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Research article

Synthesis and characterization of hesperetin derivatives and toxicity level of the zebrafish model

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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 concentration 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.

1. Introduction

Hesperetin is a flavanone class of flavonoids. It's abundant in oranges and grapefruits, tomatoes, and cherries [1]. Itis a major pharmaceutically active component contained in the peel of citrus fruit. Some citrus flavonoids such as hesperetin and hesperidin have been shown to possess cytoprotective effects by regulating cellular signaling pathways and mitogen-activated protein kinases (MAPKs) [2]. Several studies have reported that hesperetin shows anti-inflammatory, antioxidant, anti-carcinogenic, and neuroprotective effects [3, 4, 5, 6, 7]. The multiple OH groups which confer greater antioxidant potency than are possessed by other flavanones [8, 9]. It easily passes through the blood-brain barrier into the brain and exerts neuroprotective effects [10, 11]. It could reduce neuronal cell death through antioxidant properties [12]. 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. 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 [13, 14]. 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 [15, 16, 17]. 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.

2. Experimental section

2.1. Materials and methods

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

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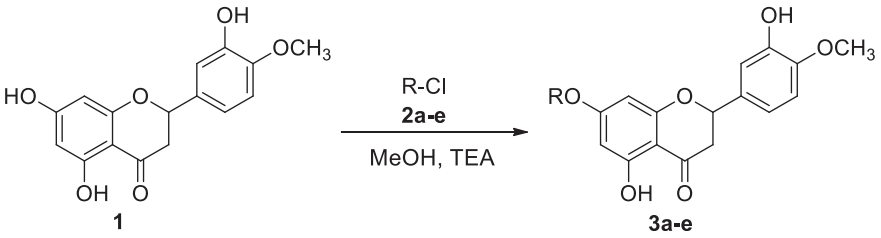


Scheme 1. Synthesis of Hesperetin derivatives (**3a-e**).

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.

2.2. General procedure for the synthesis of hesperetin derivatives (3a-e)

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.

2.2.1. Synthesis of 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-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.

2.2.2. Synthesis of 5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxochroman-7-yl 4-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.

2.2.3. 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.

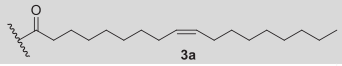
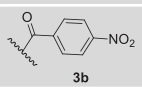
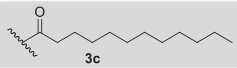
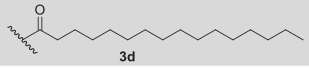
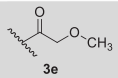
2.3. Breeding and maintenance of zebrafish embryos

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 °C [18].

2.4. 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

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

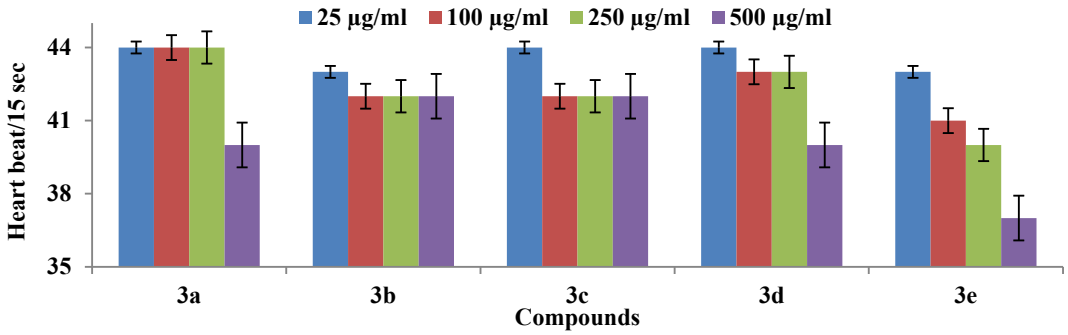


Figure 1. Heartbeat rate analysis after of 6 hpf treated embryos.

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.

