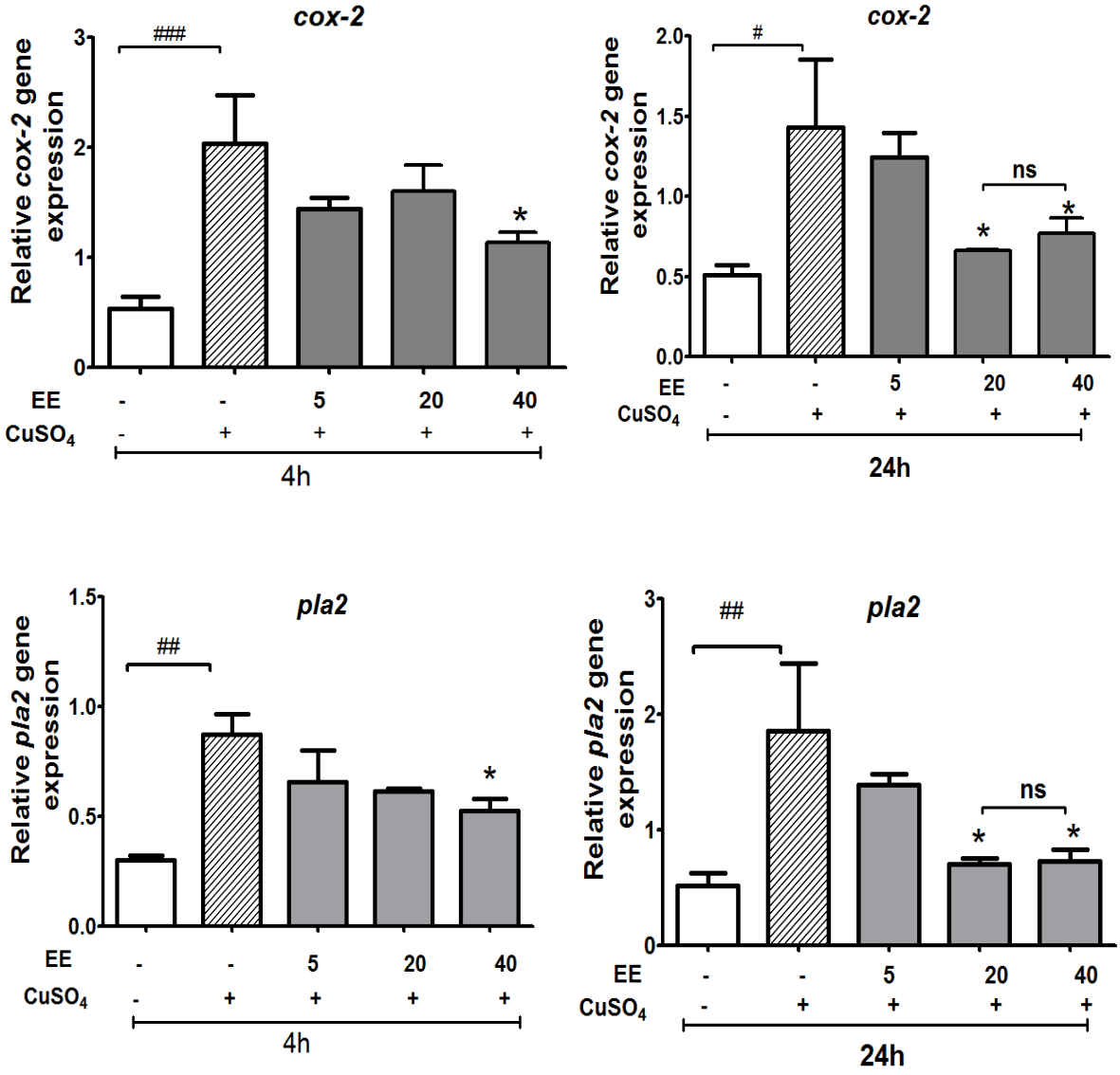


Figure 4. Effect of ethanol extract (EE) of *C. cyrtophyllum* Turcz leaves on expression of antioxidant genes: *sod (A), gpx4 (B)*, hsp70(C), and *gadd45bb*(D). The zebrafish larvae were exposure to EE for 1 h and exposure to $CuSO_4$ 10µM for 4 h. After 4 h, larvae were collected for qPCR analysis. The relative gene expression are presented as the ratio of the quantity of candidate gene/average quantity of housekeeping genes. It was used a pool of 20 larvae per group (n=3). *Each bar represents the mean ± S.E. for three different experiments performed in triplicate. *p<0.05, ** and ### p<0.01, ***p<0.001 compared to the $CuSO_4$ alone group.*

3.3 Anti-inflammatory effect of EE

3.3.1 Effect of the ethanol extract of *C. cyrtophyllum* Turcz leaves on the expression of *c3a, cox-2,* and *pla2* genes

On the expression of two genes involved in the eicosanoid pathway (*cox-2*, *pla2*), and of the complement gene *c3a*, shown in Figure 5, $CuSO_4$ markedly increased the expression of *cox-2*, *c3a*, and *pla2* genes when compared with the control group. EE at 40 µg/mL greatly suppressed the $CuSO_4$-induced mRNA levels of *cox-2*, *pla2*, and *c3a* at both 4 h and 24 h after copper sulfate exposure, while EE at 20 µg/mL only showed an effect on expression after 24 h.



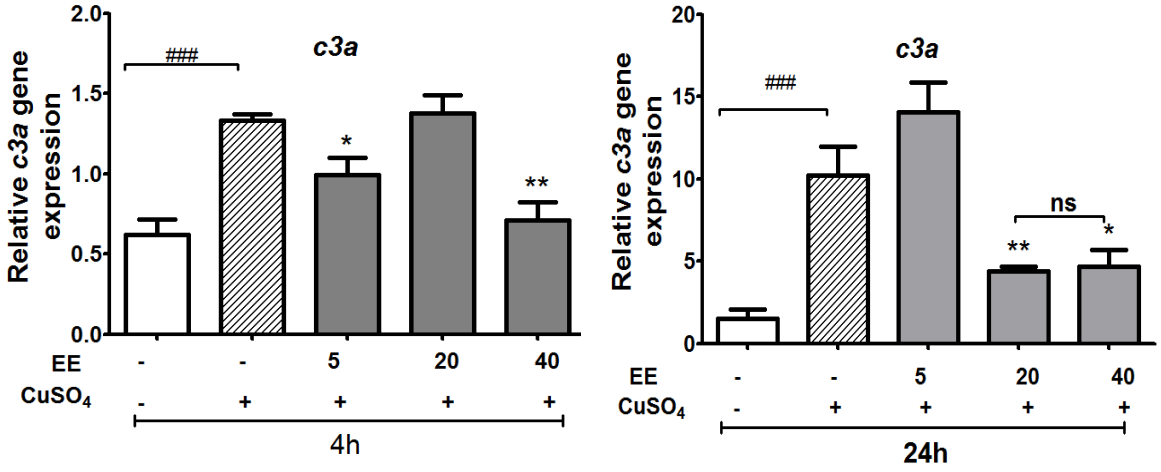


Figure 5. Effect of ethanol extract (EE) of *C. cyrtophyllum* Turcz leaves on the expression of genes in the eicosanoid pathway (*pla2*, *cox-2*) and *c3a*.). The zebrafish larvae were exposure to EE for 1 h and exposure to $CuSO_4$ 10µM for 4 h and 24 h. After 4 h or 24 h, larvae were collected for qPCR analysis. The relative gene expression are presented as the ratio of the quantity of candidate gene/average quantity of housekeeping genes. It was used a pool of 20 larvae per group (n=3). *Each bar represents the mean ± S.E. for three different experiments performed in triplicate. *p<0.05, ** and ### p<0.01, ***p<0.001 compared to the $CuSO_4$ alone group*

3.3.2 The EE inhibited the transcription of $CuSO_4$-induced pro-inflammatory cytokines in zebrafish larvae

We examined whether the expression of pro-inflammatory cytokine genes increased due to $CuSO_4$ exposure and, subsequently, whether EE inhibits $CuSO_4$-induced expression of *tnf-α*, *il-1β*, and *nf-ƙb*, *il-8* in the concentration range of 5, 20, and 40 µg/mL. Real-time RT-PCR analysis revealed that $CuSO_4$ stimulated the inflammation process by a significant increase of *il-1β* and *tnf-α* expression at 4 and 24 h and *il-8* levels at 24 h after copper sulfate exposure. The co-administration of EE at the doses of 20 and 40 µg/ml resulted in a decrease in the mRNA levels of *il-1β* at both 4 h and 24 h, and *il-8* at 24 h. No significant differences were observed for the expression of *nf-ƙb* or *tnf-α* between the different treatment groups. These results suggest that the EE at concentrations of 20 and 40 µg/mL inhibited transcription activities of pro-inflammatory cytokines induced by $CuSO_4$ in zebrafish larvae.

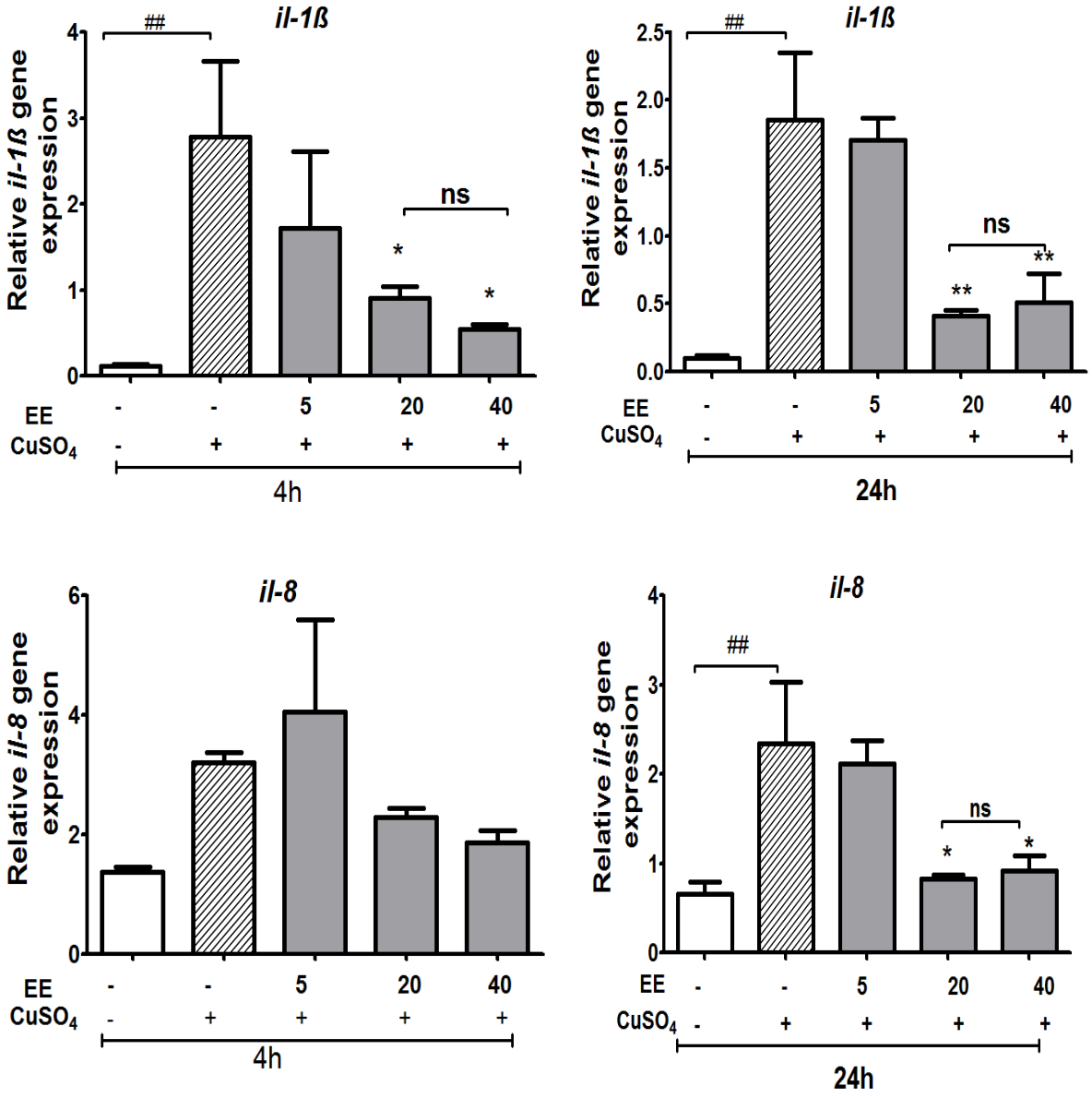


Figure 6A. Relative expression of genes involved in immune responses (*il-1ß*, *il-8*) of zebrafish larvae after exposure to $CuSO_4$ and treatment with the ethanol extract (EE) of *C. cyrtophyllum* Turcz at different doses. The zebrafish larvae were exposure to EE for 1 h and exposure to $CuSO_4$ 10µM for 4 h and 24 h. After 4 h or 24 h, larvae were collected for qPCR analysis. The relative gene expression are presented as the ratio of the quantity of candidate gene/average quantity of housekeeping genes. It was used a pool of 20 larvae per group (n=3). *Each bar represents the mean ± S.E. for three different experiments performed in triplicate. *p<0.05, ** and ### p<0.01, ***p<0.001 compared to the $CuSO_4$ alone group*

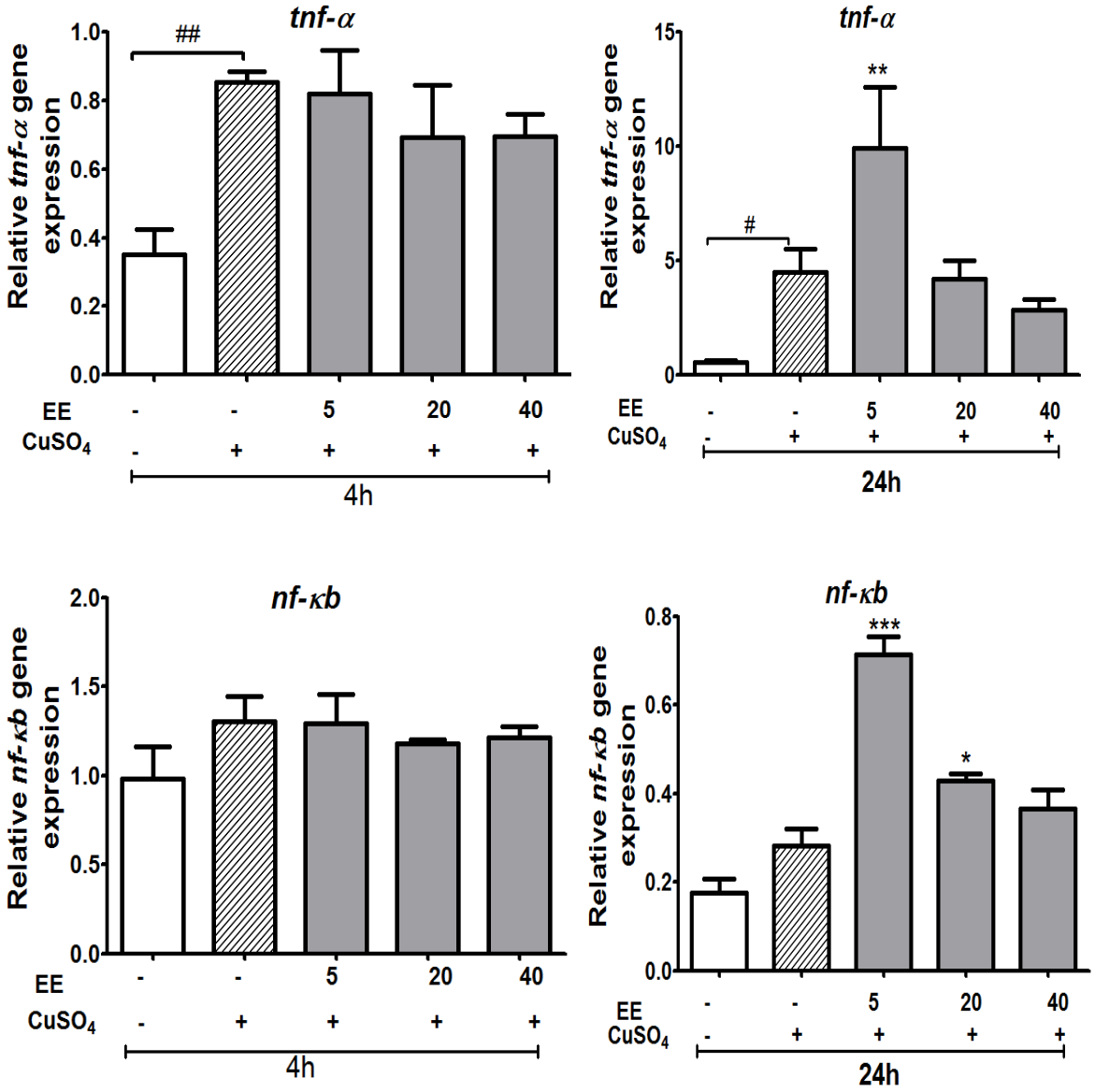


Figure 6B. Relative expression of genes involved in immune responses (*tnf-α, nf-ƙb*) of zebrafish larvae after exposure to $CuSO_4$ and treatment with the ethanol extract (EE) of *C. cyrtophyllum* Turcz at different doses. The zebrafish larvae were exposure to EE for 1 h and exposure to $CuSO_4$ 10µM for 4 h and 24 h. After 4 h or 24 h, larvae were collected for qPCR analysis. The relative gene expression are presented as the ratio of the quantity of candidate gene/average quantity of housekeeping genes. It was used a pool of 20 larvae per group (n=3). *Each bar represents the mean ± S.E. for three different experiments performed in triplicate. *p<0.05, ** and ### p<0.01, ***p<0.001 compared to the $CuSO_4$ alone group.*

3.3.3 Effect of the ethanol extract (EE) of *C. cyrtophyllum* Turcz leaves on expression of the *mpo* gene

Neutrophil migration, as indirectly measured by the mRNA level of *mpo*, displayed a significant increase after 24 h of exposure to copper sulfate. Exposing zebrafish larvae to EE resulted in a decrease in the mRNA levels of *mpo* at 20 and 40 µg/ml (p<0.05) (Figure 7).

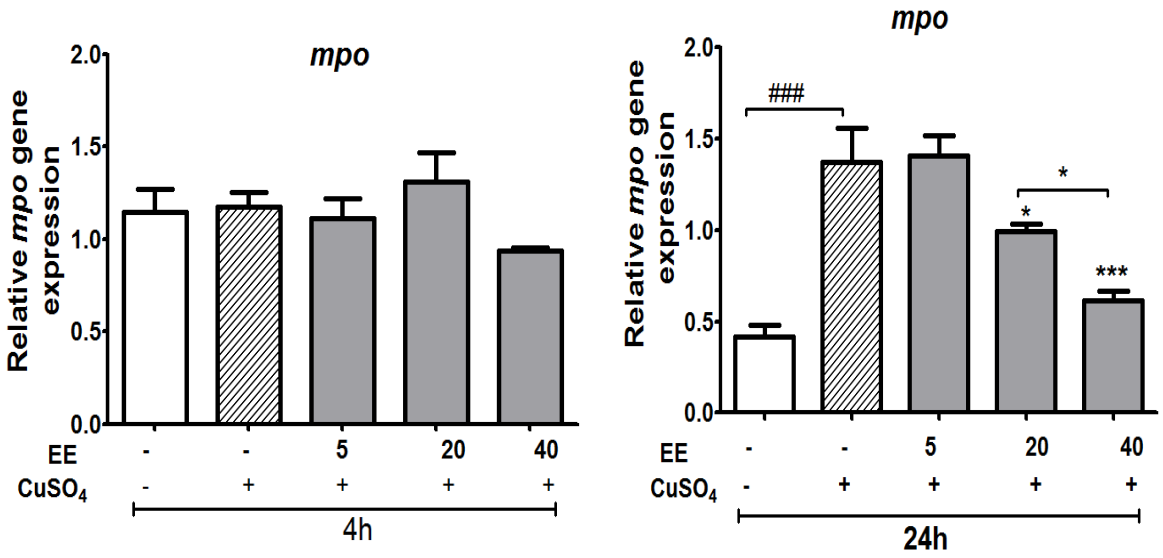


Figure 7. Relative expression of genes involved in immune responses (*mpo*) of zebrafish larvae after exposure to $CuSO_4$ and treatment with the ethanol extract (EE) of *C. cyrtophyllum* Turcz.

*The data are presented as mean ± S.E. for three different experiments performed in triplicate. *p<0.05, ** and ### p<0.01, ***p<0.001 compared to the $CuSO_4$ alone group.*

3.3.4 Effect of the ethanol extract (EE) of *C. cyrtophyllum* Turcz leaves on the expression of anti-inflammatory cytokine il-10

As shown in Figure 8, stimulating zebrafish larvae with $CuSO_4$ increased the level of *il-10* mRNA. Increased *il-10* expression in response to $CuSO_4$ stimulation alone showed the regulatory action on cells after the inflammatory reaction. The level of *il-10* was significantly suppressed by EE at 40 µg/mL at 4 h, and at both 20 and 40 µg/mL at 24 h (p<0.05). The level of *il-10* decreased the most under the EE treatment condition, which illustrated the effect of EE on reinforcing cellular immunity and inhibiting humoral immunity.

