SYNTHESIS AND CHARACTERIZATION OF HESPERETIN DERIVATIVES AND TOXICITY LEVEL OF THE ZEBRAFISH MODEL

Submitted in partial fulfillment of the requirements for the award of a
Bachelor ofTechnology degree in Biotechnology
By

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This is to certify that this Project Report is the bonafide work of **Bhuvanesh P (39230007) and Varada (39230056)**, and who carried out the project entitled **"SYNTHESIS AND CHARACTERIZATION OF HESPERETIN DERIVATIVES AND TOXICITY LEVEL OF THE ZEBRAFISH MODEL"** Under our supervision from **October 2022 to April 2023**

10/05/23

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10/5/23

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I **Bhuvanesh P (39230007)** hereby declare that the Project Report entitled **"SYNTHESIS AND CHARACTERIZATION OF HESPERETIN DERIVATIVES AND TOXICITY LEVEL OF THE ZEBRAFISH MODEL"** done by me under the guidance of **Dr. M. MASILAMANI SELVAM** (Internal) at **DEPARTMENT OF BIOTECHNOLOGY, SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** and **Dr. M. RAJASEKAR** (External) at the **CENTRE OF MOLECULAR AND NANOMEDICAL SCIENCES, INTERNATIONAL RESEARCH CENTRE**, SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY (Deemed to be University) is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE: 10/5/23

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SIGNATURE OF THE CANDIDATE

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ABSTRACT

Hesperetin derivatives were synthesized through the esterification of acid chlorides with Hesperetin under ambient reaction conditions with good yields. The product was confirmed using different spectral techniques. It was treated on zebrafish embryos to study the lethality, phenotypic deformities, and toxicity level of the compound. In that assessment, embryos showed lethality towards 3e at the minimal concentration. It assesses slow heartbeat since the compound loaded, the curvature on the back, upcurved fish, Cardiac chamber bulging, and poor survival rate in 72 h. 3a shows less toxicity more than other compounds. It shows only pericardial edema at higher concentrations and 3c induced pericardial edema and upcurved tail at a medium range of the concentration. But both compounds were shown a good survival ratio at the minimal concentration.

Keywords: Hesperetin, Acid chlorides, Zebrafish, Toxicity, Lethality

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1. INTRODUCTION

Hesperetin is a flavanone class of flavonoids. It's abundant in oranges and grapefruits, tomatoes, and cherries. It is a major pharmaceutically active component contained in the peel of citrus fruit. Some citrus flavonoids such as hesperetin and hesperedin have been shown to possess cytoprotective effects by regulating cellular signaling pathways and mitogen-activated protein kinases (MAPKs). Several studies have reported that hesperetin shows anti-inflammatory, antioxidant, anti-carcinogenic, and neuroprotective effects. The multiple OH groups which confer greater antioxidant potency than are possessed by other flavanones. It easily passes through the blood-brain barrier into the brain and exerts neuroprotective effects It could reduce neuronal cell death through antioxidant properties. Properties and mode of action of compounds are different in nature. In basic chemically synthesized compounds different from normal compounds. These properties are restricted toxicity and the binding site upon the dosage level of the compounds. It may cause many good and bad effects on host tissues and host organs. Developing derivatives of hesperetin molecule can help in identifying potential alternate compounds as an efficient anti-inflammatory, antioxidant, anti-carcinogenic, and neuroprotective effects (Figure 1). To understand the toxicity effect of hesperetin derivatives they were analyzed using the zebrafish model system. Zebrafish (*Danio rerio*) is a clinically evaluated human-animal model. It is used to find the lethal toxicity of the compounds, antibiotics, and drugs. Most of the antibiotics and compounds were screened in zebrafish embryos assessment. zebrafish is a sensitive model organism with a strong history of use in the evaluation of developmental neurotoxicity and ecotoxicology. The zebrafish embryos are rapid and well-characterized. It has many conserved biological processes, including metabolic pathways, and endocrine axes. The zebrafish genome is sequenced, with 70% overall similarity to the human genome and 80% similarity in genes related to disease, making the zebrafish a useful biomedical model. Hence in this study, we focused on the synthesis of Hesperetin derivatives, and these were evaluated for the lethality and toxicity level in zebrafish *(Danio rerio)* embryo model and further evaluation for potential drug compound.

Hesperetin is a cholesterol lowering flavanoid found in a number of citrus juices. It appears to reduce cholesteryl ester mass and inhibit apoB secretion by up to 80%.

Hesperetin may have antioxidant, anti-inflammatory, anti-allergic, hypolipidemic, vasoprotective and anticarcinogenic actions. Hespereitin reduces or inhibits the activity of acyl-coenzyme A: cholesterol acyltransferase genes (ACAT1 and ACAT2) and it reduces microsomal triglyceride transfer protein (MTP) activity. Hespereitineretin also seems to upregulate the LDL receptor. This leads to the reduced assembly and secretion of apoB-containing lipoproteins and enhanced reuptake of those lipoproteins, thereby lowering cholesterol levels.