3. Results and discussion

3.1. Synthesis of Hesperetin derivatives (3a-e)

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

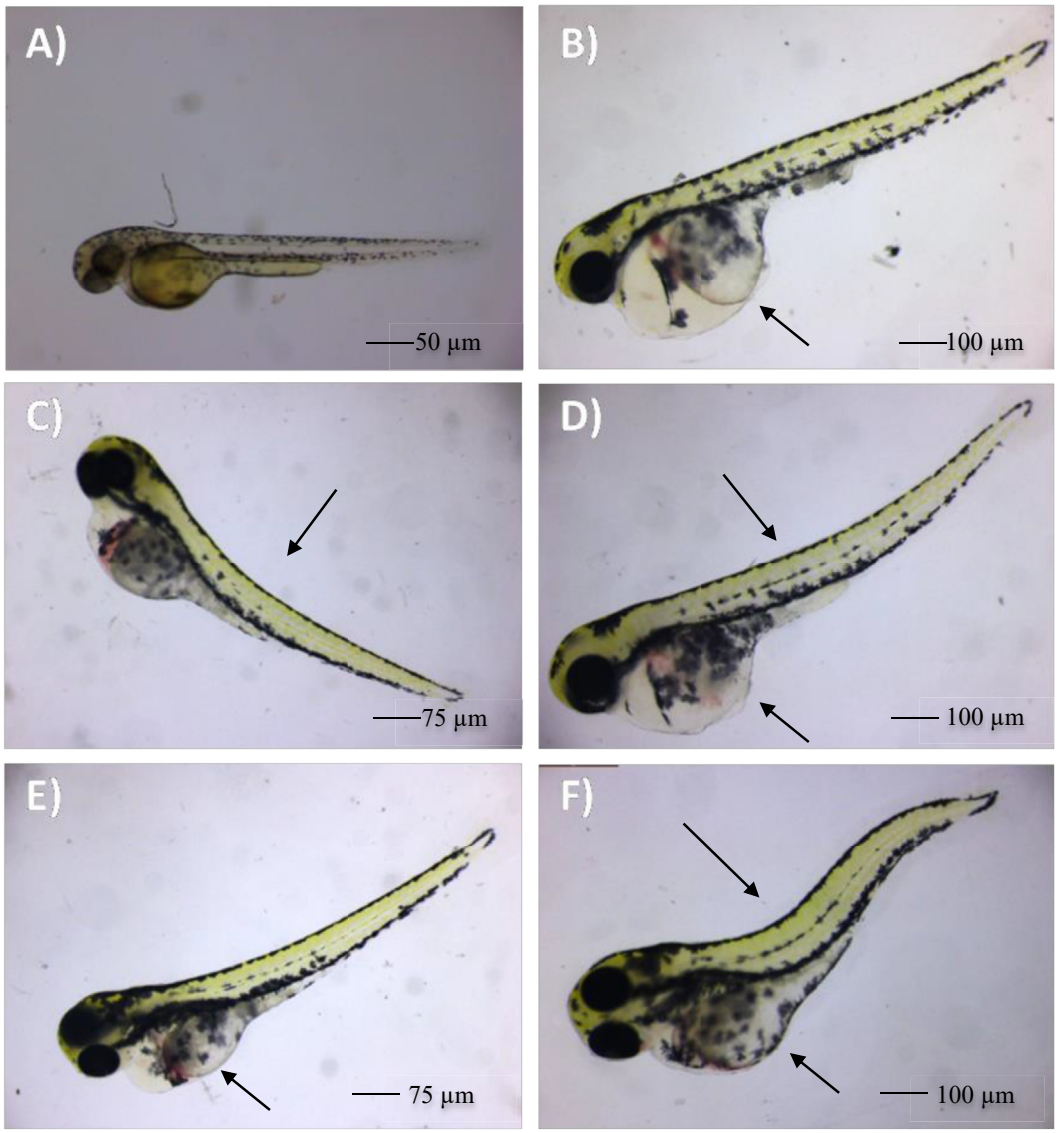


Figure 2. Phenotypic deformities in 24 hpf embryo by using Hesperetin synthesized compounds. A) Control embryo, B) **3a** causes yolk-sac edema at 100 µg/ml, C) **3b** causes up the curved tail at 100 µg/ml, D) **3c** causes pericardial edema and upcurved tail at 500 µg/ml, E) **3d** causes Curved body axis and pericardial edema at 100 µg/ml, F) **3e** upcurved fish and Cardiac chamber bulging at 25 µg/ml.

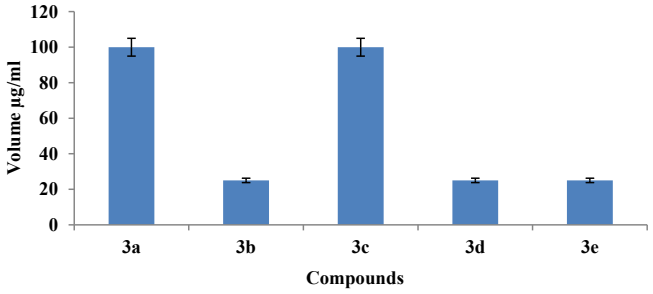


Figure 3. Lethality of 72 hpf treated embryos.

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 2). 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

Table 2. Evaluation of Heartbeat rate (HBR) of zebrafish larvae value are t-Test: Two-Sample Assuming Equal Variances and Anova factor.

ANOVA: Single Factor						
SUMMARY						
Groups	Count	Sum	Average			Variance
44	3	128	**42.66666667**			**5.333333333**
43	3	126	42			0
44	3	126	42			0
44	3	126	42			3
43	3	118	**39.33333333**			**4.333333333**
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	20.266667	4	5.067	2	0.17053	3.47805
Within Groups	25.333333	10	2.533			
Total	**45.6**	**14**				
t-Test: Two-Sample Assuming Equal Variances						
			44			43
Mean			**42.66666667**			**39.33333333**
Variance			**5.333333333**			**4.333333333**
Observations			**3**			**3**
Pooled Variance			4.833333333			
Hypothesized Mean Difference			0			
df			4			
t Stat			1.856953382			
P (T<=t) one-tail			0.068441236			
t Critical one-tail			2.131846782			
P (T<=t) two-tail			0.136882472			
t Critical two-tail			2.776445105			
The mean difference is significant at the 0.05 level.						

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.

3.2. 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 [19]. The cardiac assay results, while increasing concentrations of the compounds the embryos heartbeats showed variations (Figure 1). 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 treated antibiotics is tabulated in Figure 3.

The cardiac assay shows whether the compound is toxic or non-toxic to the zebrafish embryos [20, 21]. 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.

4. Conclusion

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 concentration. Zebrafish is a human-animal model for invent new drugs

and compounds in favor of humans and study the preclinical evaluation to investigate new drug analysis and research. As result, **3a** treated embryos have shown sedema 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.

Declarations

Author contribution statement

M. Rajasekar: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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