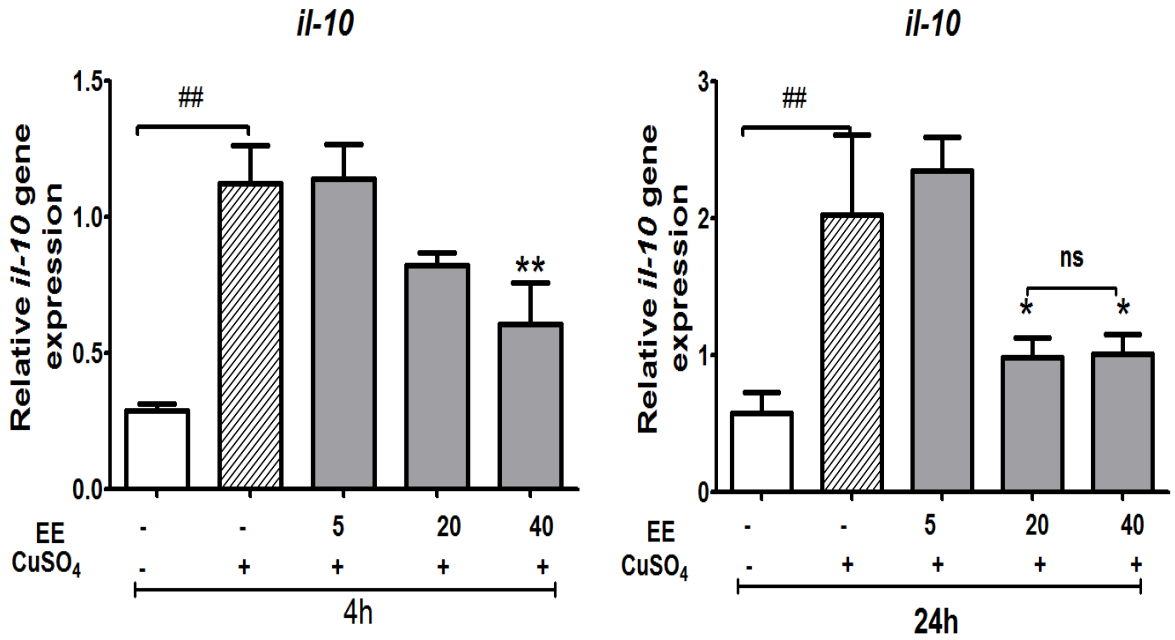


Figure 8. Relative expression of anti-inflammatory gene (*il-10)* of zebrafish larvae after exposure to $CuSO_4$ and treatment with the ethanol extract (EE) of *C. cyrtophyllum* Turcz at different doses. The zebrafish larvae were exposure to EE for 1 h and exposure to $CuSO_4$ 10µM for 4 h and 24 h. After 4 h or 24 h, larvae were collected for qPCR analysis. The relative gene expression are presented as the ratio of the quantity of candidate gene/average quantity of housekeeping genes. It was used a pool of 20 larvae per group (n=3). *Each bar represents the mean ± S.E. for three different experiments performed in triplicate. *p<0.05, ** and ### p<0.01, ***p<0.001 compared to the $CuSO_4$ alone group.*

4. Discussion

Protective activity of the ethanol extract of *C. cyrtophyllum* against $CuSO_4$ toxicity

Copper is an essential micronutrient but misregulation of intracellular levels can become toxic to many cell types. Copper generates dose-dependent responses. A previous study demonstrated that low concentrations of copper (<10 µM) did not induce mortality [16]. At the concentration of 10 µM, although it did not cause a significant decrease of zebrafish larvae survival, copper sulfate was able to induce oxidative stress and a marked increase of inflammatory markers [17]. A concentration of 25 µM induced a 75–80% death rate [18]. In the present study, $CuSO_4$ at 20 µM induced 80% mortality after 24 h of exposure. Interestingly, treatment with EE at concentrations of 20 and 40 µg/mL showed a protective effect against damage induced by $CuSO_4$.

So what is the reason for this? Which molecular mechanism of the EE helped zebrafish larvae to avoid the toxicity of copper? To better understand the protective mechanism of EE, the toxicity mechanism of copper in zebrafish larvae first needs to be identified.

It was suggested recently that copper induces oxidative stress [16,18]. Oxidative stress can occur by two mechanisms: the direct effect of copper which induces DNA and cell damage, or the increase of ROS release due to the activation of phagocytic cells and increased tissue damage [13]. The increase of ROS release might induce the activation of activator protein-1 (AP-1) and nuclear factor kappa-B (Nf-κB), both signalling pathways that upregulate pro-inflammatory cytokines and chemokines leading to inflammation. Inflammation represents the result of copper damage. Both oxidative stress and inflammation are related to the toxicity mechanism of copper. We thus investigated the antioxidant and anti-inflammatory properties of EE.

Antioxidant activity of the ethanol extract of C. cyrtophyllum

To evaluate the antioxidant activity, we first used an *in vitro* test, the DPPH assay. DPPH radical scavenging assay is one of the most common methods used to evaluate the radical scavenging activity of antioxidants because of its speed, reliability, and reproducibility. A change in colour from purple to yellow indicates a decrease in absorbance of DPPH radicals. This demonstrates that an antioxidant present in a solution interacts with the free radicals [11]. In this study, EE exhibited strong antioxidant properties against 1,1-diphenyl,2-picryl hydrazyl (DPPH), with an IC_{50} value of 16.45 ± 1.11 µg/mL. The antioxidant activity of EE found here was stronger than that reported by Liu et al. (2011) for a methanol extract of *C. cyrtophyllum* (52.74 ± 2.07 µg/mL) [9]. This can be explained by the fact that flavonoids are important antioxidants due to their high redox potential which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [19]. Antioxidant activity was correlated with total flavonoids. The higher the content of flavonoids, the stronger the antioxidant capacity *in vitro*. In our study, EE was found to present higher flavonoid contents than the methanol extract reported by Liu et al. Another antioxidant mechanism of flavonoids may result from the interactions between flavonoid and metal ions (especially iron and copper) leading to chelates formation that are only slightly active in the promotion of free-radical reaction. In our study, EE can chelate to copper, decreasing the toxicity of $CuSO_4$. However, copper chelating ability of EE is weak. It did not appear as a major mechanism for protective activity of the ethanol extract of *C. cyrtophyllum* against $CuSO_4$ toxicity.

Although the *in vitro* model is simple, fast, and inexpensive, it cannot be a complete substitute for *in vivo* animal testing. The absence of biokinetics in *in vitro* methods may lead to a misinterpretation of the data. Animal models thus are more reliable than *in vitro* tests [20]. Among animal models, zebrafish display several pathological features similar to those of humans [21]. In this study, we used a zebrafish model to confirm the antioxidant effects of EE, as well as to elucidate the molecular mechanisms of its antioxidant activity.

There are two main mechanisms of drug-induced oxidative stress: an increase in ROS production and a reduction of cellular antioxidant genes. We thus examined the effects of EE on ROS production as well as on the expression of antioxidant genes.

Regarding ROS production, the image analysis in stress-induced ROS generation showed that copper sulfate induced toxicity in the cells via the generation of reactive oxygen species (ROS). EE displayed a protective effect against stress *in vivo* by reducing ROS formation in zebrafish larvae. Regarding the expression of antioxidant genes, in our study *sod* was decreased significantly after 4 h of exposure to copper when compared to control, while groups that were treated with $CuSO_4$ plus EE at 20 and 40 µg/mL resulted in a significant increase of mRNA levels of *sod* when compared with the $CuSO_4$ group. Increasing superoxide dismutase (SOD) helps to transfer ROS to hydrogen peroxide (H_2O_2), which then forms a non-toxic compound (H_2O) under the effects of catalase, glutathione peroxidase, peroxidase**.**

Major cytotoxic roles for ROS include the activation of the apoptosis pathway [22]. Heat shock proteins are one of key players in protecting the cell against the harmful effects of oxidative stress in the cellular stress response process and are crucial for defending cells from copper toxicity [16]. Our results were similar to those previously reported where copper induces *hsp70* expression [16]. Interestingly, expression of *hsp70* decreased in the EE treatment groups. This result suggests that the presence of EE helped to protect zebrafish larvae against $CuSO_4$ toxicity, reducing oxidative stress and damage. As a result, the expression of *hsp70* decreased.

Gadd45 participates in cell growth and cell cycle control, DNA repair, apoptosis, maintenance of genomic stability, and the regulation of signalling pathways. Transcription of the *gadd45* genes is induced by DNA-damaging agents and other cellular stresses and is associated with growth arrest [23, 24]. Olivari et al. (2008) described events induced by copper via oxidative stress, such as cell death by apoptosis and necrosis in hair cells of the lateral line of zebrafish [13]. Supporting these data, our results showed that copper induced activation of the apoptosis pathway in such a way that mRNA expression level of *gadd45bb* was significantly upregulated.

EE can scavenge superoxide anions, inhibit ROS production, and defend cells from copper toxicity, decreasing cellular stresses, apoptosis, and DNA-damage. As a result, the expression of *gadd45bb* was downregulated compared to the $CuSO_4$ alone group.

Intracellular production of reactive oxygen species (ROS) is deeply involved in inflammatory responses. High levels of redox metals promote ROS formation and these metals can also act as mediators of inflammation, inducing peripheral inflammation in numerous models [25]. Leite et al. (2013) reported the gradual accumulation of copper in zebrafish larvae within 24 hours post fertilization (hpf), increased activity of NO, upregulation of pro-inflammatory cytokine-related genes *il-1β*, *tnf*, and *cox-2*, as well as increased PGE2 and myeloperoxidase (MPO) levels, which are also known for their activity as inflammatory players [18]. Similar to previous studies, our data also showed that copper induced inflammation through the upregulation of *cox-2*, *il-1β*, *tnf-α*, *mpo*, *pla2*, *il-8*, *c3a*, and *il-10*. Altogether, this study provides more evidence on the effects of copper as well as on the general scenario of the inflammatory status and response to copper exposure that corroborates copper's role in oxidative stress and inflammation.

The expression of *cox-2*, *pla2*, *c3a*, *il-1ß*, and *il-8* decreased in the EE treatment groups. COX-2 and PLA2 are involved in the synthesis of key biological mediators in inflammation. Inhibition of these enzymes is a well-established target in the discovery of anti-inflammatory drugs, with COX-inhibitors being the most prominent drug class in non-steroidal anti-inflammatory drugs (NSAIDs). Flavonoids play an important role in various medicinal applications. The C. *cyrtophyllum* leaves presents high flavonoid contents [8, 9]. Flavonoids have been reported to inhibit the activities of arachidonic acid metabolising enzymes such as PLA2, COX, and 5-LOX, to reduce the production of inflammatory metabolites from arachidonic acid and oxidative damage [26]. IL-1ß and IL-8 are pro-inflammatory cytokines. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1β, IL-2, TNF-α, Il-6, IL-8, and IFN-γ are involved in the process of pathological inflammation. Because of this, the cytokine system constitutes a very interesting target for the development of clinically relevant anti-inflammatory drugs. In this study, EE effectively inhibited the production of *il-1ß* and *il-8* at both 20 and 40 µg/mL by suppressing their mRNA expression in $CuSO_4$-stimulated zebrafish larvae. This result demonstrates that EE effectively inhibits the generation of an inflammatory response. However, the level of TNF-α transcription was not affected by EE at 4h. At 24h, expression of *tnf-α* even increased at the dose of 5µg/ml. Increased expression of *tnf-α* can explain the weak anti-inflammatory activity of EE at 5µg/ml.

The regulation of inflammatory gene transcription is controlled by specific signaling pathways and transcription factors, such as NF-κB and AP-1. In response to stimulation, NF-κB is liberated from a complex with I-κB and induces transcription of inflammatory genes that cause acute inflammation and a systemic inflammatory response syndrome [27]. In this study, $CuSO_4$ did not increase the expression of *nf-κb*. This result demonstrates that *nf-κb* did not regulate the inflammatory gene transcription stimulated by $CuSO_4$. The increase expression of inflammatory genes due to $CuSO_4$ can relate to other transcription factors with other signaling pathways that need further studies. The ethanol extract from C. c*yrtophyllum* had no effect on the expression of *nf-κb* at 4 h, however expression of *nf-κb* increased at the dose of 5 and 20 µg/ml at 24 h. In our previous study, EE alone had no effect on the expression of *nf-κb*. Increasing its expression when association with $CuSO_4$ may be related to another function of *nf-κb*. The association of NF-kB activity and inflammatory disease is not easy to interpret because both pro and anti-inflammatory mediators are produced during inflammation [28]. In 2001, Lawrence et al showed NF-kB involves in both the onset and resolution of acute inflammation in a single model system using pharmacological inhibitors. These studies confirmed the role of NF-kB in proinflammatory gene induction but also showed a role for NF-kB in the expression of anti-inflammatory genes during the resolution of inflammation [29]. So increasing expression of *nf-κb* at the dose of 5 and 20µg/ml at 24 h may be related to resolution function of NF-kB in a cute inflammation.

However, the increase in mRNA levels is not directly proportional to the amount of protein translated, due to transcriptional and post translational modifications. Although no significant differences in the mRNA levels of *tnf-α* and *nf- κb* at 4 h or increase of the expression of these genes at 24 h was observed, further studies are required in order to investigate the expression at the protein level and to verify the presence and activation of these proteins.

Recently, d'Alencon et al. demonstrated that copper sulfate exposure induces neutrophil migration to the inflammatory focus, in response to damage induced in hair cells of the lateral line of zebrafish larvae [30]. Activated neutrophils, monocytes, and some tissue macrophages release MPO at the site of inflammation. MPO catalyses the reaction between hydrogen peroxide (H_2O_2) and physiological halides (such as Cl^-) to form reactive oxidants such as hypochlorous acid (HOCl) [31]. In this study, EE effectively inhibited the expression of *mpo* at 20 and 40 µg/mL at 24 h. This suggests that EE has an anti-inflammatory role through the inhibition of leukocyte infiltration.

Interleukin-10 (IL-10) is a cytokine with potent anti-inflammatory properties that plays a central role in limiting the host immune response to pathogens, thereby preventing damage to the host and maintaining normal tissue homeostasis. IL-10 is a molecule that displays both immunostimulatory and immunoregulatory activities [32]. IL-10 can up-regulate endogenous anti-cytokines and down-regulate pro-inflammatory cytokine receptors to dampen uncontrolled production of inflammatory cytokines and excessive inflammation during infection [33]. In this study, stimulation of zebrafish larvae with $CuSO_4$ increased the level of *il-10* mRNA at 4 h and 24 h after exposure. The elevation of *il-10* displayed immunoregulatory activity on cells after the inflammatory reaction [18]. Co-administration with EE at 20 and 40 µg/ml down-regulated *il-10.* Supporting these data, a previous study conducted by Visser et al. (1998) revealed that elevated *il-10* levels were also observed in an LPS-induced inflammatory model in peritoneal macrophages, demonstrating similarities of modulation mechanisms in these two models [34].

The relation between dose and response

In most cases, EE displayed an effect on gene expression depending on the dose. At the low dose of 5µg/mL, EE generally exerted no detectable effects. However, at 20µg/mL, EE showed a higher effect. The interesting, anti-inflammatory property of EE not only depends on the dose but also on the time. At 4 h, EE at 20µg/mL indicated no effects or low effect on the inhibition expression of inflammatory genes. However, this effect increased at 24 h. One possible explanation could be that as exposure to the extract is prolonged over time, there is an increase in the accumulation of the extract until it reaches a concentration that induces anti-inflammatory effects in zebrafish. At the highest dose of 40µg/mL, EE induced the best effects at both 4 and 24 h.

The potential area of application of EE

The results of this study suggest that the ethanol extract of C. *cyrtophyllum* leaves possesses antioxidant and anti-inflammatory activities. They provide some evidence for the use of the plant extract in prevention as well as in treatment of oxidative stress and inflammatory conditions. However, before applying EE in treatment human diseases, we still need to do experiments in other models to confirm anti-inflammatory and antioxidant activities of EE. We also need to perform additional experiments to determine whether EE is safe for testing in human subjects, preclinical toxicology studies need to be performed to identify the treatment regimen associated with the least degree of toxicity and thus determine a suitable and safe starting dose for clinical trials.

5. Conclusions

In conclusion, the ethanol extract of *C. cyrtophyllum* displayed prominent antioxidant effects through radical scavenging activity *in vitro* and decreased production of ROS in a $CuSO_4$-induced zebrafish inflammation model. In addition, *in vivo* results in zebrafish suggest that the ethanol extract from *C. cyrtophyllum* also inhibits oxidative stress via the upregulation of *sod* and the downregulation of *hsp70* and *gadd45bb*. EE also inhibited inflammation via the downregulation of the inflammatory genes *cox-2*, *pla2*, *c3a*, *il-1*, *il-8*, *mpo*, and *il-10*. These *in vivo* results help to elucidate the protective mechanism against $CuSO_4$ toxicity of the ethanol extract of leaves of *C. cyrtophyllum*. Taken together, these findings provide a pharmacological basis for the use of *C. cyrtophyllum* leaves in the treatment of inflammatory disorders.

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Foreword: Pharmacology effect of the ethanol extract from *Clerodendrum cyrtophyllum* leaves was proved in previous chapters. However, it needs to assure safety before moving into the next phase for test in the human. The safety aspect has become an outstanding issue in the process of drug discovery and development. The zebrafish model is widely used in screening for teratogenicity in preclinical developmental toxicity assessment. *Clerodendrum cyrtophyllum* leaves have been used widely for the treatment of human diseases. Because of their frequent use, this chapter was designed to prove the safety of EE in zebrafish embryo model.

Chapter 7

Developmental Toxicity of *Clerodendrum cyrtophyllum* Turcz Ethanol Extract in Zebrafish Embryo

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Abstract

Ethnopharmacological relevance: *Clerodendrum cyrtophyllum* Turcz has been used in traditional medicine for the treatment of various diseases. In spite of its therapeutic applications, research on its toxicity and teratogenicity is still limited.

Aim of the study: The study aimed to investigate the developmental toxicity of the ethanol extract of *C. cyrtophyllum* (EE) in zebrafish embryo model.

Material and Methods: Major compounds from crude ethanol extract of *Clerodendron cyrtophyllum* Turcz leaves were determined using HPLC-DAD-Orbitrap-MS analysis**.** The developmental toxicity of EE were investigated using zebrafish embryo model. Zebrafish embryos at 6 h post-fertilization (hpf) were treated with EE at different concentrations. Egg

coagulation, mortality, hatching, yolk sac oedema, pericardial oedema and teratogenicity were recorded each day for during a 5-day exposure. At time point 120 hpf, body length, pericardial area, heartbeat and yolk sac area were assessed. In order to elucidate molecular mechanisms for the developmental toxicity of EE, we further evaluated the effects of the EE on the expression of genes involved on signaling pathways affecting fish embryo's development such as heart development (*gata5, myl7, myh6, has2, hand2, nkx 2.5*), oxidative stress (*cat, sod1, gpx4, gstp2*), wnt pathway (β-*catenin, wnt3a, wnt5, wnt8a, wnt11*), or cell apoptosis (*p53, bax, bcl2, casp3, casp8, casp9, apaf-1, gadd45bb)* using qRT-PCR analysis.

Results: Our results demonstrated that three major components including acteoside, cirsilineol and cirsilineol-4'-*O-β*-D-glucopyranoside were identified from EE. EE exposure during 6- 96 h post-fertilization (hpf) at doses ranging from 80 – 200 µg/ml increased embryo mortality and reduced hatching rate. EE exposure at 20 and 40 µg/ml until 72 – 120 hpf induced a series of malformations, including yolk sac oedema, pericardial oedema, spine deformation, shorter body length. Based on two prediction models using a teratogenic index (TI), a 25% lethality concentration (LD25) and the no observed-adverse-effect level (NOAEL), EE is considered as teratogenic for zebrafish embryos with TI (LC50/EC50) and LD25/NOAEC values at 96 hpf reaching 3.87 and 15.73 respectively. The mRNA expression levels of *p53*, *casp8*, *bax/bcl2*, *gstp2*, *nkx2.5*, *wnt3a*, *wnt11*, *gadd45bb* and *gata5* were significantly upregulated by EE exposure at 20 and 40 µg/ml while the expression of *wnt5*, *hand2* and *bcl2* were downregulated.

Conclusions: These results provide evidence for toxicity effects of EE to embryo stages and provide an insight into the potential toxicity mechanisms on embryonic development.

Keywords: Teratogenicity, oxidative stress, wnt pathway, apoptosis, embryonic development.

Abbreviations: EE, the ethanol extract from *Clerodendrum cyrtophyllum* Turcz leaves; HPTLC, High performance thin layer chromatography; QE, quercetin; GAE, gallic acid equivalent; quantitative polymerase chain reaction (qRT-PCR); dpf, day post fertilization; TI, Teratogenic Index; NOAEL, the no observed-adverse-effect level.

1. Introduction

Congenital abnormalities represent a severe problem, 5-10% of the congenital abnormalities of human newborns are known to be related to environmental factors including therapeutic agents and developmental toxicants (Hong and Jeung, 2013). During pregnancy, the mother can be exposed to a variety of chemicals and medicine. These compounds subsequently can be

transported through the placenta and exert adverse effects on reproductive tissues or the embryo. As a result, birth defects and biological dysfunction may be induced (Peters et al., 2008). Toxicological screening is very important for the development of new drugs and, according to international regulatory guidelines, each drug in development for administration to women of childbearing potential must be tested for developmental toxicity (Nishimura et al., 2016).