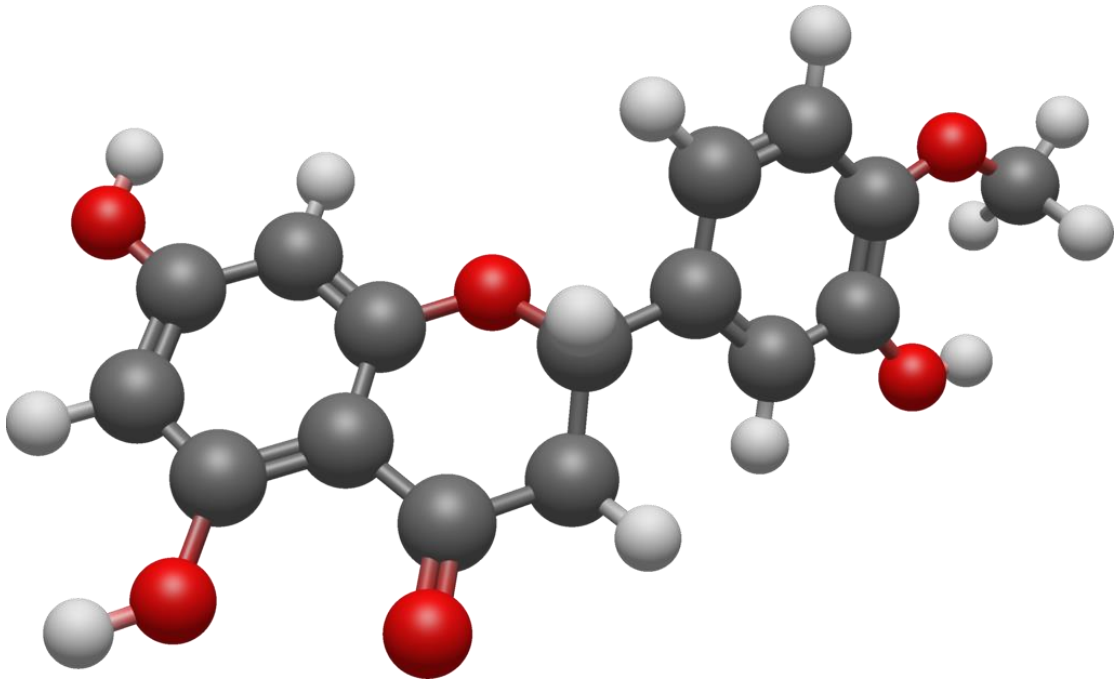


Fig.1. 3D structure of hesperetin compound

Due to their genetic closeness to humans and the possibility of studying their developmental processes in vivo, zebrafish (Danio rerio) have emerged as a crucial model organism in biomedical research. Zebrafish have become increasingly prominent in recent years in the field of toxicology as a model for researching the toxicity of various substances. One such substance is hesperetin, a flavonoid present in citrus fruits that has been demonstrated to offer possible health advantages but also potentially harmful effects. Hesperetin has been discovered to have potential therapeutic uses in the treatment of a number of illnesses, including cancer, diabetes, and cardiovascular diseases. It is also known to have antioxidant and anti-inflammatory properties. Hesperetin, including zebrafish, may, however, be hazardous to living things, according to certain studies. Hesperetin caused oxidative stress and

DNA damage, which led to developmental defects and mortality, according to a study done on zebrafish embryos exposed to it. Due to their high fecundity, transparency during the first stages of development, and the ease with which chemicals can be administered to the fish, zebrafish are a useful model for toxicity testing. Zebrafish are an excellent model for analyzing the toxicity of substances in humans since they share a great deal of genetic and physiological similarities with people. Zebrafish have a number of benefits over conventional animal models like mice when it comes to toxicity assessment. Zebrafish can be tested more quickly and with better throughput since they have a shorter reproductive cycle. Additionally, their openness throughout the earliest phases of growth makes it simple to visualize and evaluate potential hazardous effects. The use of fluorescent dyes and markers to examine particular cell types or organs is also made possible by this transparency. Zebrafish as a model for toxicity research has benefits, but there are drawbacks as well. One significant drawback is that zebrafish embryos lack a completely formed immune system, which may impact how they react to specific substances. Furthermore, zebrafish differ from humans in their metabolic pathways, which may have an impact on the metabolism and toxicity of specific substances. As a result, when interpreting the results, it is crucial to take into account the zebrafish model's limitations.

Various substances, including as medications, insecticides, and industrial chemicals, can toxicity in zebrafish. Through a variety of channels, including wastewater effluent, agricultural runoff, and industrial discharges, these substances may infiltrate the environment. These substances can accumulate in a variety of creatures, including zebrafish, and have hazardous effects when they are released into the environment. The disruption of the food chain is one of the main environmental impacts of toxicity in zebrafish. Zebrafish are a crucial part of the diets of many creatures, including birds and larger fish. Toxic effects on zebrafish populations could have a cascade effect on the entire food chain and result in the loss of other creatures that depend on zebrafish for food. Reproduction and population dynamics can be impacted by toxicity in zebrafish. For instance, developmental defects in zebrafish caused by hazardous substances may result in decreased reproductive success and population decreases. Additionally, toxicity may have an impact on other organisms that rely on zebrafish as a food supply, which could result in a drop in other species if zebrafish numbers are harmed. Toxicity in zebrafish can potentially have issues for water quality. If harmful substances get into waterways, they could have an impact on

other aquatic creatures and ecosystems. Furthermore, toxicity that affects zebrafish populations may alter the natural balance of streams and could cause to algae blooms and other problems with water quality.

In conclusion, the use of zebrafish as a model organism for toxicity testing has become increasingly popular in recent years due to their advantages over traditional animal models. The use of hesperetin as a compound to study toxicity in zebrafish has highlighted the potentially toxic effects of this compound on living organisms, and the importance of studying toxicity in animal models before testing in humans. While there are limitations to the zebrafish model, their genetic and physiological similarity to humans makes them a valuable tool in toxicity testing and drug development. the effects of toxicity in zebrafish can have implications for the environment, including the disruption of the food chain, impacts on reproduction and population dynamics, and potential effects on water quality. It is important to study the effects of toxicity in zebrafish as a model organism, as this can help to better understand the potential impacts of toxic compounds on the environment. Additionally, efforts should be made to reduce the release of toxic compounds into the environment, to help protect zebrafish and other organisms from the negative effects of toxicity.

2. REVIEW OF LITERATURE

Citrus fruits contain the flavonoid hesperetin, which has been demonstrated to possess antioxidant and anti-inflammatory properties. Hesperetin has been linked to potential health advantages. Hesperetin, including zebrafish, may be poisonous to living things, according to research, which also supports this. Hesperetin has a number of derivatives that have been synthesized and characterized in an effort to lessen its toxicity. The synthesis, characterization, and toxicity of hesperetin derivatives, as well as their level of toxicity in the zebrafish model, will be the main subjects of this literature review.

2.1. FLAVANOID COMPOUNDS

Fruits, vegetables, and herbs all include a class of natural substances called flavonoids. These substances have been investigated for their possible health advantages and are the cause of the vivid colours of these plants. Flavanones, flavonols, and flavones are just a few of the subclasses of flavonoids. The flavonoid subclass flavonols and its possible health advantages will be the main topics of this study.

2.1.1. FLAVANOLS

Flavonols are a subclass of flavonoids that are found in various plant-based foods such as onions, kale, and broccoli. The most common flavonols found in the human diet are quercetin, kaempferol, and myricetin. Flavonols have been studied extensively for their potential health benefits, including antioxidant, anti-inflammatory, and anticancer properties.

2.2. HESPERETIN

Citrus fruits including oranges, lemons, and grapefruits naturally contain hesperetin, a flavonoid component. It belongs to the flavanone subclass of flavonoids and shares structural similarities with hesperetin, another flavonoid molecule. Hesperetin has been investigated for its possible medicinal use in treating a number

of disorders as well as for its potential health advantages, such as its anti-inflammatory and antioxidant characteristics. Hesperetin has powerful antioxidant qualities, which means it can help shield cells from harm brought on by free radicals, according to research. Free radicals are unstable chemicals that have the potential to harm cells and have a role in the onset of many illnesses, such as cancer and heart disease. Free radicals have been demonstrated to be scavenged and kept from harming cells by hesperetin. Hesperetin possesses both anti-inflammatory and antioxidant effects. Inflammation is a normal reaction to injury or illness, but persistent inflammation can aid in the emergence of a number of disorders. It has been demonstrated that hesperetin decreases inflammation in the body by preventing inflammatory molecules from acting. Hesperetin may one day be used as a treatment for a number of illnesses, including diabetes, cardiovascular disease, and cancer, according to research. Hesperetin, for instance, has been demonstrated in research to have anticancer qualities by preventing the growth and spread of cancer cells. By lowering cholesterol levels and enhancing blood vessel function, it has also been demonstrated to promote cardiovascular health. Hesperetin is a substance that has the potential to be healthy, in general. Despite the fact that additional studies are required to completely comprehend its effects on the body and its potential as a medicinal agent, recent studies indicate that it might be a beneficial supplement to a balanced diet and lifestyle.