Clerodendrum cyrtophyllum Turcz (Lamiaceae), a medicinal plant, is widely used in traditional medicine of many Asian countries such as China, India, Japan, Korea, Thailand, and Vietnam for the treatment of colds, high fever, migraines, hypertension, enteritis, dyspepsia, inflammation of the throat, rheumatic arthritis, fever, jaundice, leukorrhea, syphilis, and typhoid (Kar et al., 2014, Zhou et al., 2013). Several therapeutic and pharmacological properties of *Clerodendrum cyrtophyllum* Turcz have recently been reported such as antioxidant, anti-inflammatory (Nguyen et al., 2020a, Nguyen et al., 2020b), and anticancer (Cheng et al., 2001) activities. Despite their frequent use, studies on its toxicity and its effects on reproductive tissues or embryos are currently unknown.

The zebrafish model is widely used in screening for teratogenity in preclinical developmental toxicity assessment. Compared with traditional animal models (rabbit, rat, mice), the zebrafish has many advantages such as transparency (easy for observation of malformations), small size, requirement of small amounts of test compound, low-cost, easy maintenance, similarity of major organ systems of zebrafish compared to human (Li et al., 2018, Gao et al., 2017). Due to the above advantages, zebrafish is the ideal model for testing drug's preclinical developmental toxicity today.

In this study, we investigated the developmental toxicity induced by the ethanol extract of *Clerodendrum cyrtophyllum* Turcz (EE) in the zebrafish model by focusing on the effects of EE on teratogenicity indicators and on the expression of genes involved in different signaling pathways affecting embryo development such as oxidative stress, apoptosis and the wnt pathway.

2. Materials and methods

Plant collection*:* Leaves of *Clerodendrum cyrtophyllum* Turcz were collected in June, 2018 from Vietnam. A voucher specimen (HNU 024106) was kept at the Botanical Museum of Hanoi, University of Science.

Preparation of total extract: The ethanol extract from leaves of *Clerodendrum cyrtophyllum* Turcz was prepared as described in our previous study (Nguyen et al., 2020b). After the extraction, the EE was analysed with various analytical techniques including colorometric methods, HPLC-DAD- Orbitrap-MS analysis. The concentration of total phenolic compounds and flavonoid in the extract were 23.3 ± 1.5 GAE mg/g and 2.97 ± 0.01 QE mg/g expressed in dry weight of leaves material (Nguyen et al., 2020b).

Analysis of samples by HPLC-DAD- Orbitrap-MS analysis

Analyses were performed on an Accela HPLC system (Thermo Fisher Scientific) consisting of a PDA detector connected with a Thermo Fisher Scientific LTQ Orbitrap XL mass spectrometer from the UCLouvain Massmet platform. A Phenomenex® Lichrospher C18, 4.6 x 250 mm column packed with 5 µm particles was applied. Ten µL of samples at 1 mg/mL concentration were injected in the full loop injection mode. The column was eluted at a constant flow rate of 0.8 mL/min using a binary solvent system: solvent A, MilliQ water 0.1% formic acid and solvent B, acetonitrile HPLC grade (0–10 min, 83% A and 17% B; 30 min, 50% A and 50% B; 38 min, 15% A and 85% B; 38 min, 15% A and 85% B). DAD-UV detector was set at 254 nm. HR-MS were measured with APCI source in the negative mode using full-scan MS with a mass range of 100-2000 *m/z*. The following (-) APCI conditions were applied: vaporizer temperature, 400°C; sheath gas (N_2) flow rate, 25 a.u.; auxiliary gas (N_2) flow rate, 25 a.u.; sweep gas (N_2) flow rate, 5 a.u.; capillary temperature, 250°C; capillary voltage, 10 V; tube lens, 125 V.

Fish and experimental conditions

Adult wild – type AB zebrafish (*Danio rerio*) were maintained at at 28 °C in a recirculating ZebTec housing system. Conductivity was kept at approximately 500 µS/cm, pH at 7.2 with a 12:12 h (light/dark) photoperiod. Fish were fed to apparent satiation twice daily with ZM-400 fry food for zebrafish. The evening before reproduction, males and females were placed together in spawning tanks in a ratio of 2:2. The next morning, eggs were collected within 30 min of spawning. Fertilized eggs were selected and placed in embryo medium at 26 ± 0.5 ^{o}C. The use of zebrafish was in accordance with the animal welfare act. Since zebrafish larvae below 120 hpf old are not considered animals (Lackmann et al., 2018), no animal test authorization was requested in accordance with European legislation (EU Directive, 2010/63/EU). All experiments were terminated at 120 hpf.

Preparation of test samples

The ethanol extract of *Clerodendrum cyrtophyllum* Turcz (EE) was dissolved in DMSO for stock solution at 50mg/mL and then diluted to different concentrations using fresh medium so that the final concentration in each experimental well was 1, 5, 10, 20, 40, 80, 100, 200 µg/mL.

Fish Embryo Acute Toxicity (FET) Test on Zebrafish

The evaluation of mortality and malformation in zebrafish after exposure to EE was performed according to OECD guideline 236 (OECD, 2013) and literature methods (Huang et al., 2018, Alafiatayo et al., 2019). At 6-h post fertilization (6hpf), fertilized healthy embryos were selected, washed and examined under the microscope. Fertilized embryos were incubated with EE at difference doses (1, 5, 10, 20, 40, 80, 100, 200 µg/mL) in 6-well culture plates. Twenty fertilized embryos were used per treatment per well, and each treatment was repeated three times. The control group was exposed to medium containing only 0.04% DMSO. The plates were then placed in an illuminated incubator at 26 ± 0.5^{o}C. The exposure solutions were replaced at 24, 48, 72 and 96 hpf, to maintain water quality and the concentrations of EE. We checked for abnormal development and removed dead embryos/larvae when we replaced exposure solutions. Egg coagulation, mortality, hatching, yolk sac oedema, pericardial oedema, teratogenicity were recorded each day for five days of exposure with the aid of a microscopic image acquisition system (Nikon SMZ 1270). The number of spontaneous movements during 1 min was counted at 24 hpf. Coagulated embryos are milky white and appear dark under the microscope. Sign of death is the absence of heartbeat under the microscope. When larva's head or tail breaks out of the embryo membrane, hatching is successful. The presence of fluid above or below the yolk sac was considered as yolk sac oedema. Pericardial oedema was characterised by the inflation of the pericardial cavity to twice its size due to the presence of fluid. Counting formulas of lethality, hatchability and teratogenicity at each time point are given below:

Mortality (%) = death number/total exposed number × 100

Hatching rate (%) = hatched number/total surviving embryos number × 100

Teratogenicity rate (%) = abnormal number/total surviving number × 100

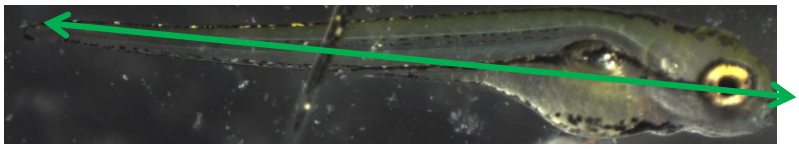
Embryo was considered abnormal if it had spinal curvature, pericardium edema, tail hypoplasia, yolk sac edema, and growth retardation.

Dead embryos or larvae were cleaned at each stage of observation. Embryo medium was changed once a day. At time point 120 hpf, larvae were anesthetized with 0.003% MS-222

(tricaine methane-sulfonate) before observation. Body length, pericardial area, heartbeat, yolk sac area were assessed using a microscope with digital camera and the support tool for measurement (NIS elements imagines software 4.4).

Measurement of body length

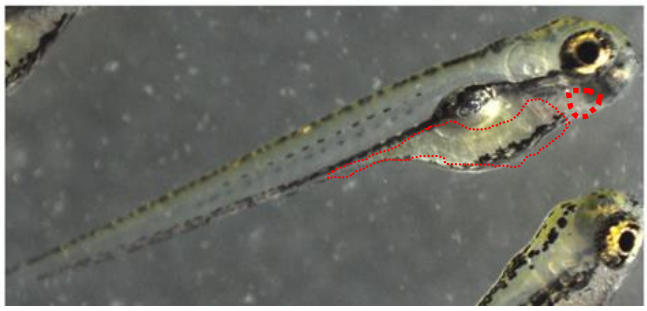
For body length, a line starting at the anterior-most point of the head and ending at the tail end was measured. All pictures were taken at the same resolution and magnification with the live fish positioned in a lateral orientation.



Pericardial sac area, yolk sac area

To measure pericardial and yolk sac areas, lateral view images of each embryo were taken at the same magnification, outline of the pericardial sac and yolk sac, respectively, was traced, and the

area within each tracing was determined by NIS elements imaging software.



The heart rate

The number of heartbeats for each larva was recorded by counting the beats per 20s under the stereomicroscope when the fish was stationary.

Classification of teratogenicity

The ratio LC50/EC50 (Teratogenic Index- TI) evaluation

A dose-response analysis was performed as previously described by Selderslaghs et al (Selderslaghs et al., 2012). Using Graph Pad prism, version 5.0, concentration-response curves for malformed, and mortality for each time point were created. The data were fitted to a sigmoidal equation with variable slope. This dose-response curve was used to determine the $EC50_{48h}$, $EC50_{72h,}$ $EC50_{96h}$ (teratogenic effect) and $LC50_{48h,}$ $LC50_{72h,}$ $LC50_{96h}$

(lethal/embryotoxic effects) values. These were derived from a four parameter equation describing the curve as follows:

Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope

Where Y is response (percentage of death or malformed individual).

X is log of concentration of EE.

(Bottom ≈ 0, Top ≈ 100).

Based on values of $LC50_{48h,}$ $LC50_{72h,}$ $LC50_{96h}$ and $EC50_{48h}$, $EC50_{72h,}$ $EC50_{96h}$, TI was calculated as the ratio LC50/EC50 for each time point. TI values allow ranking the compounds according to their teratogenic potency. The higher the TI value the greater the tetratogenic potential of a compound. Cut-off value of TI was 2, TI values higher than 2 indicating that the compound is considered as teratogenic (Selderslaghs et al., 2012).

The ratio of the NOAEL to LC25 (teratogenic index)

Based on the morphological assessments, the no observed-adverse-effect level (NOAEL) was identified as the highest dose at which there were no observed toxic or adverse effects on the development of zebrafish.

The LC25 of each compound was calculated by curve-fitting of incidence data for dead larvae. The curve-fitting model used was the 4-parameter logistic equation:

Y=Bottom + (Top-Bottom)/(1+10^((LogEC25-X)*HillSlope

The ratio of the NOAEL to LC25 (teratogenic index) was calculated as described by Brannen et al (Brannen et al., 2010). The highest concordance and predictivity were obtained when ratios greater than or equal to 10 were used to classify a compound as a predicted teratogen, whereas ratios less than 10 resulted in a classification of a predicted non-teratogen.

Analysis of mRNA expression by quantitative polymerase chain reaction (qRT-PCR)

To investigate the possible mechanisms for the developmental toxicity of EE, real-time quantitative polymerase chain reaction (qRT-PCR) was used to evaluate the mRNA levels of marker genes in whole embryos at 2 doses, 20 and 40 µg/mL. These concentrations were found to produce a fully and uniform phenotype in zebrafish embryo. At 6hpf, 20 fertilized eggs were treated with EE at the doses 20 and 40 µg/mL for 48 h. After 48h, zebrafish embryos were collected, frozen and stored at -80^{o}C for qRT-PCR analysis.

Quantitative PCR

Total RNA extraction, DNase and reverse transcription

Total RNA extraction, DNase and reverse transcription were carried out as described in previous our study (Nguyen et al., 2020a) using Trizol Reagent solution (Ambion,

Thermofisher Scientific), DNA-*free*™ DNA Removal Kit- Invitrogen and the RevertAid RT kit (Thermo Scientific).

The cDNA sample was diluted and used for real-time qRT-PCR to determine gene expression. β-actin and elongation factor 1α (*eef1a*) were used as housekeeping genes. The expression of genes related to oxidative stress (*cat, sod1, gpx4* and *gstp2*), wnt pathway (β-*catenin, wnt3a, wnt5, wnt8a* and *wnt11*), apoptosis (*p53, bax, bcl2, casp3, casp8* and *casp9, apaf-1, gadd45bb),* heart development (*gata5, myl7, myh6, has2, hand2* and *nkx 2.5*) were determined using specific primers. Primer sequences list are presented in **Table** 1.

Table 1. Primers pair used in this study

Gene name	**Full name**	**GenBank Accession No.**	**Forward and reverse primer sequences (5' -3')**
β-actin	Danio rerio beta-actin	AF057040	*Fwd:* CCCCATTGAGCACGGTATTG *Rev*: ATACATGGCAGGGGTGTTGA
eef1a	Danio rerio elongation factor 1 alpha	L23807.1	*Fwd:* CCAAGGAAGTCAGCGCATAC *Rev*: CCTCCTTGCGCTCAATCTTC
wnt3a	Danio rerio Wnt3a	AY613787.1	*Fwd:* GGTACGCAGCCCATAATGTG *Rev:* GGCCAGCTTGTCGTTGATAG
wnt8a	Danio rerio wingless-type MMTV integration site family, member 8a	NM_130946.3	*Fwd:* TGTAGACGCGCTGGAAAATG *Rev*: ACTTCCGTGCTTGATCATGC
β-*catenin*	Danio rerio b-catenin	U41081.1	*Fwd: ACCTCTGGCACCCTACACAA* *Rev*: *AGGGGAGCCGAGCATATTGA*
wnt5	Danio rerio Wnt5	U51268.1	*Fwd: CCGGAAGAATGGCGGTGTAT* *Rev*: *GGCGCTGTCGTATTTCTCCT*
wnt11	Danio rerio Wnt11 protein	AF067429.1	*Fwd: TTCGCTACTACGGCCTACAGAT* *Rev*: *ACAGAGCATGAGCCAGAAACG*
sod1	Danio rerio superoxide dismutase 1, soluble	NM_131294.1	*Fwd*: ATGGTGAACAAGGCCGTTTG *Rev*: AAAGCATGGACGTGGAAACC

gpx4b	Danio rerio glutathione peroxidase 4b	BC095133.1	*Fwd:*TGAGAAGGGTTTACGCATCCTG *Rev*: TGTTGTTCCCCAGTGTTCCT
cat	Danio rerio catalase	NM_130912.2	*Fwd:* TCCGGACATGGTTTGGGAT *Rev*: CGATCCGCTTCTTCAACAGG
gstp2	Danio rerio glutathione S-transferase pi 2	NM_001020513.1	*Fwd:* GGACTGGATGAAGGGTGACA *Rev*: ACGCTTCTTTACCGGTCTCA
casp 3b	Danio rerio caspase 3, apoptosis-related cysteine peptidase b	NM_001048066.2	*Fwd:* TCACAGTAAGTCGGCCATGTTT *Rev*: TCACCTACACCGTCACACTC
casp 9	Danio rerio caspase 9, apoptosis-related cysteine peptidase	NM_001007404.2	*Fwd:*TCAGCGGCACAGGTTAAACCTC *Rev*: AGTCTCACGCAGGGAATCAA
apaf1	Danio rerio apoptotic protease activating factor 1	BC116581.1	*Fwd:* AGTTCTTCTGACCACACGCAAT *Rev*: CCTGTTCTGGGAGTTTGTGC
bcl-2	Danio rerio Bcl2	AY695820.1	*Fwd:* GGGCGGATCATTGCATTCTT *Rev:* TCTGCTGACCGTACATCTCC
p53	Danio rerio tumor suppressor p53	U60804.1	*Fwd:* TACTTGCCGGGATCGTTTGAC *Rev:* TCAGGTCCGGTGAATAAGTGC
bax	Danio rerio Bax gene	AF231015.1	*Fwd:* CTGTGTGACCCCAGCCATAAA *Rev*: GATGACAAGGCGACAGGCAA
casp 8	Danio rerio caspase 8, apoptosis-related cysteine peptidase	NM_131510.2	*Fwd:* CCTTTTGCCGGATGCAGAAC *Rev*: TCCATCCGACGTCCAAACAC
gadd45bb	Danio rerio growth arrest and DNA-damage-inducible, beta b	NM_001012386.2	Fwd: CGCTTCAGATCCACTTCACG Rev: TCCCACTTCCTTCAGCTTGA
gata5	Danio rerio GATA binding protein 5	NM_131235.2	Fwd: CCACAGACTGGCACCGATAA Rev: GCGTACGGGCTGGAATAAGA

myl7	Danio rerio myosin, light chain 7, regulatory	NM_131329.3	Fwd: TGCACAACTAGGGAAGCTGAA Rev: GCAGCAAGGATGGTTTCCTC
myh6	Danio rerio myosin, heavy chain 6, cardiac muscle, alpha	NM_198823.1	Fwd: AAGCCACTACCGCCTCTCTA Rev: TGAGGCAAGGTCGTCCAA
has2	Danio rerio hyaluronan synthase 2	NM_153650.2	Fwd: CCTGAGCAGCGTGAGGTATT Rev: TGCAGTGGCTTCCCATGAAT
hand2	Danio rerio heart and neural crest derivatives expressed 2	NM_131626.3	Fwd: CGCGGATACGAAGCTATCCA Rev: GGCCAACCAGTTCTCCCTTT
nkx2.5	Danio rerio homeodomain protein Nkx2.5	U66572.1	Fwd: ACCCGGGTGAAGATCTGAAG Rev: TGGCTAGGTGGTCTCTCTCT

RT-PCR was performed using an ABI Step One Plus Real Time PCR system (Applied Biosystems). The thermal conditions used were 3 min at 95 °C of preincubation, followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. For analysis, a standard curve of a pool of the cDNAs of all samples was constructed to calculate the PCR efficiency and the quantity of an unknown sample. The relative gene expressions are presented as the ratio of the quantity of candidate gene/average quantity of housekeeping genes. It was used a pool of 20 larvae per group (n=3).

Data presentation and statistical analyses

Data analyses were performed using SPSS 22.0 software (SPSS Inc, Chicago) or GraphPad Prism Software 5.0. Shapiro-wilk test was used for normality check. Data are shown as mean ± SD in the case of data with a normal distribution, a *t*-test and one-way analysis of variance with LSD post hoc test were used to determine differences between the experimental groups. In case the data were not normally distributed, the non-parametric Kruskal-Wallis test was performed, followed by a Mann-Whitney test to determine significant differences between the experimental groups. A p-value less than 0.05 was considered statistically significant. LD50, EC50, LD25 was calculated using GraphPad Prism Software 5.0 (San Diego, CA, USA).

3. Results

3.1 Chemical constituent identification from EE by HPLC-DAD-Orbitrap-MS analysis

Identification of major compounds from the ethanol extract from *C. cyrtophyllum* Turcz leaves was performed by HPLC-DAD-Orbitrap-MS analysis**.** Major constituents of the ethanol extract were identified based on characteristic properties of the peaks in the chromatograms such as the retention times, UV absorption spectra, experimental mass data, best matching molecular formula provided by the Xcalibur software of the OrbitrapMS fragments and comparison with the reported data of other samples of *C. cyrtophyllum* Turcz leaves.

The results show that three major compounds were present in the the crude ethanol extract from *C. cyrtophyllum* (Figure 1). These compounds could be identified, by comparison of MS molecular formulas as acteoside (Zhou et al., 2020), cirsilineol and cirsilineol-4'-*O*-*β*-D-glucopyranoside (Cong Nhuong et al., 2006, Zhou et al., 2020) which were also previously isolated from other samples of this plant and reported in the literature (Cong Nhuong et al., 2006, Zhou et al., 2020). The HR-MS data of these compounds are summarized in Table 2 and their structures are presented in Figure 2.

Table 2. Identification of major compounds from *C. cyrtophyllum* leaves using HPLC-DAD-Orbitrap-MS method.