2.3. BIOACTIVITY APPLICATIONS OF HESPERETIN COMPOUNDS

2.3.1. Anti-cancer Properties

The pharmacological activity of hesperetin is responsible for the significance of citrus bioflavonoids. Cancer is not totally curable, and standard treatment regimens are linked to short- and long-term adverse outcomes. This is true despite extensive treatment protocols and regimes for cancer patients, including surgery, chemotherapy, and immunotherapy. In light of their potential as antioxidants, researchers are currently exploring the use of bio-flavonoids like hesperetin, for example, in the treatment of cancer. The powerful antioxidant properties of hesperetin and its prospective use as an anticancer drug have been highlighted in recent literature from various research groups. With a focus on its molecular mode of action, this review reveals the anti-tumor properties of hesperetin in several malignancies. Experimental research highlighting how Hespereitin affects oxidative stress, inflammation, and cancer cell death (hallmarks of cancer) makes it clear how important hespereitin is as an anti-

cancer agent. Additionally, Hespereitin has been shown to promote apoptosis in cancerous cells by activating the NF-jB, mTOR, and PI3K/AKT pathways.111 Additionally, hesperetin has been shown to enhance antioxidant defense mechanisms and down-regulate pro-inflammatory mediators and enzymes (IL-1/6, TNF, and COX-2) in cancer. Hesperetin also considerably lessens the pharmacological symptoms of other illnesses like arthritis, myocardial infarction, and infertility.112–116 Due to their low toxicity and tolerability, flavonoids are therefore preferred over chemotherapy treatments. It is also important to note that even at the greatest dose, hesperetin does not have cytotoxic effects or acute oxidative damage. However, further studies are required to unravel the therapeutic effects of Hesperetin/hesperidin in cancer treatment. Though, Hesperetin is presently in pre-clinical trials, promising data from clinical trials are warranted to increase the translation applicability of Hesperetin in cancer treatment. From the future perspective, future in vitro and in vivo studies focusing on the following dimensions of Hesperetin need to be studied to translate the practical applicability of Hesperetin as anticancer agent.

2.3.2. Anti-Oxidant Properties

The antioxidant hesperidin, a major flavonoid in sweet orange and lemon, was evaluated using chemical and biological systems. The chemical assay evaluates the hesperidin capacity to sequester 1,1-diphenyl-2-picrylhydrazyl (DPPH,). Biological studies were done using the eukaryotic cells of superoxide-dismutase proficient and deficient strains of Saccharomyces cerevisiae treated with hesperidin and the stressing agents hydrogen peroxide or paraquat (methylviologen; 1,1′-dimethyl-4,4′-bipyridinium dichloride). Hesperidin was able to reduce significantly the level of the free radical DPPH, with similar efficacy of trolox (positive control). When the yeast cells were exposed to the flavonoid hesperidin before the stressing agents, there was a significant increase in the survival of all strains. Paraquat induced higher catalase and superoxide dismutase than did hydrogen peroxide, which only increased catalase activity. Previous addition of hesperidin to these treatments was able to reduce significantly both enzymatic levels. These observations clearly demonstrate that hesperidin provides strong cellular antioxidant protection against the damaging effects induced by paraquat and peroxide hydrogen. Regulation of melanogenesis has been the focus of treatment for hyperpigmentary skin disorders. Although hesperidin is one of the most well-known, naturally occurring flavonoids with antioxidant and anti-

inflammatory effect, its anti-melanogenic effect is not known. The present study aims to determine the anti-melanogenic effect of hespiridin as well as its underlying molecular mechanisms. Melanin contents were measured in normal human melanocytes and B16F10 melanoma cells. Protein and mRNA levels of tyrosinase, microphthalmia-associated transcription factor (MITF), tyrosinase related protein-1 (TRP-1) and TRP-2 were determined. Melanogenesis-regulating signals were examined. In results, hesperidin strongly inhibited melanin synthesis and tyrosinase activity. Hesperidin decreased tyrosinase, TRP-1, and TRP-2 protein expression but increased phospho-extracellular signal-regulated kinase 1/2 (p-Erk1/2) expression. Specific inhibitor of Erk1/2 or proteasome inhibitor reversed the inhibition of melanogenesis induced by hesperidin. Taken together, hesperidin, a popular antioxidant, stimulated Erk1/2 phosphorylation which subsequently degraded MITF which resulted in suppression of melanogenic enzymes and melanin synthesis.

2.3.3. Anti-Viral Properties

Hesperetin are flavonoid that are abundantly present as constituents of citrus fruits. These compounds have attracted attention as several computational methods, mostly docking studies, have shown that hesperidin may bind to multiple regions of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (spike protein, angiotensin-converting enzyme 2, and proteases). Hesperidin has a low binding energy, both with the SARS-CoV-2 “spike” protein responsible for internalization, and also with the “PLpro” and “Mpro” responsible for transforming the early proteins of the virus into the complex responsible for viral replication. This suggests that these flflavonoids could act as prophylactic agents by blocking several mechanisms of viral infection and replication, and thus helping the host cell to resist viral attack. Inflammation and oxidative stress are two major causes of various life-threatening diseases. Hesperidin (Hsd) and its aglycone, hesperetin (Hst), are two flavonoids from citrus species that have numerous biological properties, particularly antioxidant and anti-inflammatory. New findings showed that the antioxidant activity of Hsd/Hst was not only limited to its radical scavenging activity, but it augmented the antioxidant cellular defenses via the ERK/Nrf2 signaling pathway as well. Various in vitro and in vivo studies have been conducted to evaluate Hsd, its metabolites, or its synthetic derivatives at reducing inflammatory targets including NF-κB, iNOS, and COX-2, and the markers of chronic inflammation. In this review, new findings regarding the

molecular targets of Hsd and Hst in the reduction of oxidative stress are discussed. Also, in the anti-inflammatory section, we provide a summary of significant investigations concerning the mechanisms of action based on the studied inflammation models.