Nº	**Retention time**	**λmax (Metwally et al.)**	**Molecular ion *m/z* [M-H]$^-$**	**Mw**	**Formula**	**Error (ppm)**	**Compound**	**Reference**
1	20.58	243, 230	623.19690	624	$C_{29}H_{36}O_{15}$	-0.235	Acteoside (Verbascoside)	(Zhou et al., 2020)
2	26.62	245,275,335	505.13547	506	$C_{24}H_{26}O_{12}$	+2.806	Cirsilineol-4´-*O*-*ß*-D-glucopyranoside	(Cong Nhuong et al., 2006),(Zhou et al., 2020)
3	36.45	245,275,343	343.08237	344	$C_{18}H_{16}O_7$	+3.325	Cirsilineol	(Cong Nhuong et al., 2006),(Zhou et al., 2020)

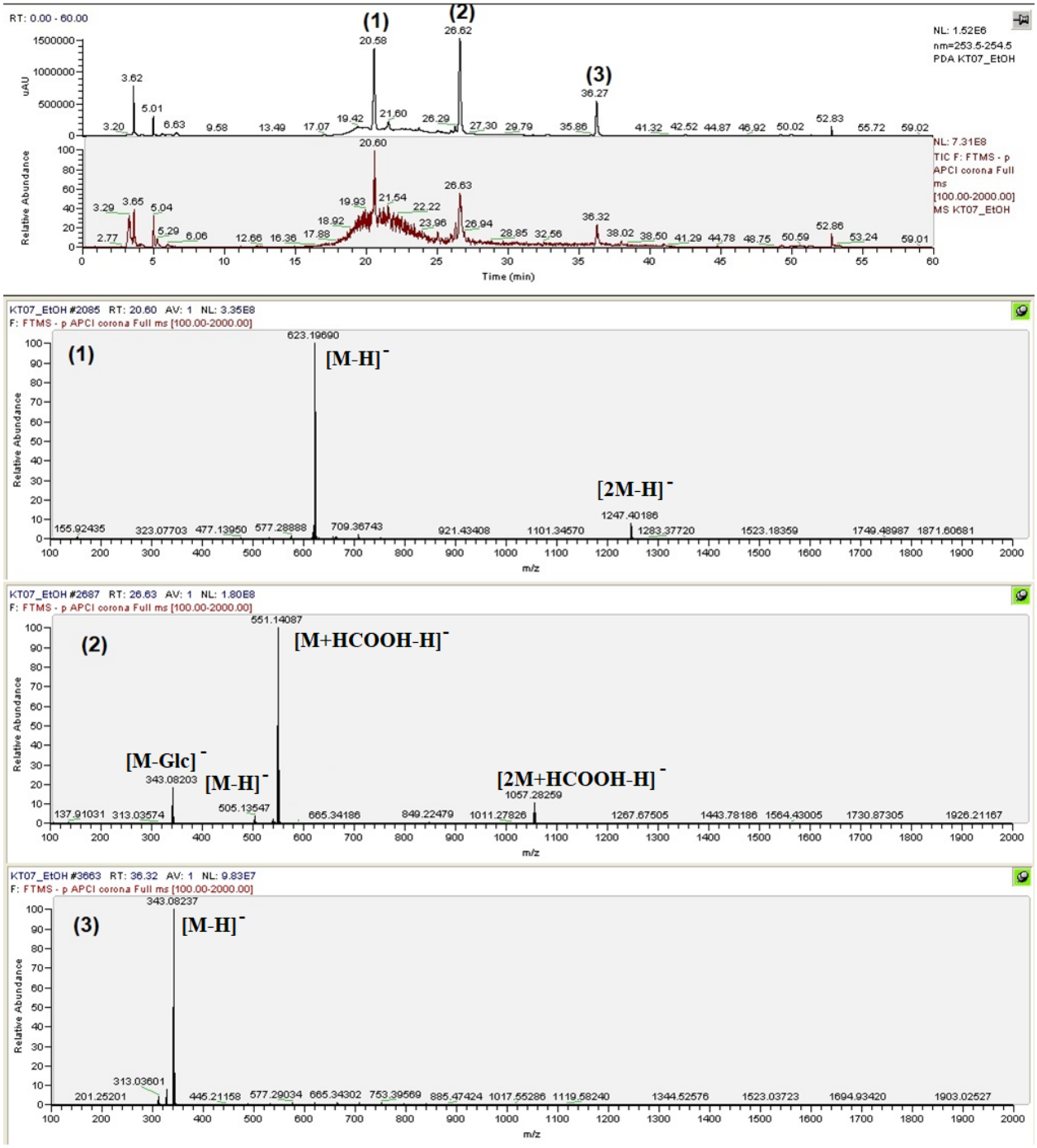


Figure 1. HPLC-DAD (upper) and HPLC-Orbitrap-MS-TIC (lower) chromatograms of the crude ethanolic extract of *C. cyrtophyllum* in negative ion mode.

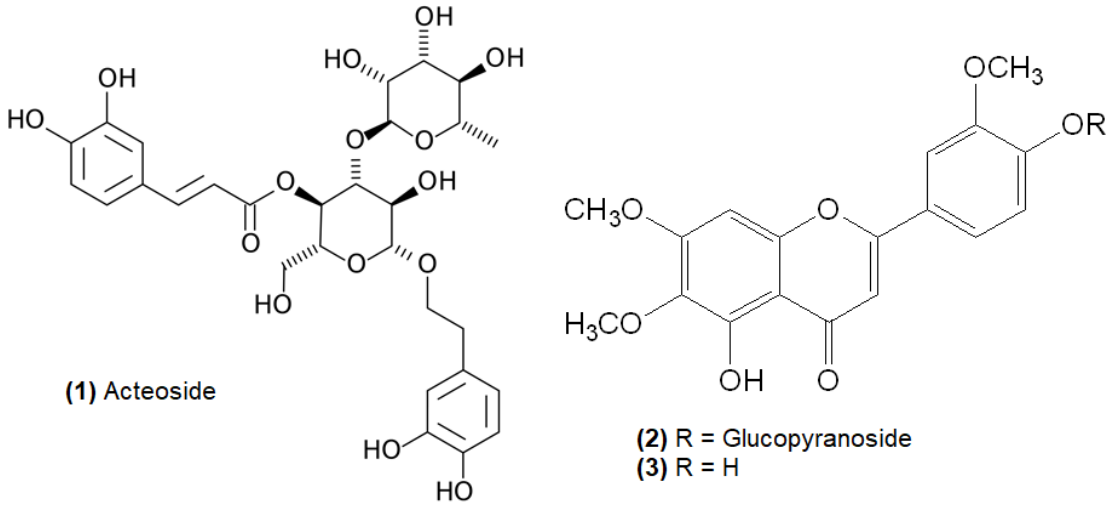


Figure 2. Structures of major compounds identified from *Clerodendron cyrtophyllum* Turcz leaves.

Embryo mortality and hatchability

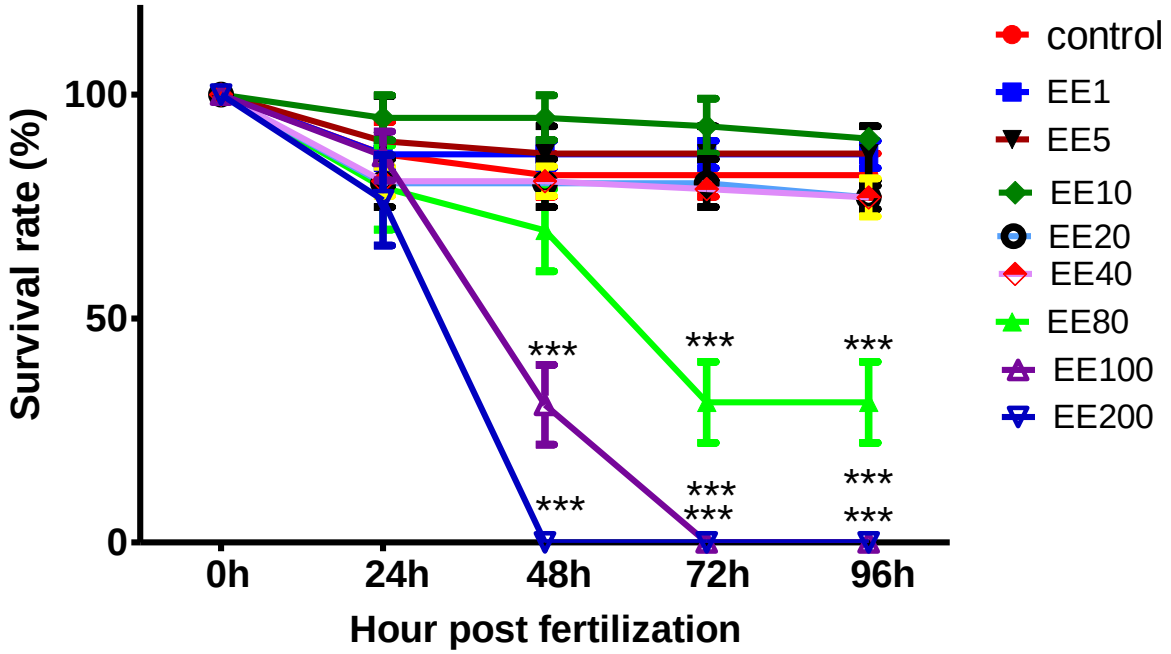


Figure 3. Survival rate of zebrafish embryo and larvae after treatment with the ethanol extract of *C. cyrtophyllum* (EE) at different concentrations. *The data are presented as mean ± SD. for three different experiments performed in triplicate, ***p<0.001 compared to the control group.*

The percentage of surviving embryo decreased as the concentration of EE increased (Figure 3). EE at concentrations below 40µg/mL had no obvious effects on the survival of embryo and

larvae. At 72 hpf, 31.3% of the embryos exposed to EE at 80µg/mL survived. Meanwhile, no embryo survival was observed at concentrations of 100 and 200µg/mL at 72 and 96 hpf.

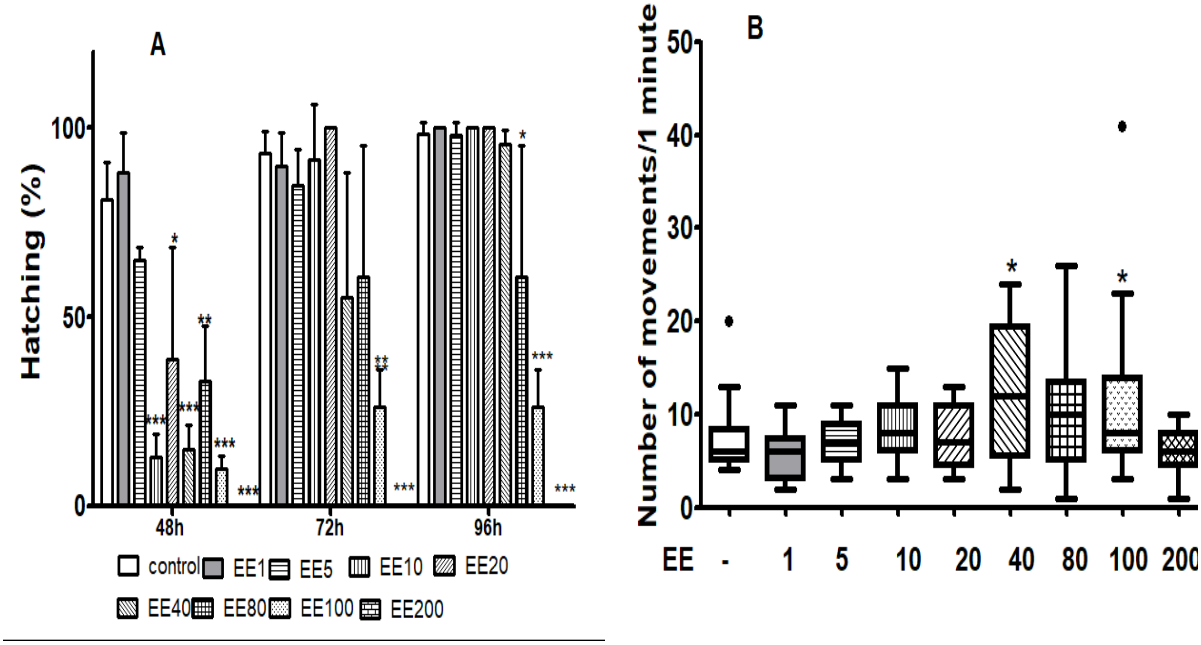


Figure 4 **A.** Hatching rate of surviving embryos at 48, 72 and 96 hpf after exposure to EE. *The data are presented as mean ± SD. for three different experiments performed in triplicate. * p<0.05, **p<0.01, and ***p<0.001 compared to the control group.*

B. Number of movements by surviving embryos at 24 hpf after exposure to EE (*Each bar represents median (interquartile range) for 15 different larvae, n=15,* p< 0.05; ** p<0.01;***p< 0.001 compared to control group).*

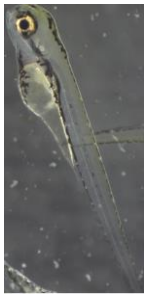
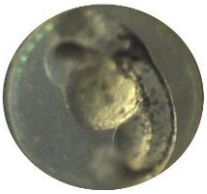
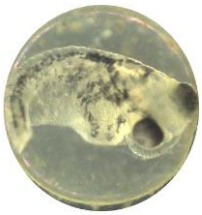
The effects of EE on the embryo hatching are shown in Figure 4A. Our results show that 81 and 93% of the control zebrafish embryos hatched after 48 hpf and 72 hpf, respectively. However, a significant decrease ($p<0.05$) in the hatching rate was observed at all concentrations in the range from 10 – 100µg/mL of EE as compared to control at 48 hpf. By 96 hpf, at concentrations of EE 80 and 100µg/mL, hatching rates were 61 and 26% respectively while no hatching was observed at 200µg/mL. These data indicated a remarkable dose- and time-dependent decrease in the hatching rate in the EE-treated groups compared with those of the control.

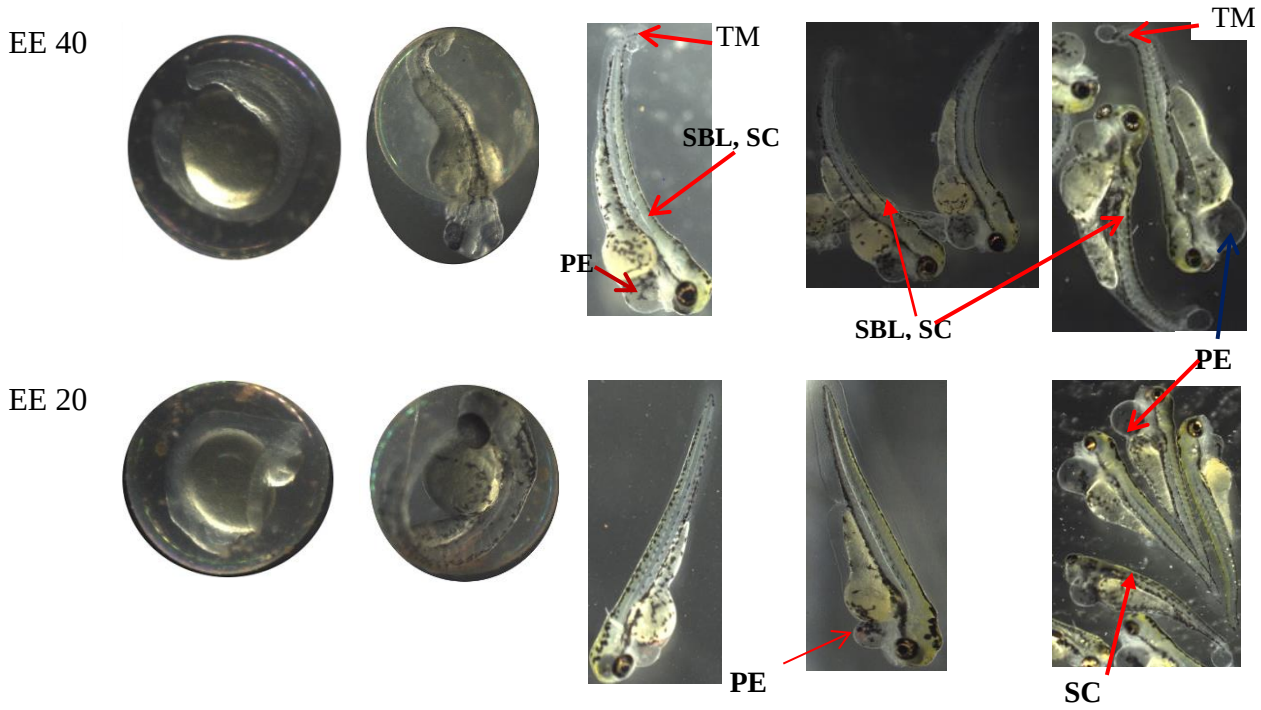
The hatching of embryos depends, among other processes, on the embryo movements. To further investigate the mechanism of the low hatchability rate, we measured the effects of EE on the embryo movements at 24 hpf. As shown in Figure 4B, at 24 hpf, the number of spontaneous movements in normal embryos was 6 movements/min. Exposure to EE had no

effects on the spontaneous movement of zebrafish embryo at low concentrations 1, 5, 10 and 20µg/mL. However, at high concentrations 40 and 100µg/mL EE significantly increased the number of coiling contractions in the embryos compared with the control group whereas the number of movements was not affected by EE at 200µg/mL.

3.2 Teratogenic effects of EE in zebrafish embryos and larvae

Table 3. Representative images of teratogenic effects of EE in zebrafish embryos and larvae

Group	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf
control					
EE 200					
EE 100					
EE 80					



PE: pericardial edema, SBL: short body length, SC: Spinal curvature, TM: tail malformation

At 24 hpf, no hatchings of embryos were observed. At 48 hpf, hatching of embryos was observed but no observable effects were noticed in 1, 5, 10 and 20 µg/mL concentrations, while unhatched darkened embryos were observed in EE 100 and 200 µg/mL. At 72, 96 and 120 hpf dead hatched larvae were observed at 80 µg/mL and morphological deformities such as spinal curvature, short body length, yolk retention, pericardial edema, and tail malformation were seen at EE 20 and 40 µg/mL (Tables 3).

3.3 Classification of teratogenicity

Table 4. LC50, EC50 and TI values for EE

	LC50 (µg/mL)	EC50 (µg/mL)	TI (LC50/EC50)
48 hpf	93.68	60.31	1.55
72 hpf	79.59	29.23	2.72
96 hpf	79.61	20.57	3.87

In this study, we used 3 criteria for risk classification of EE: LC50, the ratio LC50/EC50 and ratio LC25/NOAEL. The toxicity effect of EE was dose-dependent. The LC50 (for embryotoxic effects/lethality) and EC50 (for particular teratogenic effects) data were obtained from the dose-response curves (Table 4). According to OECD guidelines, toxicity of pollutants against zebrafish is categorized as harmful (10 mg/L < LC50 < 100 mg/L), toxic (1 mg/L < LC50 < 10

mg/L), and highly toxic (LC50 < 1 mg/L). Based on this, EE was found harmful. Based on LC50 and EC50 values, a teratogenic index (TI) was calculated as the ratio LC50/EC50 for each time point. TI values allowed ranking the compounds according to their teratogenic potency. In this study, with TI values of EE higher than 2, the plant extract is considered as a teratogenic compound.

Table 5. LC25, NOAEL and ratio LC25/NOAEL values for EE

	LC25	**NOAEL**	**LC25/NOAEL**
48 hpf	81.82	20	4.1
72 hpf	78.65	5	15.73
96 hpf	78.68	5	15.73

To further confirm the teratogenicity risk related to the use of EE, we also used the ratio LC25/NOAEL for the classification. Ratio LC25/NOAEL greater than or equal to 10 were used to classify a compound as a predicted teratogen. The LC25/NOAEL value of EE was 15.73 at both 72 and 96hpf (Table 5). Therefore, EE was correctly classified as a teratogen in the zebrafish embryo assay.

3.4 Cardiovascular toxicity

Heart rates, pericardial sac area, pericardial edema rate were recorded to determine the effects of EE on cardiac function. As shown in Figure 5, at 120 hpf, EE at 20 and 40 µg/mL produced a significant increase in the pericardial sac area and rate of larvae with pericardial edema compared with the control (Figure 5 A, B). However, no significant differences were observed for heart beat between the different experimental groups (Figure 5 C).

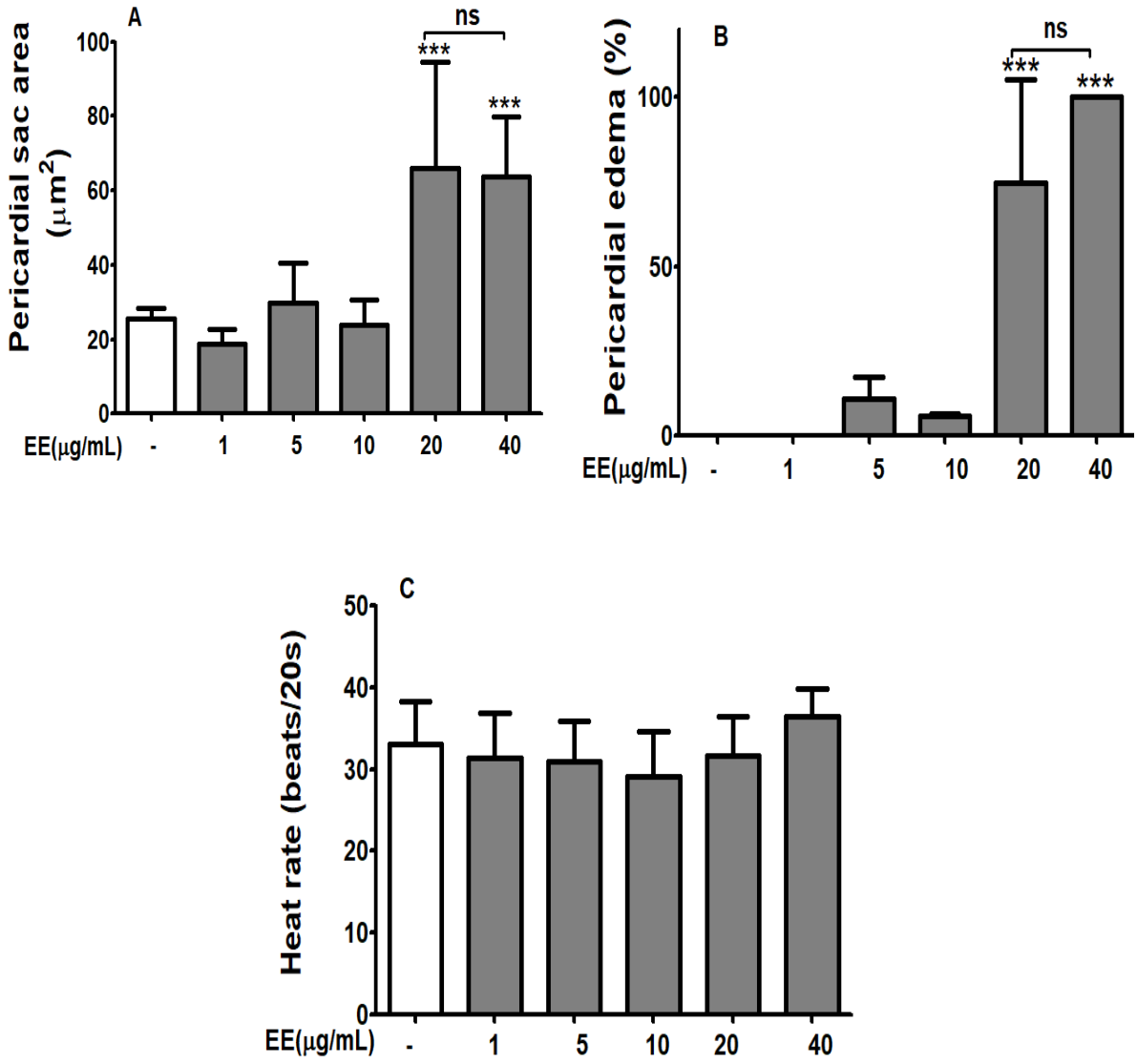


Figure 5. Effect of ethanol extract from leaves of *C. cyrtophyllum* (EE) on the cardiovascular system of zebrafish larvae at 120 hpf. Effects of EE on pericardial sac area (**A**) (*each bar represents the mean ± SD for 10 different larvae, n=10 * p< 0.05; ** p<0.01;***p< 0.001 compared to control group)* and rate of larvae with pericardial edema (**B**) (*each bar represents the mean ± SD. for three different experiments performed in triplicate. *p<0.05, ** p<0.01, ***p<0.001 compared to control group),* and heart rate (**C**) (*each bar represents the mean ± SD for 12 different larvae, n=12, * p< 0.05; ** p<0.01;***p< 0.001 compared to control group)*

3.5 Skeletal deformities and yolk sac

As shown in Figure 6 A, B, compared with the control group, the groups treated with EE 20 and 40 µg/mL increased the skeletal deformity rate and decreased the body length of zebrafish larvae at 120 hpf. At 40 µg/mL, EE even delayed nutrient absorption from yolk sac (Figure 6C).