2.3.4. Anti-microbial properties

Antibacterial activities of hesperetin, hesperidin and hesperidin glucoside with different solubility were compared in vitro. Hesperetin was prepared by enzymatic hydrolysis from hesperidin, and hesperidin glucoside composed of hesperidin monoglucoside was prepared from hesperidin through enzymatic transglycosylation. Solubility of the compounds was different: the partition coeffificient (log P) was 2.85 ± 0.02 for hesperetin, 2.01 ± 0.02 for hesperidin, and −3.04 ± 0.03 for hesperidin glucoside. Hesperetin showed a higher effect than hesperidin and hesperidin glucoside on radical scavenging activity in antioxidant assays, while hesperidin and hesperidin glucoside showed similar activity. Cytotoxicity was low in the order of hesperidin glucoside, hesperidin, and hesperetin in murine macrophage RAW264.7 cells. Treatment of the cells with each compound reduced the levels of inflflammatory mediators, nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). Hesperetin was most effective at relatively low concentrations, however, hesperidin glucoside was also effective at higher concentration. Hesperetin showed higher antibacterial activity than hesperidin in both Gram-positive and -egative bacteria, and hesperidin glucoside showed similarly higher activity with hesperetin depending on the bacterial strain. In conclusion, hesperetin in the form of aglycone showed more potent biological activity than hesperidin and hesperidin glucoside. However, hesperidin glucoside, the highly soluble form, has been shown to increase the activity compared to poorly soluble hesperidin.

3. AIM AND OBJECTIVES

3.1. AIM

The aim is to study the toxicity effect of hesperetin compounds in zebrafish model.

3.2. OBJECTIVES

- Synthesis of Hesperetin derivatives
- To study the characterization of Hesperetin derivatives (IR, NMR, Mass spectroscopy, CHN Analysis)
- To study breeding and maintenance of zebrafish embryos
- To study zebrafish toxic effects assessment

4. METHODOLOGY

4.1. MATERIALS REQUIRED

Hesperetin, Oleoyl chloride, Lauroyl chloride, Palmitoyl chloride, 4-nitrobenzoyl chloride, and Methoxyacetyl chloride, were bought from Sigma-Aldrich Chemicals Pvt. Ltd, USA. Triethylamine, Sodium sulphate, and solvents were bought from SRL, India. It comes with high purity so we can use it without any further purification. Column chromatography was performed on Silica Gel 60 (100–200 mesh). ^{1}H &^{13}C NMR spectra were recorded on Bruker DRX 500. Elemental analyses were performed using Perkin-Elmer 2400 elemental analyzer and optical rotations were determined by using a Rudolph Autopol II digital polarimeter.

4.2. GENERAL PROCEDURE FOR THE SYNTHESIS OF HESPERETIN DERIVATIVES

To a solution of Hesperetin (**1**, 1 mmol) in dry MeOH was added TEA (25% mol) and acid chlorides (**2a-e**, 1 mmol). After stirring at room temperature for a given period of time, the reaction mixture was evaporated under reduced pressure and extracted by EtOAc-water. The ethyl acetate layer was dried over anhyd Na_2SO_4 and concentrated to dryness. The product was further purified by flash column chromatography.

4.2.1. Synthesis of 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochro- man-7-yl oleate (3a).

Compound **3a** was obtained by the reaction of Hesperetin (**1**, 1 mmol, 0.30 g), and Oleoyl chloride (**2a**, 1 mmol, 0.30 g) as a yellow solid. Yield: 0.41 g (73%); $[\alpha]_D^{20}$ + 54.6 (c 0.2, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$+ DMSO-d_6): δ 1.23 (t, 3H, *J* = 4.2 Hz), 2.55 (s, 5H), 2.70 (q, 8H, *J* = 5.2 Hz), 3.08 (t, 7H, *J* = 5.5 Hz), 3.83 (s, 3H), 3.85 (q, 6H, *J* = 8.2 Hz), 5.32 (d, 5H, *J* = 9.5 Hz), 5.89 (s, 2H), 6.82 (d, 3H, *J* = 10.5 Hz),

7.26 (d, 2H, *J* = 10.5 Hz), 9.32 (s, 2H). ^{13}C NMR (125 MHz, $CDCl_3$+ DMSO-d_6): δ 15.9, 22.6, 24.2, 24.5, 39.1, 47.7, 55.8, 78.8, 100.4, 101.0, 107.4, 118.5, 120.7, 129.6, 132.5, 134.0, 151.5, 162.8, 167.79, 168.8, 171.8, 198.8. Anal. Calcd for $C_{34}H_{46}O_7$: C, 72.06; H, 8.18. Found: C, 72.08; H, 8.16.

4.2.2. Synthesis of 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl4-nitrobenzoate (3b)

Compound **3b** was obtained by the reaction of Hesperetin (**1**, 1 mmol, 0.30 g), and 4-nitrobenzoyl chloride (**2b**, 1 mmol, 0.18 g) as a yellow solid. Yield: 0.30 g (67%); $[\alpha]_D^{20}$ + 48.2 (c 0.2, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$+ DMSO-d_6): δ 3.82 (s, 3H), 6.55 (d, 1H, *J* = 7.2 Hz), 6.59 (s, 2H), 6.85 (d, 1H, *J* = 7.2 Hz), 7.59 (q, 6H, *J* = 7.2 Hz), 7.88 (t, 1H, *J* = 7.2 Hz), 8.05 (d, 3H, *J* = 7.2 Hz). ^{13}C NMR (125 MHz, $CDCl_3$+ DMSO-d_6): δ 42.6, 56.5, 70.9, 90.3, 95.7, 99.5, 105.5, 106.1, 126.7, 128.9, 130.9, 132.4, 161.0, 163.6, 164.6, 181.7. Anal. Calcd for $C_{23}H_{17}NO_9$: C, 61.20; H, 3.80; N, 3.10. Found: C, C, 61.22; H, 3.82; N, 3.12.

4.2.3. Synthesis of 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl 2-methoxyacetate (3e)

Compound **3e** was obtained by the reaction of Hesperetin (**1**, 1 mmol, 0.30 g), and methoxyacetyl chloride (**2e**, 1 mmol, 0.18 g) as a yellow solid. Yield: 0.24 g (65%); $[\alpha]_D^{20}$ + 45.6 (c 0.2, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$+ DMSO-d_6): δ 3.98 (d, 2H, *J* = 4.5 Hz), 4.00 (s, 6H), 6.42 (d, 1H, *J* = 7.2 Hz), 6.55 (s, 1H), 6.74 (d, 1H, *J* = 7. 2 Hz), 6.82 (d, 1H, *J* = 7.4 Hz), 7.36 (q, 2H, *J* = 7.2 Hz), 7.88 (d, 2H, *J* = 7.2 Hz), 8.05 (t, 2H, *J* = 7.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3$+ DMSO-d_6): δ 51.3, 52.3, 68.7, 71.4, 105.5, 109.2, 114.6, 126.3, 127.1, 129.3, 129.9, 132.8, 143.3, 145.4, 155.5, 157.6, 162.4, 164.9, 165.1, 167.4, 182.2. Anal. Calcd for $C_{19}H_{18}O_8$: C, 60.96; H, 4.85. Found: C, C, 60.94; H, 4.83.