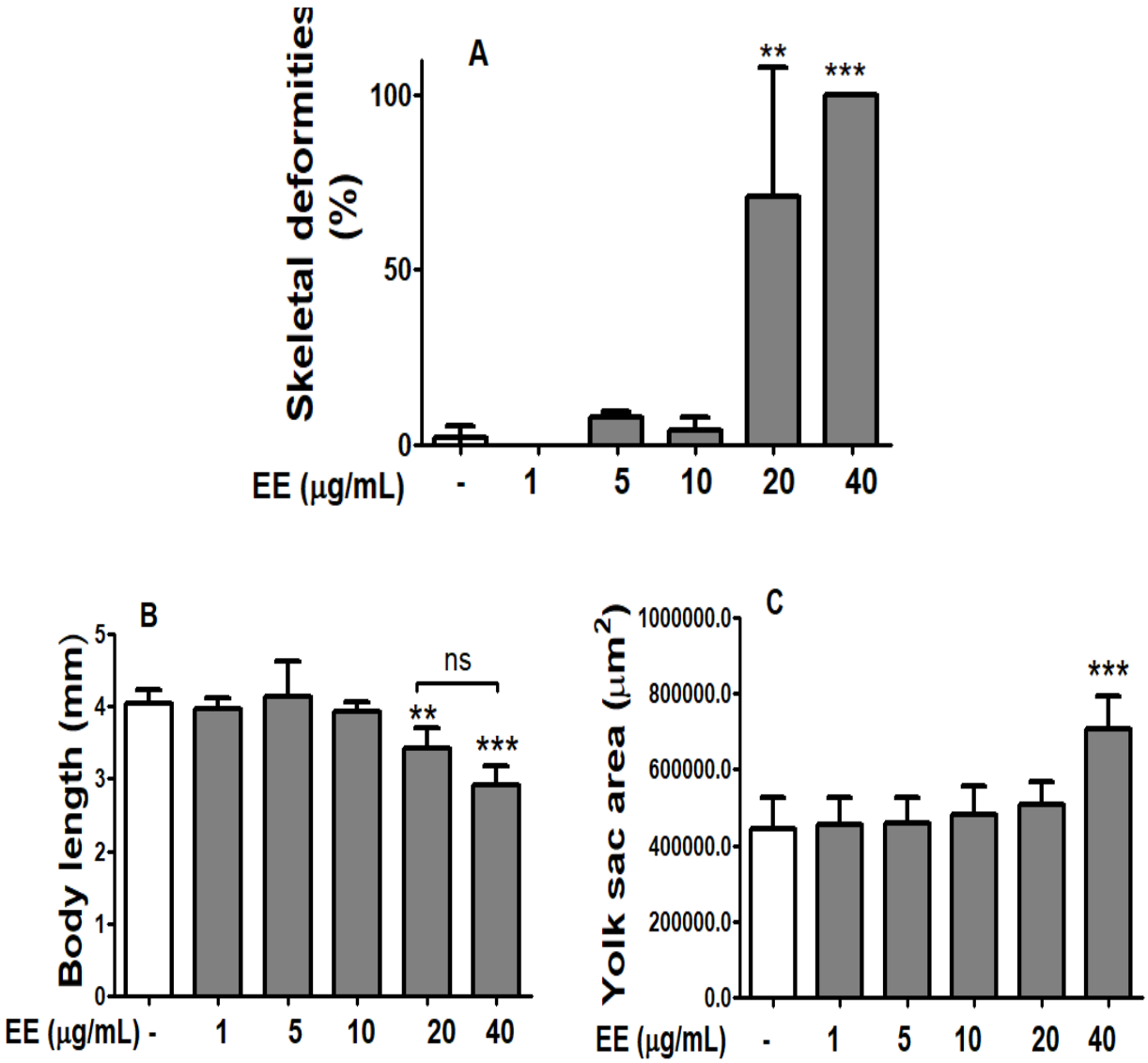


Figure 6. Effects of EE on rate of skeletal deformities (**A**) (*each bar represents the mean ± SD for three different experiments performed in triplicate. *p<0.05, ** p<0.01, ***p<0.001 compared to control group)* at 120 hpf ; body length (**B)** and yolk sac area (**C**) (*each bar represents the mean ± SD for 12 different larvae, n=12, * p< 0.05; ** p<0.01;***p< 0.001 compared to control group).*

3.6 Effect of EE on gene expression

Oxidative stress

To investigate the possible mechanisms of the toxic effects induced by EE, we used qRT-PCR to examine the mRNA expression levels of antioxidant genes in zebrafish embryo after exposure to different EE concentrations from 20 to 40 µg/mL. We observed that the mRNA levels of the oxidative stress-related gene *gstp2* were upregulated upon EE exposure compared with the control group while the level of other antioxidant genes (*sod1*, *cat* and *gpx4*) was not affected (Figure 7).

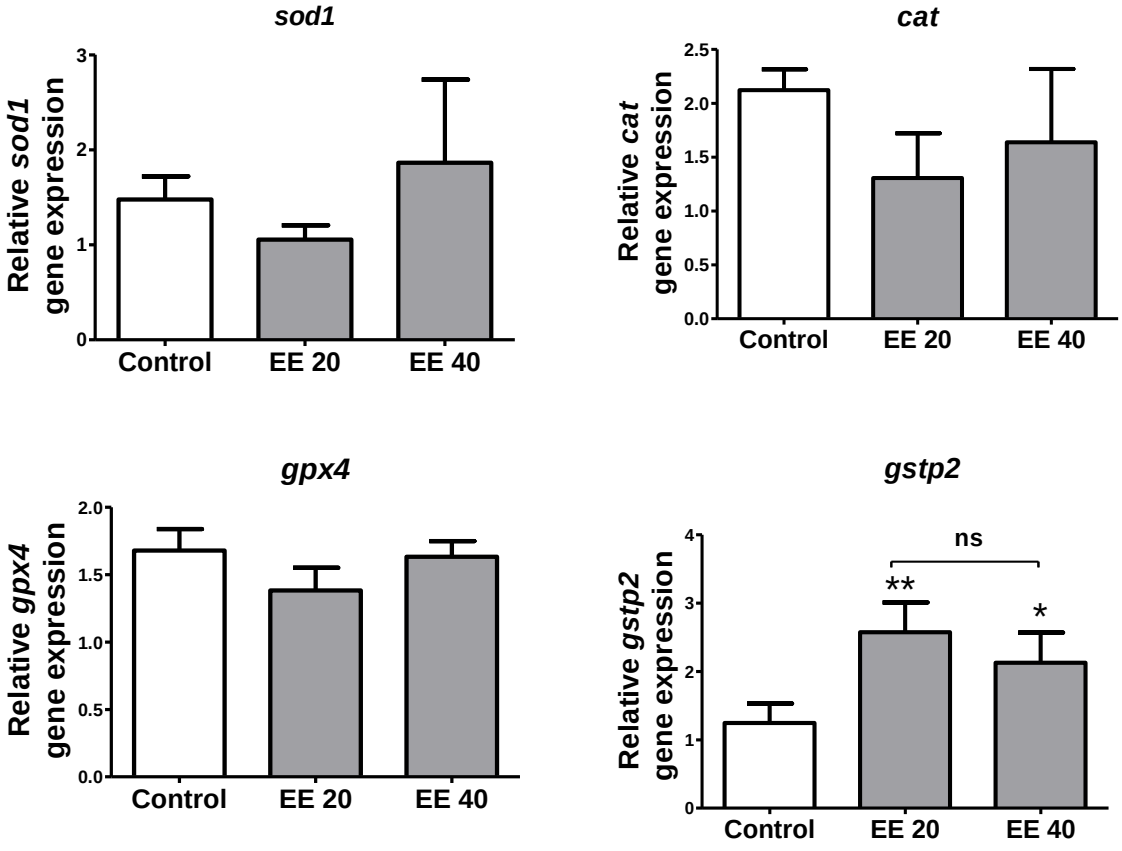


Figure 7. Effects of EE on antioxidant genes expression.

*Each bar represents the mean ± SD. for three different experiments performed in triplicate. *p<0.05, ** p<0.01, ***p<0.001 compared to the control group.*

Development of body patterning

EE induced skeletal deformities with bending of the body, spinal curvature, and bent tails. To investigate the possible mechanisms of abnormal body patterning induced by EE, we evaluated the effects on the expression of Wnt pathway-related genes. The mRNA level of *wnt3a, wnt11*

increased significantly upon EE exposure, while *wnt5* mRNA level decreased. The mRNA levels *ß- catenin* and *wnt8a* were not affected by the EE exposure (Figure 8).

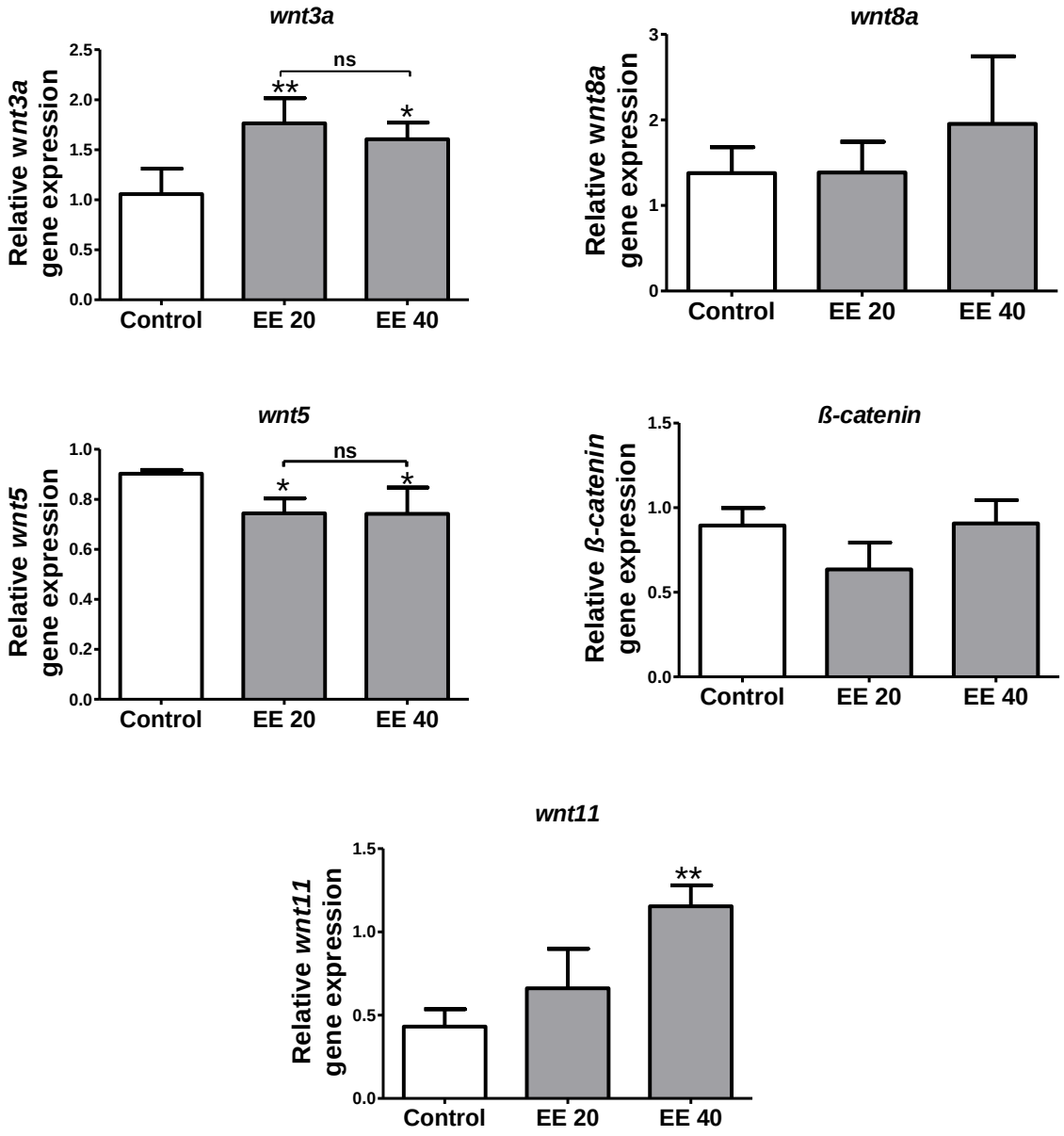


Figure 8. Effects of EE on the expression of genes related to the development of body patterning

*Each bar represents the mean ± SD. for three different experiments performed in triplicate. *p<0.05, ** p<0.01, ***p<0.001 compared to the control group.*

Cardiac development

Compared to control, EE at 20 and 40 µg/mL induced a significant increase in the pericardial sac area and in the rate of larvae with pericardial edema. To elucidate molecular mechanism for

heart defects, we evaluated the effects of EE on the expression of genes related to heart development, including atrial myosin heavy chain (*myh6*), cardiac myosin light chain (*myl7*), GATA-binding protein 5 (*gata5*), heart and neural crest derivatives expressed transcript 2 (*hand2*), hyaluronan synthase 2 (*has2*) and homeodomain protein nkx2.5 (*nkx2.5*). The mRNA levels of *gata5* and *nkx2.5* increased significantly after exposure to EE at 40 μg/mL. The mRNA levels of *myh6* also increased in the larvae exposed to EE at 20 μg/mL. On the other hand the mRNA levels *hand2* was decreased significantly with EE exposure at 20 and 40 μg/mL. The mRNA levels of *myl7* and *has2* were not affected by EE exposure (Figure 9).

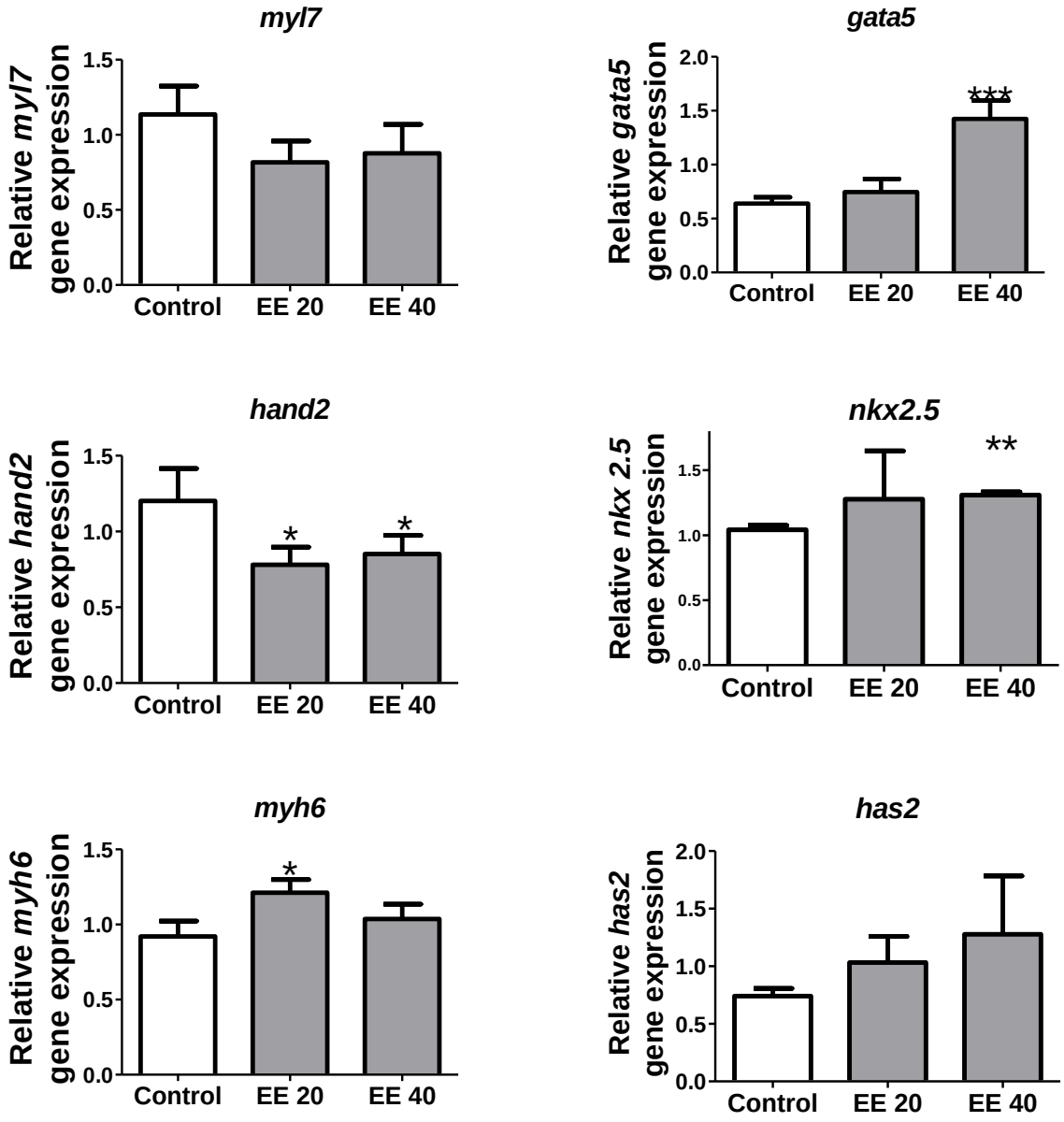
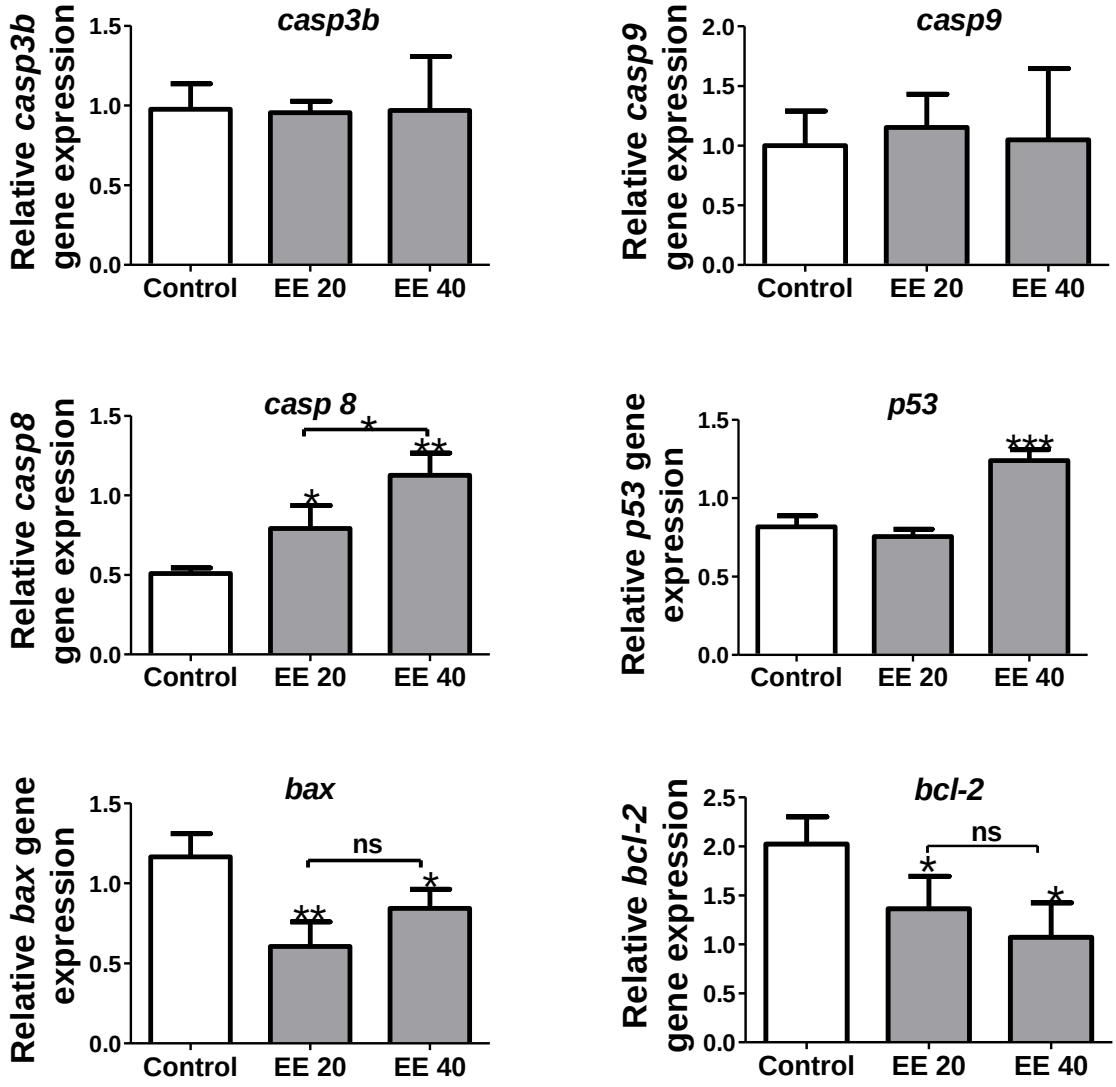


Figure 9. Effects of EE exposure on the expression of cardiac program related genes.