4.3. BREEDING AND MAINTENANCE OF ZEBRAFISH EMBRYOS

Adult and healthy zebrafish were obtained and kept in the standalone system (Aquaneering, USA). These Zebrafish were maintained in a light/ dark cycle (14 h light/10 h dark) in the zebrafish culture facility of Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology. Adult zebrafish are bred in spawning tanks with the male-female ratio of 2:1 according to established protocols and embryos are collected immediately after the breeding interval. Embryos are raised in E3 medium at 28℃.

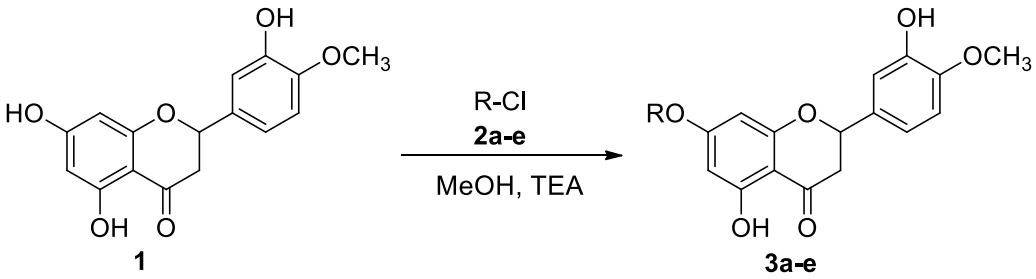
4.4. ZEBRAFISH TOXIC EFFECTS ASSESSMENT

Toxicity assay was performed in 4 hpf developing embryo to find the toxicity of the hesperetin synthesized compounds (**3a-e**). The embryos were treated with compounds dissolved in E3 medium to make a final concentration of 25, 100, 250–500 µg/ml. 12 well plate was used in which 1 ml of E3 medium makeup with the compounds was examined with 10 embryos in each well. Treated embryos deformities and abnormal activities were monitored with control embryos for 96 h. Embryos were monitored under the microscope for any abnormalities on each day.

5. RESULTS AND DISCUSSION

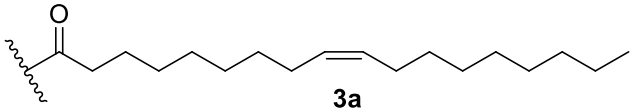
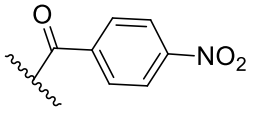
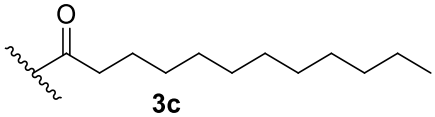
5.1. SYNTHESIS OF HESPERETIN DERIVATIVES

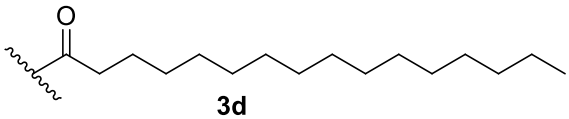
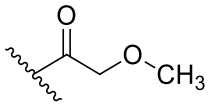
Hesperetin (**1**) reacts with different acid chlorides **2 (a-e)** in the presence of TEA as an organic catalyst in the MeOH solvent and results in a 59-73% yield of the respective Hesperetin derivatives **3 (a-e)** as shown in Scheme 1. The structure of reaction time and product yields are given in Table 1. The efficient molecule **3a** is further studied for structure prediction using NMR. The ^{1}H NMR spectra of 3a showed the methyl proton appeared in the range of 1.0-1.5 ppm and aromatic proton at 6.00-8.5 ppm. However, ^{13}C NMR studies show peaks around 17-41 ppm, 110-162 ppm, and 169-200 ppm corresponding to the alkyl carbons, aromatic carbons, and carbonyl group respectively.



Scheme 1. Synthesis of Hesperetin derivatives (**3a-e**)

Table 1. Synthesis of Hesperetin derivatives **(3a-e)**

No	R	t (h)	Yield (%)
1	 **3a**	8	73
2	 **3b**	7	67
3	 **3c**	8	70

4	 3d	8	59
5	 3e	8	65

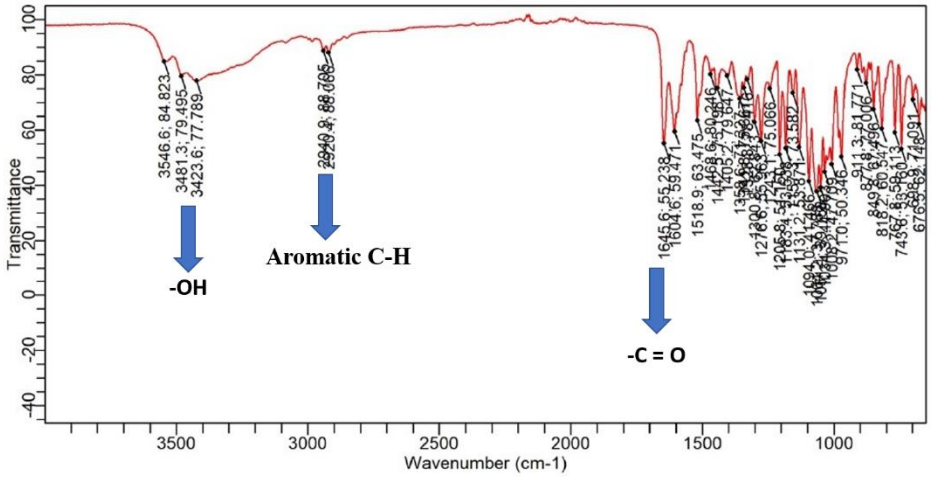


Fig. 2. IR spectra of Compound (3a)

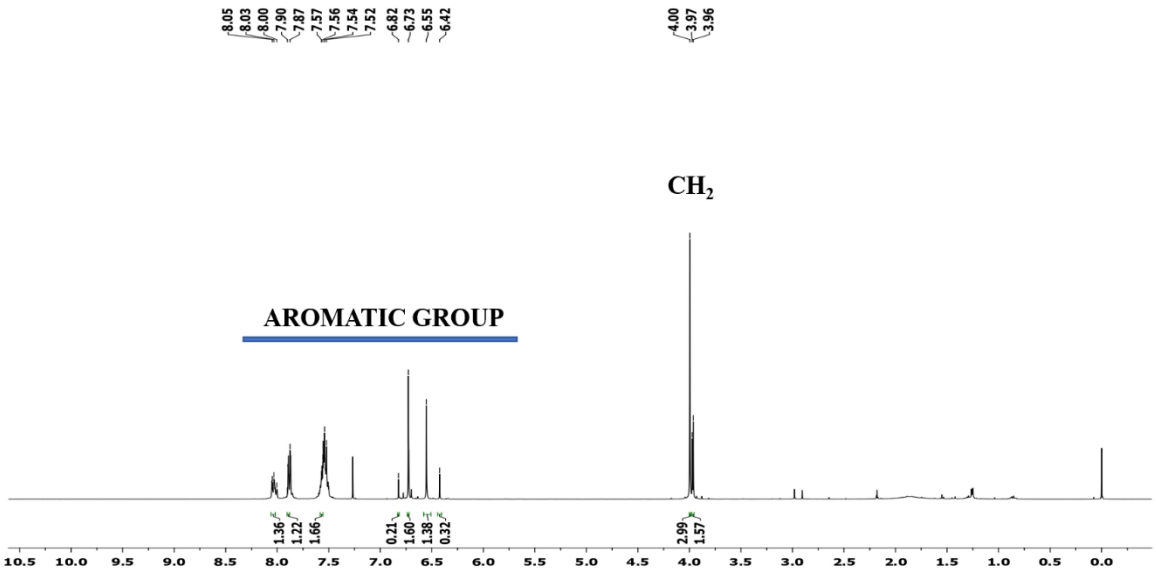


Fig. 3. ^{1}H NMR spectra of Compound (3a)

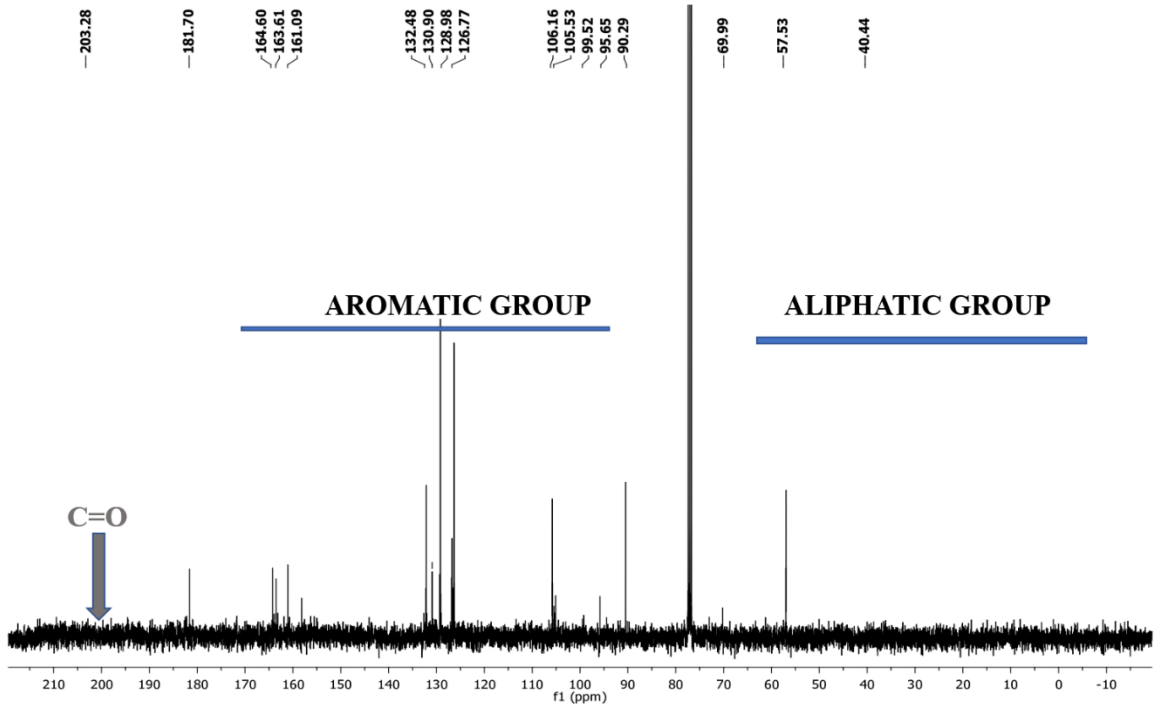


Fig. 4. ^{13}C NMR spectra of Compound (3a)

5.2. ADVERSE DRUG EFFECTS IN ZEBRAFISH EMBRYOS

We obtained different types of morphological defects, treated embryos starting at 24 h, and analyze the effects to 72 h for lethality and phenotypic deformities. The cardiac assay results, while increasing concentrations of the compounds the embryos heartbeats showed variations (Figure 5). The heartbeat ratio was determined with the control embryo. The treated embryos were showed a slow heartbeat and phenotypic deformities at different ratios of compounds. 3a and 3c was showed better results than other compounds. But both compounds showed some deformities at higher concentrations, in lower concentrations 25 µg/ml there are no deformities and it shows full survival rate.3a and 3c was found to be less toxic up to 25 µg/ml, 50µg 3a showed yolk-sac edema and lethality. Up to 25 µg, 3c showed pericardial edema, upcurved tail deformities, 3b showed up the curved tail, and presents a small amount of blood that can be located, nearby heart.3d showed curved body axis and pericardial edema. 3e exhibited slow heartbeat are two slightly different phenotypes (Figure 6). There is a curvature on the back of the embryo for “upcurved fish” and cardiac chamber bulging and poor survival ratio also. All the compounds cause damages towards the heart, tail,

and in size at higher concentrations. LC_{50} analysis of all the treated antibiotics is tabulated in Figure 7. The cardiac assay shows whether the compound is toxic or non-toxic to the zebrafish embryos. It depends on the dosage level of the compound. Heartbeat rate was found to be normal up to 25 µg/ml compared with untreated normal embryos, and when the dosage level was increased 500 µg/ml the compounds were toxic to embryos and it gets deformities, finally embryos were dead between 24 to 48 h. These values were considered to be statistically significant in the t-test with the p-value of 0.17053 are shown in Table 2.