*Each bar represents the mean ± SD. for three different experiments performed in triplicate. *p<0.05, ** p<0.01, ***p<0.001 compared to the control group.*

Cell apoptosis-related genes

To assess whether EE induced apoptosis, the expression levels of apoptosis-related genes *p53, bax, casp9, casp3*, *casp8* and *bcl-2, apaf-1* were examined. The mRNA expression levels of *casp8, p53* and *bax/bcl-2* significantly increased with increasing EE exposure dose while mRNA levels of *bax, bcl-2* decreased (Figure 10).



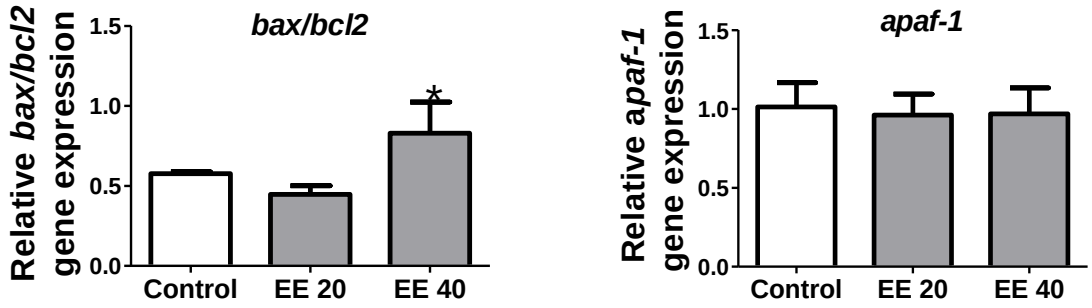


Figure 10. Effects of EE on the expression of cell apoptosis-related genes.

*Each bar represents the mean ± SD. for three different experiments performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 compared to the control group.*

Expression of gadd45bb gene

As shown in Figure 11, the mRNA level of *gadd45bb* was significantly upregulated by the exposure to EE at 40 μg/mL.

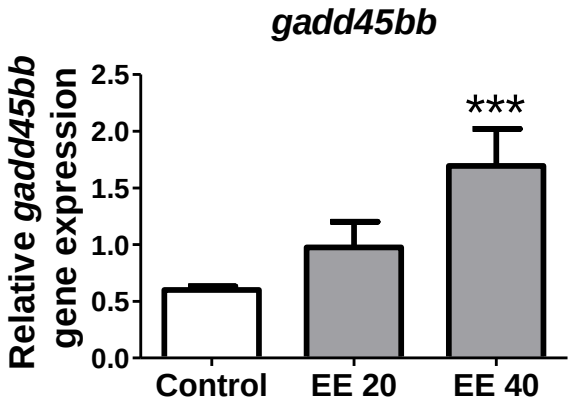


Figure 11. Effects of EE on the expression of *gadd45bb.*

*Each bar represents the mean ± SD. for three different experiments performed in triplicate. *p<0.05, **p<0.01, ***p<0.001 compared to the control group.*

4. Discussion

4.1 Identification of compounds from *Clerodendron cyrtophyllum* Turcz leaves

Three main compounds (acteoside, cirsilineol and cirsilineol-4'-*O*-*β*-D-glucopyranoside) were identified from EE in this study. The results are consistent with previous reports for phytochemical investigations from *C. cyrtophyllum* Turcz. Acteoside was found to be a major

component (0.803 g, 0.54%) in *Clerodendrum cyrtophyllum* Turcz leaf extracts (Zhou et al., 2020). Cirsilineol and Cirsilineol-4´-*O*-*β*-D-glucoside also were isolated from the ethyl acetate fraction of *Clerodendrum cyrtophyllum* Turcz leaves (Cong Nhuong et al., 2006, Zhou et al., 2020). These compounds are the main active ingredients and maybe responsible for the pharmacological effects but also the toxicological ones of *C. cyrtophyllum*. More research studies are needed to confirm the safety of the combination between cirsilineol or cirsilineol-4´-*O*-*β*-D-glucoside with verbascoside or other compounds in the crude extract, in order to evaluate the possible additive, synergistic or antagonist effects of this mixture.

4.2 Developmental Toxicity of *Clerodendrum cyrtophyllum* Turcz

In the present study, we reveal that the exposure of zebrafish embryos to ethanol extract of *Clerodendrum cyrtophyllum* impaired their early development by particularly decreasing the survival rate, inducing morphological abnormalities, and delaying the hatching rate. EE treatment at doses of 80 - 200 µg/mL obviously reduced hatching rate at 72 hpf. Hatching is a key step in the transformation of embryos into larvae and is one of the most important indices to evaluate the developmental toxicity in zebrafish. Delayed hatching may be due to retarded development or the inability of embryos to break the chorion. Hatching of embryos depends on the hatching enzyme activity and embryo movements (Zhang et al., 2015, Qian et al., 2018). In our study, the number of spontaneous movements during embryogenesis in groups exposed to EE from 40 – 100 µg/mL increased at 24 hpf, while the hatching rate decreased at all time points of observation. It can be deduced that hatching enzyme activity might be affected by EE, but this requires further studies.

The exposure of embryos to EE caused several developmental abnormalities, such as yolk sac retention, pericardial oedema, spine deformation and bent tails. The deformities were observed to be dose-dependent and increased with the time of exposure. Among the malformations, the most pronounced morphological alterations were pericardial edema and yolk sac edema. Congenital heart disease is the most common birth defect (Zaidi and Martina Brueckner, 2018). Damage to the heart may lead to inhibition of blood transport in embryos and then affect energy transport, further influencing the development of embryos (Qian et al., 2018). The yolk sac plays an important role during the early developmental stage, because it is the only source of nutrition for embryos. The yolk size will decrease along with the embryonic development (Zhang et al., 2015). In this study, exposure to EE at the dose of 40 µg/mL delayed nutrient absorption from yolk sac. Decreased absorption of nutrients can delay or impair growth of larvae.

Body length is an important indicator of embryo growth, and the loss of nutrients may induce a shorter body length. In this study, exposure to EE at the doses of 20 and 40 µg/mL exhibited a significant reduction in the body length of larvae. In addition, yolk lipids are the source of triacylglycerol and cholesterol, which are required for the synthesis of cell membranes, bile acids and steroid hormones. Unabsorbed yolk thus can affect the proper development of embryos (Anderson et al., 2011). Other morphological abnormalities observed in EE treated groups were spinal curvature, and bent tails. Spinal deformities are commonly identified as being one of the major pathological traits among fish dwelling in areas polluted with toxic chemicals (Cheng et al., 2000). Spinal curvature, and bent tails can be associated with early defects in somite formation that in turn leads to deformities of the muscle and skeleton. Spinal deformities also might be associated with a reduction in myosin because myotome formation critical for a robust musculoskeletal system formation (Zoupa and Machera, 2017). Taken together, these results demonstrated that developmental toxicity of zebrafish was induced by EE exposure.

Nkx2.5 expression is the earliest marker of precardiac cells and plays a critical role in differentiation and proliferation of myocardial cell and heart morphogenesis (Han et al., 2015). Knockdown of *nkx2.5* has been shown to cause a variety of cardiovascular deformities in heart chamber development as well as pericardial edema (Targoff et al., 2008). In this study, the expression of *nkx2.5* was significantly up-regulated with increasing EE concentration, which might reflect a compensatory response to repair or rescue heart development after its impairment. *Gata5* is a transcription factor which is necessary for the development of the heart (Han et al., 2015). Overexpression of *gata5* induces the expression of a few myocardial genes including *nkx2.5* and can produce ectopic foci of beating myocardial tissue (Reiter et al., 1999). In this study, the expression of *gata5* was significantly up-regulated at EE 40 µg/mL, which may enhance the regenerative proliferation of cardiomyocytes in response to the injury. *Hand2* is associated with cardiomyocyte formation, plays pivotal roles in heart morphogenesis and cardiac-specific transcription. Overexpression of *hand2* can enhance cardiomyocyte production, resulting in an enlarged heart with a striking increase in the size of the outflow tract. In contrast, Zebrafish *hand2* mutants can cause a striking cardiac phenotype that features a dramatic deficit of cardiomyocytes (Schindler et al., 2014). In this study, the expression of *hand2* was significantly downregulated at EE 20 and 40 µg/mL. The downregulation of this gene expression provides a potential mechanism for EE-induced cardiotoxicity. These results further confirm cardiotoxicity by EE induction at the gene expression level.

The Wnt signaling pathway, plays an important role in the early stages of vertebrate embryo development. It regulates key events during embryonic patterning, morphogenesis, and skeletogenesis (Li et al., 2018). In this study, treatment with EE up-regulated *wnt3a* and downregulated *wnt5*. Because Wnt signaling plays an important role in body patterning, the up- or down-regulations of *wnt* genes could consistent with the developmental defects in the EE treated groups. Wnt11 participates in the regulation of cardiac circulation and differentiation (Pandur et al., 2002). In our study, the expression of *wnt11* significantly increased at 40 μg/mL, explaining for the heart development defects of zebrafish in the EE treated groups.

In multicellular organisms, normal development depends upon the balance between cell proliferation, differentiation, and death. Apoptosis is the most common mechanism employed during development to remove damaged, misplaced, or otherwise unwanted cells (Eimon and Ashkenazi, 2010).There are two key apoptotic signaling mechanisms in mammals and other vertebrates: the cell-intrinsic pathway and the cell-extrinsic pathway. The intrinsic pathway is regulated by the *bcl-2* gene family and functions through the initiator protease caspase-9 (Eimon and Ashkenazi, 2010, Youle and Strasser, 2008). The extrinsic pathway is triggered by death receptor ligation and functions through a death-inducing signaling complex (DISC) that includes the initiator proteases caspase-8 and -10. The p53 pathway induces apoptosis by up-regulating the transcription of pro-apoptotic genes, and down-regulating anti-apoptotic genes, including *bcl-2*(Zhang et al., 2015). All pathways converge at the level of the effector caspases (caspase-3, -6 and -7), which carry out the molecular mechanics of programmed cell death (Eimon and Ashkenazi, 2010). We show that exposure of zebrafish to EE significantly decreased the expression levels of *bcl-2, bax* while the expression level of *caspase 8* and *p53* was up-regulated. These results indicate that apoptosis was induced by EE in zebrafish which is consistent with the phenotype of developmental toxicity.

It has been reported that embryonic development is especially sensitive to Reactive Oxygen Species (ROS). ROS can induce cell apoptosis, contributing to abnormal embryonic development (Zhang et al., 2015, Shi and Zhou, 2010). Drugs can induce oxidative stress by the reduction of cellular antioxidant defenses. In our study, the expression levels of *gstp2* were upregulated while the transcription of *sod1*, *cat* and *gpx4* was not affected. The increase of *gstp2* could be explained by a self-protection mechanism to antioxidative stress in fish. These results show that oxidative stress can play an important role in the developmental toxicity of EE.

Gadd45 participates in cell cycle arrest, cell survival or apoptosis and the regulation of signaling pathways. DNA-damaging agents and cellular stresses can induce transcription of the *gadd45bb* gene. In our study, the mRNA levels of *gadd45bb* were upregulated in the EE treated group. These results indicate that DNA-damaging and growth arrest were activated in zebrafish embryos exposed to EE and played an important role in its developmental toxicity.

4.3 Classification of teratogenicity

The teratogenic index (TI- LC50/EC50) is a popular approach to classify compounds according to their teratogenic potential. In our study, TI value of EE at 96 hpf was 3.87 (>2) classifying EE as teratogen. Recent studies found that LC25 also was adequate for defining a toxic concentration and the teratogenicity index, which included the LC25, successfully discriminated between teratogenic and non-teratogenic compounds (Brannen et al., 2010). In this study, LC25/NOAEL value of EE at 96 hpf was 15.73 (>10) suggesting that EE is teratogenic. LC25/NOAEL further confirm classify for teratogenic potential of EE. EE therefore should be avoided the use during pregnancy.

4.4 The developmental toxicity of acteoside, cirsilineol-4´-O-ß-D-glucopyranoside and cirsilineol in zebrafish embryo model

In this study, the exposure of embryos to EE caused decreasing the survival rate, inducing morphological abnormalities, and delaying the hatching rate. Molecular mechanisms for the developmental toxicity of EE were proved to involve on signaling pathways affecting fish embryo's development such as heart development, oxidative stress, wnt pathway or cell apoptosis. Acteoside, cirsilineol-4´-O-ß-D-glucopyranoside and cirsilineol are major compounds of EE and can contribute to the development toxicity of EE. A previous study showed that acteoside exerted prooxidant short-term effects, increased catalase activity and reactive oxygen species (ROS) signals and reduced blastocyst formation rate with long time exposure (Dell'Aquila et al., 2014). Reactive oxygen species (ROS) production could impair biomolecules like DNA, lipids and proteins, and manifesting unsettled signal transduction that can lead to inappropriate apoptosis and necrosis. Therefore, the effects of EE on the developmental toxicity in zebrafish embryo can be explained by presence of acteoside. The results on pregnant mice also showed that although acteoside exerted no significant toxicity on the mothers however there was still small rate of fetus with skeletal abnormalities (< 2 %) of cases include vertebral column deformity (<1%) and limb deformity (<1%) are observed in acteoside -treated groups (Etemad et al., 2016).

Cirsilineol-4´-O-ß-D-glucopyranoside and cirsilineol were identified in EE. The toxicity risk analysis showed that cirsilineol possesses a mild risk for skin and ocular irritancy but is non-mutagenic (Pathak et al., 2020)**.** In previous study, Cirsilineol has anti-inflammatory effect through cyclooxygenase inhibitory activity (Kelm et al., 2000). They attributed the effect to the inhibition of zebrafish cyclooxygenase enzyme (z-cox/ptgs) is necessary for the embryo early stage development (Grosser et al., 2002). Two different COX isozymes (COX-1 and COX-2) were widely expressed during development, COX-1 transcripts were detected in all tissues except brain. The most robust COX-2 signals were detected in gills, intestine, and testes, followed by heart, skeletal muscle, and the brain. Knockdown of COX-1 causes growth arrest during early embryogenesis (Grosser et al., 2002). Nonselective inhibition COX of cirsilineol in the embryo stage can explain for the development toxicity of EE.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most prescribed medications for treating inflammatory diseases. NSAIDs work by inhibiting the activity of cyclooxygenase enzymes (COX-1 or COX-2). The recent studies revealed that NSAIDs such as ibuprofen and diclofenac induced the developmental toxicity in zebrafish embryos. At low concentrations, ibuprofen and diclofenac impair the cardiovascular development of zebrafish (Zhang et al., 2020). Exposure to high concentration of ibuprofen and diclofenac significantly decreased hatch rate, the spontaneous movement and the free swimming distance (Xia et al., 2017). Diclofenac induces short body length, smaller eye, pericardial and body edema, lack of liver, intestine and circulation, muscle degeneration, and abnormal pigmentation in zebrafish larvae. Transcriptional analysis of genes involved in cardiovascular physiology, such as *nppa* and *nkx2.5* significantly up-regulated by ibuprofen. The portion of the diclofenac transferred into the embryo altered the expression of certain genes, e.g. down-regulation of *Wnt3a* and *Gata4* and up-regulation of *Wnt8a* (Chen et al., 2014). These results are similar to EE, so the development toxicity of EE may be relate to cyclooxygenase inhibitory activity of cirsilineol.

Cirsilineol-4´-O-ß-D-glucopyranoside has not been reported previously for toxicity or pharmacology effect. However, the presence of Cirsilineol-4´-O-ß-D-glucopyranoside can contribute for toxicity of EE, too.

4.5 The relationship between therapeutic use and toxicity

Women are the primary consumers of traditional medicines, and usually continue using them during pregnancy. Pregnant women usually use tradtional medicines as a safe and natural alternative to conventional drugs and often use them to improve their wellbeing or for the treatment of non-life threating conditions (Illamola et al., 2019). However, data on the safety

of these herbal treatments during pregnancy are generally insufficient. *Clerodendrum cyrtophyllum* Turcz is widely used in traditional medicine for the treatment of many diseases. Pregnant women also are consumers of *Clerodendrum cyrtophyllum.* The results of this study showed that using EE for pregnant women can induce defects or even mortality for embryo. Due to its teratogenic effects, *Clerodendrum cyrtophyllum* EE should not be recommended during pregnancy.

5. Conclusion

Our results demonstrate that EE caused developmental toxicity to zebrafish embryos/larvae. EE exposure at doses ranging from 80 to 200 µg/mL increased the mortality of embryos and reduced the hatching rate. Exposure at 20 and 40 µg/mL until 72 hpf – 120 hpf induced a series of symptoms of malformation including yolk sac oedema, pericardial oedema, spine deformation, shorter body length. The mRNA expression levels of *p53, casp8, bax/bcl2, gstp2, nkx2.5, gata5, wnt3a, wnt11, gadd45bb* were significantly upregulated upon EE exposure at 20 and 40 µg/mL while the expression of *wnt5, hand2* and *bcl2* were downregulated. These results provide evidence of toxicity of EE to embryo stages and provide an insight into the potential toxic mechanisms on embryonic development. As zebrafish embryo development is sensitive to EE, this extract could also have potential risks for the development of human embryos, therefore caution should be taken when consuming it during pregnancy.

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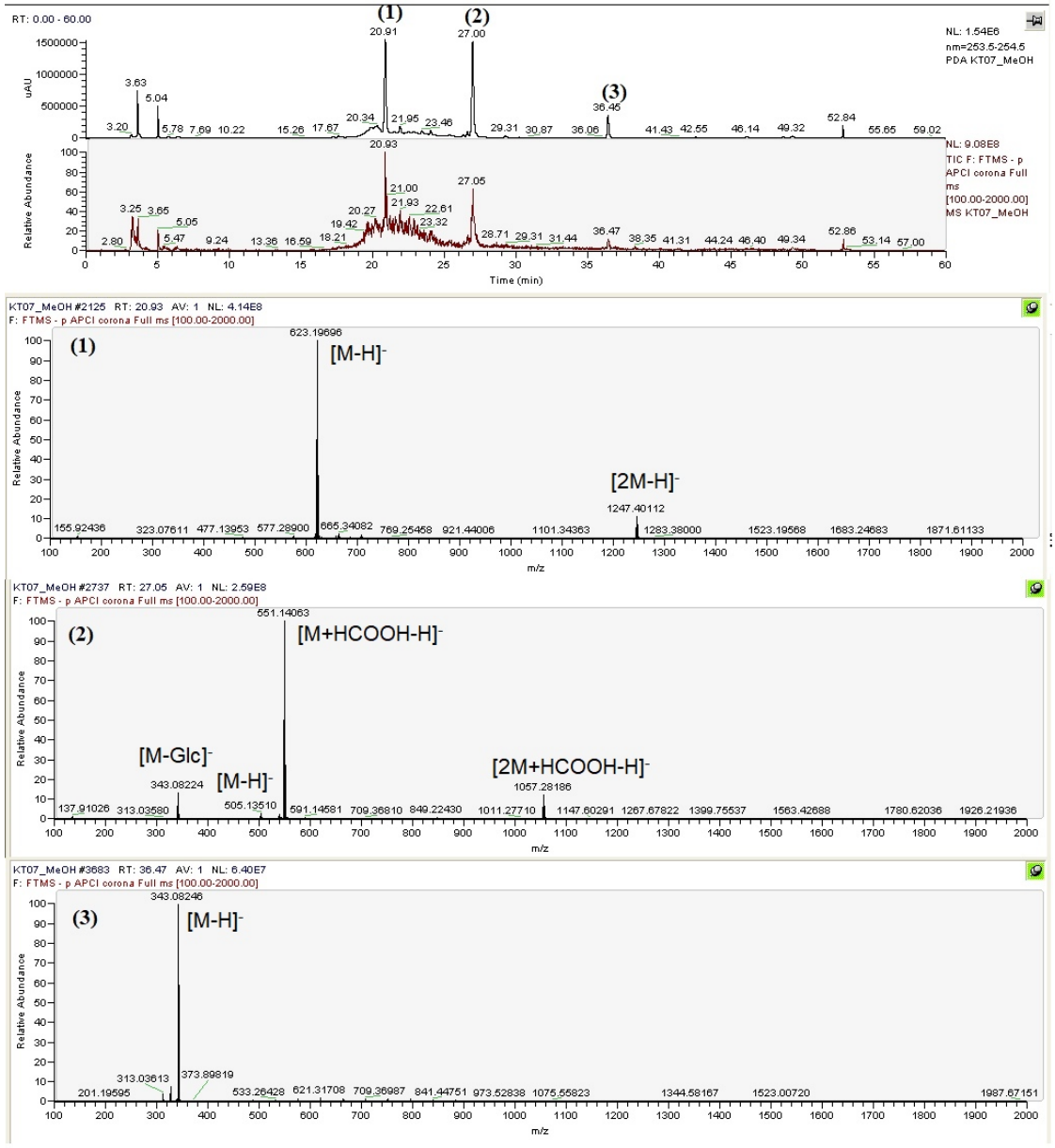

Figure 8. HPLC-DAD (upper) and HPLC-Orbitrap-MS-TIC (lower) chromatograms of the methanol extract of *C. cyrtophyllum* in negative ion mode and MS spectra of the three peaks.

Table 5. Identification of major compounds from *C. cyrtophyllum* leaves using HPLC-DAD-Orbitrap-MS method.

Nº	Retention time	λ_{max}	Molecular ion *m/z* [M-H]$^-$	Mw	Formula	Error (ppm)	Compound
1	20.91	244,230	623.19696	624	$C_{29}H_{36}O_{15}$	-0.139	Acteoside (Verbascoside)
2	27.00	243,276,335	505.13510	506	$C_{24}H_{26}O_{12}$	+2.074	Cirsilineol-4'-O-*β*-D-glucopyranoside
3	36.45	245,275,342	343.08246	344	$C_{18}H_{16}O_7$	+3.587	Cirsilineol

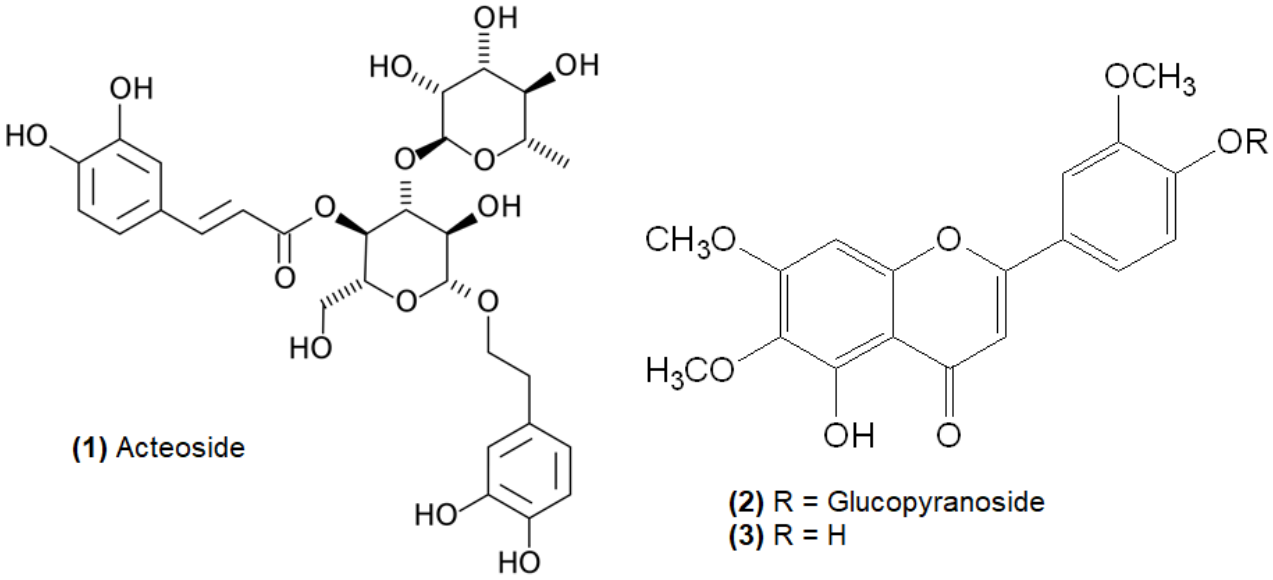

Figure 9. Structures of major compounds identified from the methanol extract of *Clerodendrum cyrtophyllum* leaves.

4. Discussion

4.1 Anti-inflammatory activity of the extracts from leaves of *C.cyrtophyllum* Turz

In the membrane stabilization model, the ethyl acetate and methanol extracts showed strong activity against heat-induced hemolysis, while the *n*-hexane and dichloromehthane extracts showed lower activity. This suggests that methanol and ethyl acetate are good solvents for the extraction of the active compounds present in the leaves of *C. cyrtophyllum* such as flavonoid glycosides and phenolic acids [12]. Phenolic compounds act as antioxidants and are responsible for stabilizing the cell membrane [13]. Supporting these data, a previous study of Babbar et al (2014) revealed that the methanol and ethyl acetate showed higher efficiency than *n*-hexane and chloroform solvents in extraction of phenolic compounds of different vegetables [12]. In another study, *n*-butyl alcohol extract of *C. cyrtophyllum* showed the highest phenolic and flavonoid contents while the ethyl acetate extract showed the highest antioxidant activity [11]. This accounts for the potent activity of methanol and ethyl acetate extract on cell membrane stabilization.

Nitric Oxide is a molecule that is involved in a variety of biological functions in the body. In the inflammation response, NO is considered as a key mediator, its overproduction can lead to tissue damages associated with acute and chronic inflammation. Therefore, NO inhibitors are potential compounds for the treatment of inflammatory diseases [14]. Our results revealed that the inhibition of NO generation between the different extracts was in the order ethyl acetate >

Chapter 9

General discussion

1. Inflammation response: benefits and risks

Inflammation is the response of the immune system to recognize and remove harmful and foreign stimuli and begins the healing process [1]. It is a part of the body's defense mechanism.

Benefits of inflammation:

- In response to inflammatory stimuli, innate immune cells are activated and migrate to the inflamed site. At the site of inflammation, they phagocyte and remove harmful stimuli such as pathogens and cellular debris [2]. Therefore, inflammation can prevent the spread of tissue injury and infection to other areas of the body.
- Inflammatory response also includes resolution and healing process. Pro-resolving mediators block leukocyte trafficking to the inflamed site, the induction of leukocyte apoptosis, reverse vasodilation, restore of normal function and integrity of the inflamed tissue [3]. Resolution process may prevent excessive tissue damage, the progression from nonresolving acute inflammation to chronic inflammation.

Risk of inflammation?

Inflammation is a protective response of the body. However, it also can induce some *symptoms such as* pain, heat, redness, swelling, and loss of function at an inflamed site. Depending on the precise nature of the initial stimulus and its location in the body, inflammation can induce different clinical signs such as fever, shortness of breath and cough in pneumococcal pneumonia, or swollen and joint pain, joint stiffness in arthritis; congestion, runny nose or sneezing in rhinitis, etc. In some cases, the uncontrolled systemic inflammatory reaction can lead to a harmful homeostatic process, even to patient’s death for example cytokine storm in COVID -19 [4]. The persistent or excessive inflammatory symptoms can affect the patient’s quality of life, using anti-inflammatory drugs or drugs to reduce inflammatory symptoms are necessary in these cases.

Inflammation is harmful in autoimmune diseases. The immune system can trigger inflammation when there are no invaders to fight off in autoimmune diseases. In these cases, the inflammatory

reaction is not beneficial and needs to be inhibited. Anti-inflammatory drugs help to reduce symptoms and the rate of disease progression.

Inflammation is the body's way of dealing with infections and tissue damage. In most cases, the inflammatory response eventually subsides. However, uncontrolled inflammation may contribute to the pathogenesis of a variety of chronic diseases such as the cardiovascular, metabolic, musculoskeletal, nervous and immune systems[5]. Recent evidence showed that increases in IL-6 and IL-6sR are linked to prostate cancer progression and metastasis [6]. Serum TNF- and IL-6 concentrations correlated with the severity of left ventricular dysfunction [7]. Ulcer recurrence is caused by *Helicobacter pylori* and NSAIDs through the accumulation of IL-1 and TNF-α at the ulcer scar [8]. High levels of TNF-α, IL-1, and IL-6 are associated with worse mental and physical morbidity in patients with sleep disorders, depression, and schizophrenia [9], [10]. In rheumatoid arthritis, cytokines (TNF-α, IL-1, IL-6 and IL-8) play a critical role in synovial inflammation and joint destruction. Chronic inflammation is a central characteristic of rheumatoid arthritis [11]. Fifteen (15 %) of human cancers are estimated to be associated with chronic infection and inflammation[1]. In these cases, the inflammatory response will require pharmacological intervention[12]. The need for anti-inflammatory drugs arises when the inflammatory response is inappropriate, over-vigorous, sustained, persists longer than is necessary, or destroys tissue.

What are good anti-inflammatory drug targets?

Treatment of inflammatory diseases today is largely based on an inhibitory/blocking action or the synthesis of inflammatory mediators. The important inflammatory mediators that are therapeutic targets for anti-inflammatory drugs include eicosanoids, biological oxidants, cytokines [12]. Cytokines (TNF-α, IL-1, IL-6, IL-2) and cytokine receptors are current targets of anti-inflammatory drugs (Table 1) [5]. Anti-cytokine drugs have been widely used as immunosuppressive agents to treat autoimmune diseases such as anti-IL1 (anakinra), anti-IL6 (tocilizumab), anti-TNF- α (etanercept). Actemra (tocilizumab), Kevzara (sarilumab) block IL-6 release. These drugs have been approved in the treatment of COVID-19 patients with systemic inflammation [13]. Biological oxidants such as nitric oxide (NO), hydroxyl radical (OH)… are potent bacterial killers but also contribute to tissue injury. Inhibition of production or inactivation of these oxidants is an important strategy for the treatment of inflammatory disorders [12]. COX-2 and PLA2 are important enzymes. The inhibition of these enzymes is the major target for conventional anti-inflammatory drugs (NSAIDs) [14]. However, drugs used to treat inflammatory disease might act either by preventing further inflammation (anti-

inflammatory) or by promoting inflammation resolution [2]. Pro-resolving mediators have been shown to exert powerful anti-inflammatory effects in various experimental models of inflammatory diseases[3]. Inflammatory resolution can be a new opportunity for drug discovery.

In our study, treatment with the ethanol extract of leaves from *C. cyrtophyllum (EE)* interrupted the synthesis or release of key mediators. EE inhibited the release of NO and TNF-α. In addition, the expression of genes related to inflammatory processes such as *cox-2*, *pla2*, *c3a*, cytokines (*il-1ß*, *il-8*, *tnf-α*), and of *nf-ƙb* were downregulated in the presence of EE in the cut-tail zebrafish moded and the expression of the anti-inflammatory cytokine *il-10* gene was upregulated (chapter 3). The treatment with EE also significantly decreased the expression of *cox-2*, *pla2*, *c3a*, *mpo*, *il-8*, and *il-1ß* in the copper sulfate-induced inflammatory zebrafish model (chapter 4).

Table 1. Cytokine-based therapeutics [5]

Cytokine-based therapeutics (approved drugs)		
Cytokine	**Therapeutic**	**Disease**
Interferon α2a	Intron® A Roferon® Pegintron® Pegasys®	Hepatitis B, hepatitis C
Interferon β1	Rebif® Avonex® Betaseron®	Multiple sclerosis
Interferon γ	Actimmune®	Chronic granulomatous disease
TNF-α (antagonists)	Remicade® Enbrel® Humira®	Rheumatoid arthritis, Crohn's disease, ankylosing spondylitis, Psoriatic arthritis Rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, Rheumatoid arthritis
IL-1β (antagonist)	Kineret®	Rheumatoid arthritis
IL-2 (antagonist)	Zenapax®	Transplantation
IL-2R (receptor antagonist)	Simulect®	Transplantation
IL-6R (receptor antagonist)	Actemera® (MRA–IL-6R mab)	Castleman's disease (approved in Japan, June 2005)
IL-11	Neumega	Chemotherapy-induced thrombocytopenia

2. Time-course of changes in inflammatory response

The inflammatory response changes with time and can be divided into phases. The acute phase often triggers within seconds to minutes after exposing stimuli. Acute inflammation lasts for a few days. If this process is unresolved, acute inflammation develops to subacute and lasts from 2 to 6 weeks. After 6 weeks, chronic inflammation appears with the migration of T lymphocytes and plasma cells. Chronic inflammation may last for months or even years if inflammatory stimuli persist and can't be eliminated.

In our study, cut tail zebrafish and copper sulfate-induced inflammation zebrafish model are acute inflammatory models. In the cut tail zebrafish model, neutrophil recruitment is one of the first events of the inflammatory response [15]. Neutrophils migrate to tissue injury and initiate phagocytosis or removal of damaged tissues. Macrophages often present later at 1-h post-wounding. The neutrophils hit the peak at 4 h post-wounding. The macrophages hit the peak within 24 to 48 h upon injury and are drastically reduced over the following three days [16]. Upon wounding, pathways NF-κB and MAPK are activated. The inflammatory mediators such as cytokines, chemokines, acute phase proteins are produced. Several hours after the wounding, the response enters the resolution phase. In the resolution phase of the inflammatory response, neutrophils leave the wounded area or undergo apoptosis. Tissue repair can be observed gradually and complete regeneration can be established 3 days later. Depending on the purpose of the study, the inflammatory response can be evaluated at different times. To evaluate the effect of drug on the neutrophil migration response, neutrophils are observed at 1 – 6 h post cut tail, neutrophil numbers peak at 4 h post-wounding [15]. For inflammation-resolution assays, larvae were treated drugs at 6 h post-injury, neutrophil numbers were counted at 12 h post-injury [2]. To observe the change in pro-inflammatory genes, larvae were collected at 6 or 24 h post-injury [17]. In our study, the inflammatory response was evaluated at 24 h after cut tail zebrafish. It is still in range of time-course of inflammatory response in the cut tail zebrafish model. Cut tail increased significantly the expression of *cox-2, pla-2, c3a, il-1, il-8, tnf-α* and *nf-κB* in zebrafish at 24 h post cut tail.

In copper sulfate-induced inflammation in the zebrafish model, excessive copper from the environment could lead to an inflammatory response mediated by damaged hair cells in the neuromasts and oxidative stress. The damage of the neuromasts results in the infiltration of macrophages and neutrophils and increased the expression and release of pro-inflammatory mediators [18]. Leite et al (2013) observed the oxidative stress at different time-points of 0, 4, 6 and 24 h. The results showed that SOD and CAT activities and GSH content were reduced after 24 h of exposure to copper. NO and PGE2 levels increased at 24 h. Neutrophil migration increased after 4 h of exposure to copper. The RT-qPCR quantification showed a significant increase of *il-1β, cox-2* and *tnf-α* expression at 4 and 24 h after copper exposure[19]. In our study, oxidative stress and inflammatory responses were also evaluated at 4 and 24h of copper exposure. The results of copper exposure in antioxidant enzyme activities, the expression of antioxidant genes and pro-inflammatory genes showed similar results. EE reduced or prevented the reactive oxygen species formation induced by copper sulfate in zebrafish model and increased the activity

of endogenous antioxidants including glutathione (GSH*)*, glutathione *S*-transferase (GST), and catalase (CAT) at 24 h after copper exposure (chapter 5). The increase in these antioxidant activities contributes to the elimination of ROS from the cell, indicating a protective role of EE against oxidative damage of $CuSO_4$. Regarding the expression of genes related to oxidative stress, co-administration with EE inhibited oxidative stress via the upregulation of *sod* and the downregulation of *gstp2, hsp70* and *gadd45bb* at 4 h after copper exposure. The treatment with EE also significantly decreased the expression of *cox-2, pla2, c3a, mpo, il-8,* and *il-1ß* in the copper sulfate-induced inflammatory zebrafish model at both 4 and 24 h after copper exposure (chapter 4).

3. The timing of administration of the therapeutic agent: pre-treatment, co-treatment or post-treatment?

One of the important decisions that can be made when evaluating the effect of a drug in animal models of disease is the timing of administration of the therapeutic agent. In fact, the large majority of the studies used pretreatment protocols to test the ability of a drug to prevent or attenuate the induction of disease[20]. There may be some reasons for pretreatment. First, pretreatment has the advantage that the drugs are absorbed and reach a steady-state before the inflammation process was stimulated. It is suitable for drugs with poorly water-soluble, slowly absorbed drugs or low bioavailability. Second, the acute inflammation phase occurs very fast within seconds to minutes after being stimulated by an inflammatory reagent. During this phase, moderate amounts of inflammatory mediators are produced such as like cytokines, acute phase proteins, and chemokines to promote the migration of neutrophils and macrophages to the area of inflammation. If the administration of the therapeutic agent after disease onset, it is difficult to reverse the preexisting inflammation response. It is difficult to observe the anti-inflammatory effect of drugs, especially with traditional medicine. Therefore, treatment is often initiated either before or shortly after the onset of disease in animal models. The literature is full of examples reporting the beneficial effects of different therapeutics when administered prior to the onset of inflammation. In the research of Kim et al (2017), RAW 264.7 macrophages were pre-incubated with an aqueous extract of *Stauntonia hexaphylla* fruit for 2h and then stimulated with LPS for 18 h. The treatment reduced protein levels of iNOS and the activity of cyclooxygenase enzyme as well as the production of NO, (IL)-1β, IL-6 and prostaglandin E2 in LPS-activated RAW 264.7 cells [21]. Also in this study, administered aqueous extract of *Stauntonia hexaphylla* three days before the carrageenan injection reduced the volume of carrageenan-induced paw edema in rats[21]. Even with cortisol, a strong anti-inflammatory

drug which is the most widely used and effective therapy for many inflammatory diseases. Dong et al (2018) used the co-treatment method cortisol with LPS (1 μg/mL) for 6, 12 and 24 h to evaluate the effect of cortisol on LPS-induced inflammatory responses in macrophage cell line[22]. However, this is in contrast to the clinical situation in which treatment is normally started after the onset of symptoms and clear diagnosis (therapeutic treatment). Thus, a potential pharmacological effect could be overestimated in an animal model, simply because therapeutic intervention occurs earlier in the disease process as compared to the clinical situation[23]. In our study, EE was administered before disease onset in RAW 264.7 cell model and copper sulfate-induced inflammation zebrafish model. EE also was administered after disease onset in cut tail zebrafish model. Therefore, EE can be considered both a prophylactic and therapeutic agent.

4. The extraction procedure

Extraction is the first step in the drug discovery process from plants. The purpose of extraction procedures is to attain the therapeutically desired portions and to eliminate unwanted material by using selective solvents. The basic parameters affect the quality of an extract include plant part used as starting material; solvent used for extraction; extraction procedure.

Plant parts are used as starting material for the extraction. Any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc may contain active components. Roots and whole plants of C. *cyrtophyllum* have been used in traditional medicine for the treatment of diseases related to inflammation. In this study, we used leaves of *C. cyrtophyllum* as material for the extraction. Collecting leaves samples is easier than root or the whole plant. Some *in vitro* studies indicated that the crude extract and the fraction from leaves of this plant possess anti-oxidant activity through scavenging DPPH, ABTS and superoxide anion radicals [24]. The methanol extract from the plant also demonstrated anti-inflammatory through the inhibition of NO release and the downregulation of the expression of COX-2 and iNOS in RAW 264.7 cells [25]. Furthermore, the ethanol extract from *C. cyrtophyllum* leaves was proved to have anti-inflammatory activity in our pilot study using carrageenan-induced paw edema rat model. Therefore, leaves of *C. cyrtophyllum* may be a potential material for anti-oxidant and anti-inflammatory activity. The literature is full of examples reporting the anti-inflammatory activity of the extract from leaves of herbal medicine. The methanol extract of leaf *Mallotus repandus* was shown to have anti-inflammatory activity in xylene induced ear edema, cotton pellet-induced granuloma, and tail immersion models[26].The root and bark of *Ficus iteophylla* have

been used in ethnomedicine for dysentery, rheumatic pain and as pain killer. However, the ethanol extract of *Ficus iteophylla* leaves also was found to decrease local inflammatory in carrageenan induced paw oedema model [27].

Solvent used for extraction: Extraction yield and bioactivity not only depend on the extraction method but also the type of solvent used in the extraction procedure. A solvent is good if it has some properties: low toxicity, ease of evaporation, preservative action, inability to cause the extract to complex or dissociate[28]. In this study, we used ethanol for the extract process. Because ethanol is easier to penetrate the cellular membrane than water, it can induce a high extraction yield. It is less toxic to humans and animals. Phenols have been found to be the major constituents of *C. cyrtophyllum* in recent studies[29]. Ethanol has been proved as a good solvent for polyphenol extraction. Some studies have reported that ethanol is more effective than water in extracting phenolic compounds, and therefore exhibits higher antioxidant activity than aqueous extracts [30]. The presence of enzyme polyphenol oxidase in water extract can degrade polyphenols and therefore decrease the activity of aqueous extract whereas in ethanol they are inactive[28]. A large of studies used ethanol as a solvent for the extraction process especially with the plant which phenols are major constituents. The propolis powder from *Apis mellifera* was extracted with different solvents: water, ethanol 25, 50, 75, 95, and 100 wt.%. The results showed that extraction yields increased with an increase in ethanol concentration. The highest total phenol and flavonoids were observed in ethanol 75%, and the lowest in water[30]. As the result, the ethanol 75% extract also showed the strongest antioxidant properties. In our study, we used reflux for the extraction of *C. cyrtophyllum* leaves. This method is a popular method that has been widely used for the preparation of herbal medicine, and it is close to the traditional extraction method of an herbal formula[31].