Table.2. Adverse drug effect studies using Heart beat rate analysis and LC_{50} analysis for Hesperetin

S.No	Name of the Antibiotic	Heart Beat Rate/15 Sec				Lauroyl Chloride
		Oleoyl Chloride	Methoxy Chloride	4-Nitrobenzoyl chloride	Palmitoyl Chloride	
1	25 (µg/ml)	44	43	44	44	43
2	100 (µg/ml)	44	42	42	43	41
3	250(µg/ml)	44	42	42	43	40
4	500 (µg/ml)	40	42	42	40	37

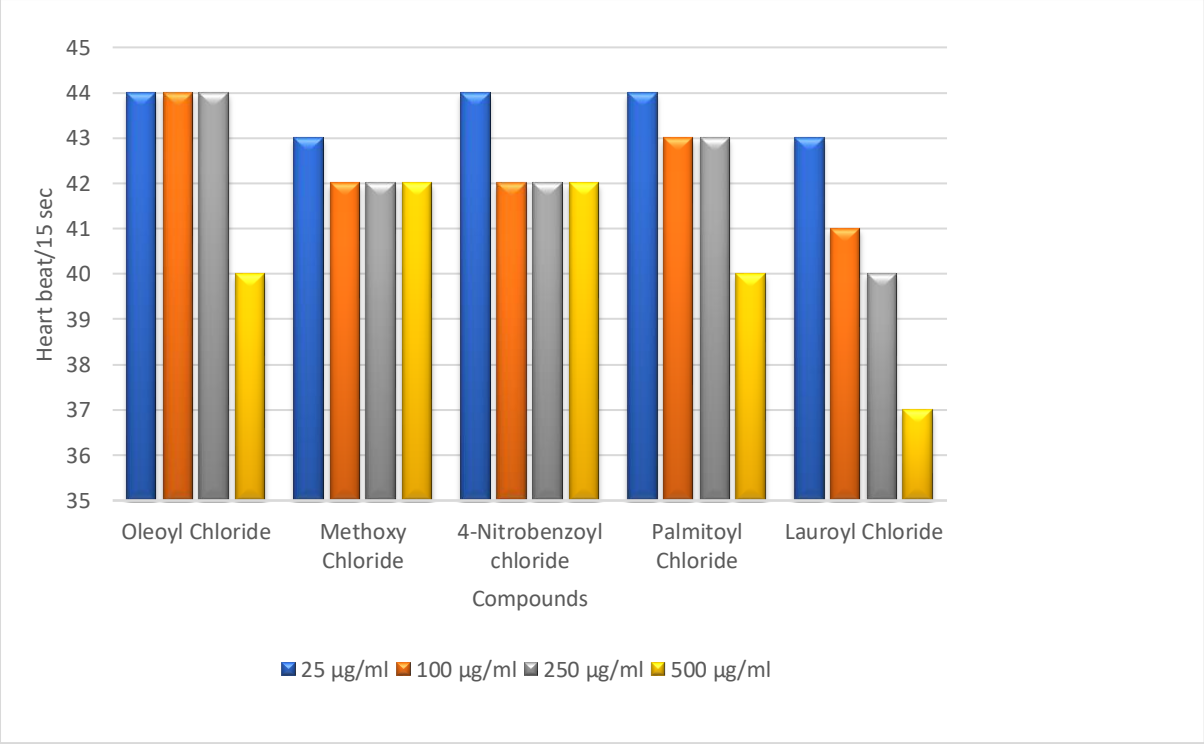


Fig.5. Heartbeat rate analysis after of 6 hpf treated embryos

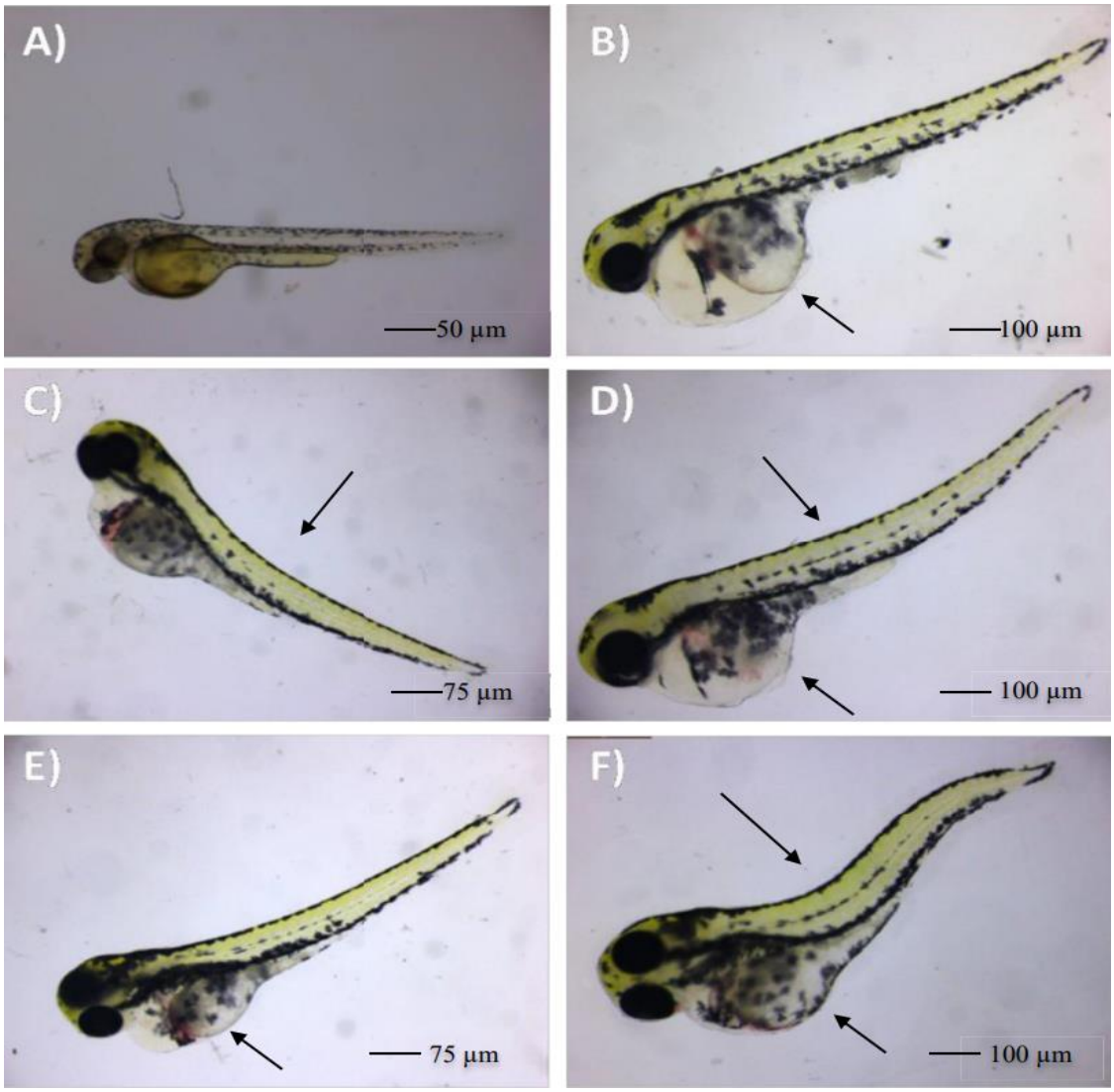


Fig.6. Phenotypic deformities in 24 hpf embryo by using Hesperetin synthesized compounds. A) Control embryo, B) 3a causes yolk-sac edema at 100 µg/ml, C) 3b causes up the curved tail at 100 µg/ml, D) 3c causes pericardial edema and upcurved tail at 500 µg/ml, E) 3d causes curved body axis and pericardial edema at 100 µg/ml, F) 3e upcurved fish and Cardiac chamber bulging at 25 µg/ml.

Table.3. Deformities of Hesperetin

1	**Oleoyl Chloride**	**yolk-sac edema**
2	**Methoxy Chloride**	**Slow heart beat rate, Up Curved Tail**
3	**4-Nitrobenzoyl chloride**	**Pericardial edema, Slow heart beat rate, Up Curved Tail**
4	**Palmitoyl Chloride**	**Curved body axis and pericardial edema, Slow heartbeat rate,**
5	**Lauroyl Chloride**	**Slow heartbeat, is two slightly different phenotypes. There is a curvature on the back of the embryo for "Up Curved Fish" and Cardiac chamber bulging. poor survival ratio**

Table 4: Lethality

1	2	3	4	5
100	**25**	**100**	**25**	**25**

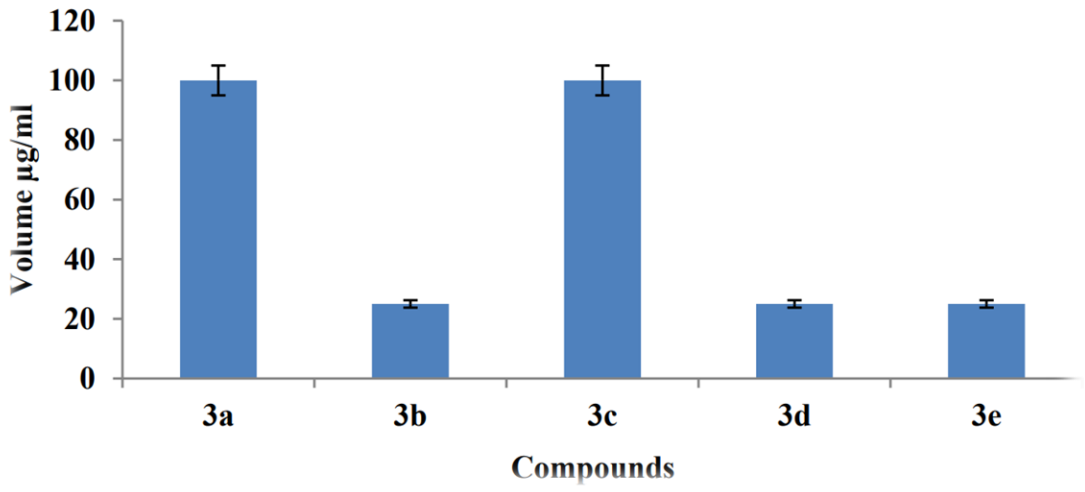


Fig.7. Lethality of 72 hpf treated embryos

SUMMARY AND CONCLUSION

A flavonoid molecule called hesperetin is commonly present in many fruits and vegetables and has been known to have anti-inflammatory, antioxidant, and anticancer activities. Hesperetin has been altered by researchers in an effort to create novel derivatives with increased bioactivity and therapeutic promise. Hesperetin's basic structure is altered during the synthesis of its derivatives by the addition or replacement of functional groups. Numerous chemical processes, including esterification, acetylation, glycosylation, methylation, and halogenation, among others, can be used to carry out this process. Hesperetin's physicochemical characteristics and pharmacological actions may change as a result of these alterations, producing compounds with increased bioactivity or improved drug-like qualities. To confirm their structure and purity after synthesis, the hesperetin derivatives are characterised. Characterization can be accomplished using a number of methods, such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), high-performance liquid chromatography (HPLC), and infrared (IR) spectroscopy, among others. The chemical composition, molecular weight, purity, and stability of the derivatives of hesperetin are all well-understood thanks to these investigations. Since it develops quickly, has a high genetic similarity to humans, and possesses transparent embryos that make it simple to see how organs develop and how drugs are distributed, the zebrafish (Danio rerio) has gained popularity as a model organism for toxicity assessment. In comparison to mammalian models, zebrafish eggs and larvae are smaller, more affordable, and require less ethical consideration. This makes them a popular choice for toxicity investigations. Zebrafish embryos or larvae are exposed to various concentrations of hesperetin derivatives in a controlled environment, and various endpoints, including mortality, developmental abnormalities, heart rate, behavioural changes, and gene expression, are evaluated. The hesperetin derivatives' potential toxicity profile and detrimental effects on multiple organ systems, such as the neurological, immunological, and cardiovascular systems, are both well-understood thanks to the zebrafish model. Depending on the particular derivative and its chemical makeup, as well as the concentration, length of exposure, and developmental stage of the zebrafish, the toxicity levels of hesperetin derivatives in the zebrafish model can change. Overall, it has been demonstrated that hesperetin derivatives are low to moderately hazardous to zebrafish, with the majority of these

derivatives exhibiting negligible or no side effects at physiological concentrations. The toxicity evaluation of several hesperetin derivatives in zebrafish has been documented in a number of research. For instance, Wang et al. produced and tested the toxicity of hesperetin-3',7-O-diglucoside and hesperetin-7-O-glucoside in zebrafish embryos, and they discovered that both derivatives had mild toxicity with no appreciable mortality or developmental defects at doses up to 50 µM. Similarly, Chang et al. produced hesperetin-7-O-acetate and examined its toxicity in zebrafish embryos and larvae. They found that at concentrations up to 50 M, the derivative showed low toxicity and had no appreciable effects on heart rate, body length, or gene expression.

In conclusion, our observations are consistent with chemically synthesized compounds and its broad-spectrum activity, toxicity, binding site, and dosage level. Most of the compounds caused yolk-sac edema, Slow heartbeat rate, upcurved tail, Pericardial edema, curved body axis, cardiac chamber bulging, and poor survival ratio in host tissues at higher concentrations. Zebrafish is a human-animal model for inventing new drugs and compounds in favor of humans and study the preclinical evaluation to investigate new drug analysis and research. As a result, 3a treated embryos have shown edema in the yolk sac at 100 µg/ml in 24 h, At the same time, a lower concentration of the compounds shows a good survival ratio at 25 µg/ml in 72 h since the embryo was treated. The compound 3e was found to be organ toxic based on phenotypic assays. Slow heartbeat and phenotypic changes were also observed in the higher concentrations. There is a curvature on the back of the embryo for “upcurved fish” and cardiac chamber bulging at starting 25 µg/ml concentration and it happened in all of the above concentration.

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