5. Limitations of the research

Any part of the plant may contain active components. Roots and whole plant of C. *cyrtophyllum* have been used in traditional medicine for the treatment of diseases related to inflammation. Due to the time and the limitation of cost, roots samples and whole plant of C. *cyrtophyllum* were not collected in our research. It is possible that the extract from roots or whole plant of C. *cyrtophyllum* also can be a potent anti-inflammatory agent that needs to be investigated in future studies.

In this study, we used ethanol 95% as solvent and heat-reflux method for the extraction. Many parameters can affect extraction yield including extraction time, the number of extractions, ratio of solvent, extraction temperature and pressure[31]. However, a study to optimize the extraction

from C. *cyrtophyllum* leaves was not performed in this thesis. The optimized extraction process may develop in the future and contribute to improving active compound's content and bioactivity of the extract from C. *cyrtophyllum* leaves.

The cut tail zebrafish model was used in my thesis to evaluate anti-inflammatory activity of the extract from C. *cyrtophyllum* leaves. The inflammatory response was evaluated at 24 h after zebrafish cut tail in my research. However, the inflammatory response changes with time. It is therefore important to observe the inflammatory response at different time-points of 0, 4, 6 and 24 h post zebrafish cut tail. Several hours after cut tail, the response enters the resolution phase. Pro-resolving mediators have been shown to exert powerful anti-inflammatory effects [3]. A study on the resolution phase has not already been performed in our research. The development of a study to assess the effect of EE on the resolution of acute inflammation can be a topic for future research.

The zebrafish larvae model has been extensively used in biomedical research and proved to have some advantages. However, zebrafish larvae survive with only innate immune responses. The adaptive immune system is morphologically and functionally mature only after 4-6 weeks postfertilization[32]. Therefore, the effect of EE on the adaptive immune system was not observed in this study. This is also a limitation of the thesis. Further studies in adult zebrafish or rodents are required to confirm the effect of the extract from *C. cyrtophyllum* on the adaptive immune system.

Many phytochemicals were identified in the ethanol extract of this plant. However, due to the limitation of time, bioassays on the major compounds have not been implemented yet. Further *in vitro* and *in vivo* studies on bioactivity, the mechanisms of action of the pure bioactive compounds are needed. These studies can contribute lead compounds to drug discovery.

6. Hypothesis mechanisms for the antioxidant and anti-inflammatory activity of the ethanol extract from *C. cyrtophyllum* leaves

The main objective of this thesis was to investigate antioxidant, anti-inflammatory activity and safety of the extract from *C. cyrtophyllum* leaves. In zebrafish model, cut tail and copper sulfate stimulated the inflammatory response. Subsequently, immune cells are recruited to tissue injury. Activated immune cells secrete a variety of substances such as chemokines and cytokines (IL-1β, IL-2, IL-6 and TNF-α), complement components, proteases, NO, prostaglandins (PGs), leukotrienes (LTs) and reactive oxygen metabolites which are considered as the primary sources of tissue injury. The extract from *C. cyrtophyllum* leaves has anti-

inflammatory activity through downregulate pro-inflammatory genes (*cox-2, pla2, c3a, il-1, il-8, mpo, nf-ƙb)* induced by both physical and chemical injuries. It also stabilize the lysosome membrane, the inhibition of NO, and the TNF-α release from activated macrophages. These effects inhibited the synthesis of inflammatory mediators and therefore reduced the symptoms of inflammation and prevented the spread of tissue injury (Figure 1).

At the site of inflammation, amounts of reactive oxygen species (ROS) such as superoxide anion (·O2⁻), hydroxyl radical (HO·), and hydrogen peroxide (H_2O_2) are produced. Our results showed that EE prevented oxidative stress. EE can directly scavenge free radicals: superoxid ($O_2^{\bullet-}$), DPPH, ABTS radical ($ABTS^{\bullet+}$) and hydroxyl radical ($OH^{\bullet-}$) or chelating (Fe^{2+}) and reduce the ability of Fe^{3+} reduction to Fe^{2+}. EE also reduced or prevented the reactive oxygen species formation induced by copper sulfate in zebrafish model. In response to oxidative stress stimuli, Nrf2 will be released from a complex with Keap1. It then initiates the cellular defense by induction of genes for phase II detoxifying and antioxidant enzymes, *e.g.* heme oxygenase-1 (Ho-1), glutathione peroxidase (Gpx), superoxide dismutase (Sod), and catalase (Cat) [6]. In our research, EE increased the activity of endogenous antioxidants including glutathione (GSH*)*, glutathione *S*-transferase (GST), and catalase (CAT) (chapter 5) and regulate the expression of genes involving Nrf2/keap/ARE signalling pathway. The increase in these antioxidant activities contributes to the elimination of ROS from the cell, indicating a protective role of EE against oxidative damage (Figure 1).

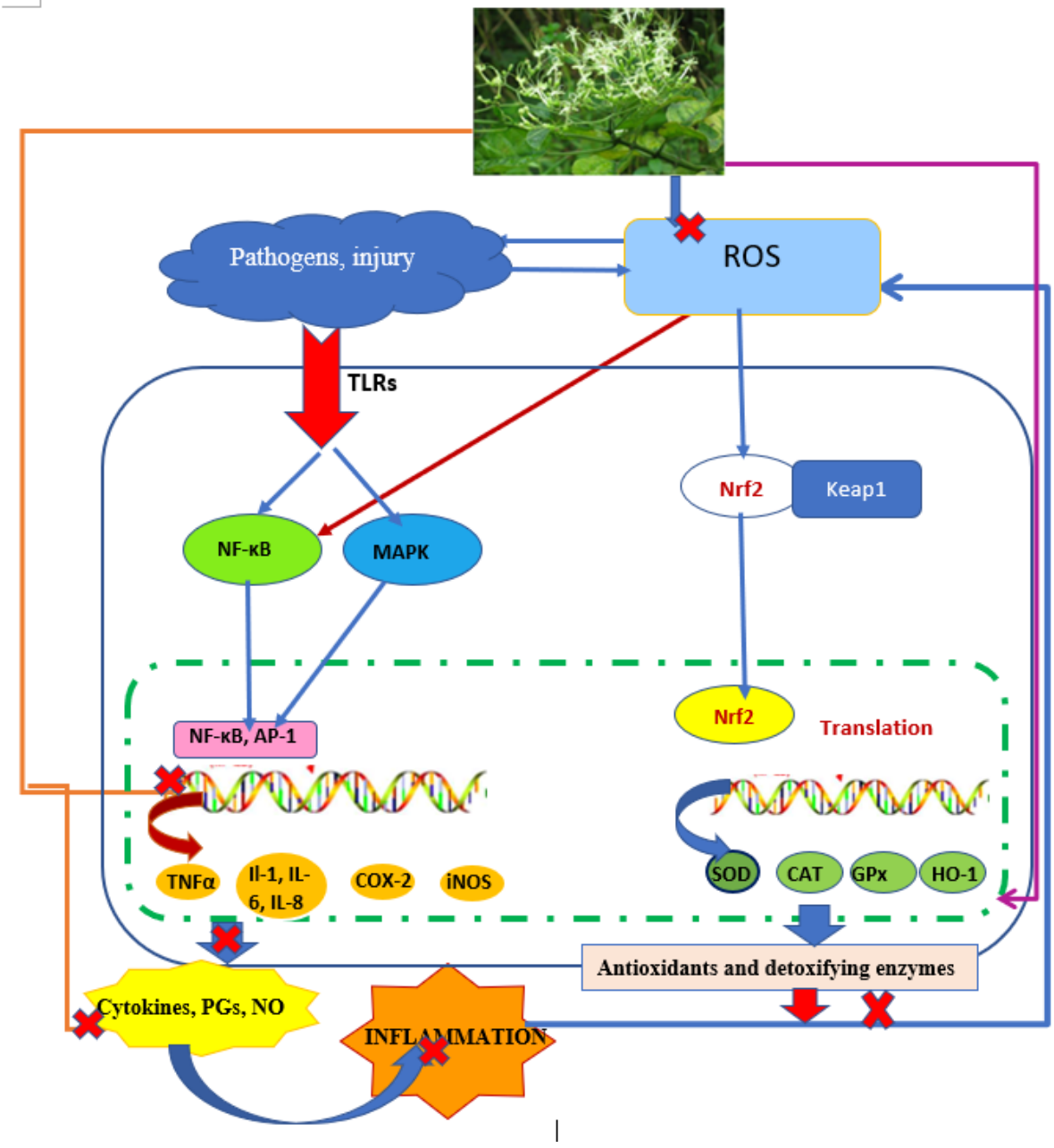

Figure 1. Hypothesis mechanisms for the antioxidant and anti-inflammatory activity of the ethanol extract from *C. cyrtophyllum* leaves.

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Chapter 10

Conclusions and Perspectives

1. Conclusions

This thesis aimed to investigate the efficacy, phytochemistry and toxicity as well as underlying molecular mechanisms of action of the extract from *C. cyrtophyllum* leaves. The results suggested that the crude ethanol extract has anti-inflammatory activity through the stabilization of lysosome membrane, the inhibition of NO, TNF-α release from activated macrophages, and the downregulation of inflammatory genes (*cox-2, pla2, c3a, il-1, il-8, mpo, nf-ƙb)* induced by both physical and chemical injuries. The ethanol extract also displays antioxidant activity through scavenging radicals such as $DPPH^{\bullet+}$, $ABTS^{\bullet+}$, $O_2^{\bullet-}$, $OH^{\bullet-}$ and chelating with Fe^{2+} or Cu^{2+}. It also increases the activity of endogenous antioxidants such as glutathione (GSH), glutathione *S*-transferases (GST), and catalase (CAT) and regulates the expression of genes related to oxidative stress. Besides that, the ethanol extract protects zebrafish larvae against the toxicity of copper sulfate. The effects can be related to antioxidant or anti-inflammatory activities but also through the effects on proteins of some signalling pathways such as ribosome, pyruvate metabolism and glycolysis/gluconeogenesis pathways or the regulation of copper metabolism. However, the ethanol extract also displays some embryotoxicity, it increases the embryo mortality and reduces the hatching rate, and induces a series of malformations. The mechanism for malformations can be related to the change in the expression of genes involved in oxidative stress (*gstp2*, *gadd45bb)*, cell apoptosis *(p53, casp8, bax/bcl2)*, heart development (*nkx2.5*, *gata5)* and wnt pathway (*wnt3a, wnt11*). The phytochemical analysis indicated that acteoside and cirsilineol and cirsilineol-4´-*O*-*β*-D-glucoside are the major compounds in the ethanol extract and methanol fraction, they can be considered as potential compounds for the discovery and development of *C. cyrtophyllum*-derived drugs. Taken together, our results provide scientific evidence to validate the ethnopharmacological use of this plant as well as the orientation for further research in order to develop this plant as botanical drugs or plant-derived drugs at the benefit of human health.

2. Perspectives

The available data in our study provided substantial evidence to support the ethnopharmacological uses of *C. cyrtophyllum* in the treatment of conditions related to inflammatory and oxidative stress. However, to develop this plant to botanical drugs or plant-derived drugs, further investigations on quality, efficacy and safety need to be performed. Considering efficacy, further studies in rodents using acute inflammation and chronic inflammation models are required to confirm the anti-inflammatory activities of the extract from *C. cyrtophyllum*. Subsequently, the pharmacokinetics and clinical studies should be executed. In addition, animal toxicity studies including acute, subacute, and subchronic toxicity studies need to be conducted according to the relevant Organisation for Economic Co-operation and Development (OECD) guidelines to ensure its safety. Specific toxicity studies such as genotoxicity, reproductive toxicity and carcinogenicity study of the same extract also need to be conducted. To ensure the quality of products from this plant, quality control and standardization of the bioactive compounds of a plant-based product are necessary. Due to the inherent nature of these products containing multiple bioactive and chemical markers, the quality control process to meet stringent regulatory standards of safety considerations is time-consuming and lengthy. Several factors can affect the extract composition: the collecting season, geographical area, extraction method, plant variety, among others. Hence, it would be of great applicability to standardize the parameters involved, whenever possible.

The limitations of the research also point out the researches need to do in the future such as optimized extraction process, the investigation bioactivity of the extract from root and the whole plant of *C. cyrtophyllum*, studies on the major compounds (acteoside and cirsilineol and cirsilineol-4´-*O*-*β*-D-glucoside) are needed to ensure the safety and effectiveness of this plant-derived drugs.

APPENDIX -1

Well	Sample Name	Target Name	CT	CT Mean		SDCt (ΔCt)	Fofd different (2^-ΔCt)
	X/5		14.957466				
A1	X/5	EF-alpha	15.95474	15.32476			
A2	X/5	EF-alpha	15.062073				
A3	X/25	EF-alpha	17.297174				
A4	X/25	EF-alpha	17.374243	17.36529			
A5	X/25	EF-alpha	17.424448				
A6	X/125	EF-alpha	19.679974				
A7	X/125	EF-alpha	19.751455	19.73013			
A8	X/125	EF-alpha	19.758951				
A9	X/625	EF-alpha	22.768892				
A10	X/625	EF-alpha	22.854305	22.85079			
A11	X/625	EF-alpha	22.929173				
Control 1	Control - CuSO4	EF-alpha	17.445274	17.62585			
Control 1	Control - CuSO4	EF-alpha	17.756989	17.62585	17.626	0.195655	0.8731767
Control 1	Control - CuSO4	EF-alpha	17.675282	17.62585			
Control 2	Control - CuSO4	EF-alpha	17.261494	17.30462			
Control 2	Control - CuSO4	EF-alpha	17.337831	17.30462	17.305		
Control 2	Control - CuSO4	EF-alpha	17.314543	17.30462			
Control 3	Control - CuSO4	EF-alpha	17.625776	17.65876			
Control 3	Control - CuSO4	EF-alpha	17.698984	17.65876	17.659		
Control 3	Control - CuSO4	EF-alpha	17.651524	17.65876			
Model 1	Control +CuSO4	EF-alpha	17.861046	17.82206			
Model 1	Control +CuSO4	EF-alpha	17.83243	17.82206	17.822	0.295474	0.8148048
Model 1	Control +CuSO4	EF-alpha	17.772709	17.82206			
Model 2	Control +CuSO4	EF-alpha	17.245274	17.236			
Model 2	Control +CuSO4	EF-alpha	17.151962	17.236	17.236		
Model 2	Control +CuSO4	EF-alpha	17.310764	17.236			
Model 3	Control +CuSO4	EF-alpha	17.426167	17.46336			
Model 3	Control +CuSO4	EF-alpha	17.420723	17.46336	17.463		

Model 3	Control +CuSO4	EF-alpha	17.543182	17.46336			
Model 4	Control +CuSO4	EF-alpha	17.407076	17.53495	17.535		
Model 4	Control +CuSO4	EF-alpha	17.712238	17.53495			
Model 4	Control +CuSO4	EF-alpha	17.48554	17.53495			
Ethanol 40-1	Ethanol 40	EF-alpha	17.294313	17.29934			
Ethanol 40-1	Ethanol 40	EF-alpha	17.306458	17.29934	17.299		
Ethanol 40-1	Ethanol 40	EF-alpha	17.297251	17.29934		0.093998	0.936923
Ethanol 40-2	Ethanol 40	EF-alpha	17.282566	17.3027	17.303		
Ethanol 40-2	Ethanol 40	EF-alpha	17.296614	17.3027			
Ethanol 40-2	Ethanol 40	EF-alpha	17.328922	17.3027			
Ethanol 40-3	Ethanol 40	EF-alpha	17.415321	17.4638	17.464		
Ethanol 40-3	Ethanol 40	EF-alpha	17.435854	17.4638			
Ethanol 40-3	Ethanol 40	EF-alpha	17.540232	17.4638			
Ethanol 20-1	Ethanol 20	EF-alpha	17.297001	17.3666			
Ethanol 20-1	Ethanol 20	EF-alpha	17.382921	17.3666	17.367		
Ethanol 20-1	Ethanol 20	EF-alpha	17.419889	17.3666			
Ethanol 20-2	Ethanol 20	EF-alpha	17.242531	17.27388	17.274	0.067449	0.9543237
Ethanol 20-2	Ethanol 20	EF-alpha	17.258522	17.27388			
Ethanol 20-2	Ethanol 20	EF-alpha	17.32058	17.27388			
Ethanol 20-3	Ethanol 20	EF-alpha	17.210733	17.23539	17.235		
Ethanol 20-3	Ethanol 20	EF-alpha	17.223345	17.23539			
Ethanol 20-3	Ethanol 20	EF-alpha	17.272091	17.23539			
Ethanol 5-1	Ethanol 5	EF-alpha	17.244307	17.32297	17.323		
Ethanol 5-1	Ethanol 5	EF-alpha	17.313711	17.32297			
Ethanol 5-1	Ethanol 5	EF-alpha	17.410881	17.32297		0.141481	0.9065882
Ethanol 5-2	Ethanol 5	EF-alpha	17.657286	17.44507	17.445		

Ethanol 5-2	Ethanol 5	EF-alpha	17.280146	17.44507			
Ethanol 5-2	Ethanol 5	EF-alpha	17.397787	17.44507			
Ethanol 5-3	Ethanol 5	EF-alpha	17.53417	17.60508	17.605		
Ethanol 5-3	Ethanol 5	EF-alpha	17.636341	17.60508			
Ethanol 5-3	Ethanol 5	EF-alpha	17.644733	17.60508			

Well	Sample Name	Target Name	CT	CT Mean		ΔCt	fold different (=2^-ΔCt)
	X/5		17.27739				
A1	X/5	actin	18.20182				
A2	X/5	actin	18.79667				
A3	X/25	actin	19.68417				
A4	X/25	actin	19.52202				
A5	X/25	actin	20.17034				
A6	X/125	actin	20.45408				
A7	X/125	actin	22.8161				
A8	X/125	actin	21.9607				
A9	X/625	actin	23.96022				
A10	X/625	actin	24.42267				
A11	X/625	actin	23.94377				
Control1	Control - CuSO4	actin	19.77202	19.83864			
	Control - CuSO4	actin	19.79024	19.83864	19.83864		
	Control - CuSO4	actin	19.95366	19.83864		0.4541	0.72998231
Control2	Control - CuSO4	actin	20.45803	20.01135			
	Control - CuSO4	actin	19.32907	20.01135	20.01135		
	Control - CuSO4	actin	20.24696	20.01135			
Control3	Control - CuSO4	actin	20.6532	20.69711			
	Control - CuSO4	actin	20.65133	20.69711	20.69711		
	Control - CuSO4	actin	20.78679	20.69711			
Model 1	Control +CuSO4	actin	19.35773	19.35038			
	Control +CuSO4	actin	19.37466	19.35038	19.35038		

	Control +CuSO4	actin	19.3261	19.56063			
Model 2	Control +CuSO4	actin	19.52296	19.56063		0.1051	0.92971006
	Control +CuSO4	actin	19.83283	19.56063	19.56063		
	Control +CuSO4	actin	19.17679	19.45917			
Model 3	Control +CuSO4	actin	19.30634	19.45917	19.45917		
	Control +CuSO4	actin	19.89439	19.45917			
Model 4	Control +CuSO4	actin	19.54438	19.51832			
	Control +CuSO4	actin	19.87773	19.51832	19.51832		
	Control +CuSO4	actin	19.13286	19.51832			
Ethanol 40-1	Ethanol 40	actin	19.23275	19.31421			
	Ethanol 40	actin	19.39568	19.31421	19.31421	0.1553	0.89792561
Ethanol 40-2	Ethanol 40	actin	19.42956	19.53193			
	Ethanol 40	actin	19.77055	19.53193	19.53193		
Ethanol 40-3	Ethanol 40	actin	19.60895	19.615			
	Ethanol 40	actin	19.49607	19.615	19.615		
	Ethanol 40	actin	19.73998	19.615			
Ethanol 20-1	Ethanol 20	actin	19.59318	19.65912			
	Ethanol 20	actin	19.7374	19.65912	19.65912		
	Ethanol 20	actin	19.64679	19.65912			
Ethanol 20-2	Ethanol 20	actin	19.28026	19.59995		0.1252	0.91687494
	Ethanol 20	actin	19.60897	19.59995	19.59995		
	Ethanol 20	actin	19.91062	19.59995			
Ethanol 20-3	Ethanol 20	actin	19.56791	19.41882			
	Ethanol 20	actin	19.10489	19.41882	19.41882		
	Ethanol 20	actin	19.58367	19.41882			
Ethanol 5-1	Ethanol 5	actin	19.99874	19.82381			
	Ethanol 5	actin	19.63384	19.82381	19.82381		
	Ethanol 5	actin	19.83887	19.82381			
Ethanol 5-3	Ethanol 5	actin	19.51771	19.59608		0.1604	0.8947871
	Ethanol 5	actin	19.59923	19.59608	19.59608		
	Ethanol 5	actin	19.6713	19.59608			
Ethanol 5-3	Ethanol 5	actin	19.95027	19.90558			
	Ethanol 5	actin	19.86905	19.90558	19.90558		
	Ethanol 5	actin	19.89742	19.90558			