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

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A R T I C L E   I N F O

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

A B S T R A C T

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]. It's a major pharmacologically 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 Methoxycetyl 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). 1H & 13C 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.

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 Na2SO4 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 4-nitrobenzoate (3a)

Compound 3a was obtained by the reaction of Hesperetin (1, 1 mmol, 0.30 g), and Oleoyl chloride (2a, 1 mmol, 0.18 g) as a yellow solid. Yield: 0.41 g (73%); [α]20 D + 54.6 (c 0.2, MeOH). 1H NMR (500 MHz, CDCl3 + DMSO-d6): δ 1.23 (t, 3H, J = 7.2 Hz), 7.59 (q, 6H, J = 7.2 Hz), 7.26 (d, 2H, J = 7.2 Hz), 6.82 (d, 1H, J = 10.5 Hz), 7.26 (d, 2H, J = 10.5 Hz), 9.32 (s, 2H).13C NMR (125 MHz, CDCl3 + DMSO-d6): δ 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 C34H46O7: 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 oleate (3b)

Compound 3b was obtained by the reaction of Hesperetin (1, 1 mmol, 0.30 g), and methoxyacetyl chloride (2b, 1 mmol, 0.18 g) as a yellow solid. Yield: 0.30 g (67%); [α]20 D + 48.2 (c 0.2, MeOH). 1H NMR (500 MHz, CDCl3 + DMSO-d6): 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).13C NMR (125 MHz, CDCl3 + DMSO-d6): 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 C23H17NO9: C, 61.22; H, 3.82; N, 3.10. Found: 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 (3c)

Compound 3c was obtained by the reaction of Hesperetin (1, 1 mmol, 0.30 g), and methoxycetyl chloride (2c, 1 mmol, 0.18 g) as a yellow solid. Yield: 0.24 g (65%); [α]20 D + 45.6 (c 0.2, MeOH). 1H NMR (500 MHz, CDCl3 + DMSO-d6): δ 3.98 (d, 2H, J = 5.2 Hz), 4.00 (s, 6H), 4.52 (d, 1H, J = 7.2 Hz), 4.62 (d, 1H, J = 7.2 Hz), 6.42 (d, 1H, J = 7.2 Hz), 6.74 (d, 1H, J = 7.2 Hz), 6.82 (d, 1H, J = 7.2 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).13C NMR (125 MHz, CDCl3 + DMSO-d6): δ 51.3, 52.3, 63.7, 71.8, 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 C19H18O8: C, 60.96; H, 4.85. Found: C, C, 60.94; H, 4.83.

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

| No | R                   | t (h) | Yield (%) |
|----|---------------------|------|-----------|
| 1  | ![OH](O)           | 8    | 73        |
| 2  | ![NO2](O)          | 7    | 67        |
| 3  | ![CH3](O)          | 8    | 70        |
| 4  | ![O](O)            | 8    | 59        |
| 5  | ![OH](O)           | 8    | 65        |
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 1. Heartbeat rate analysis after of 6 hpf treated embryos.](image)

![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.](image)
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. LC50 analysis of all the

![Figure 3. Lethality of 72 hpf treated embryos.](image)

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       | 25.3333333 | 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 |
| 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 1H 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 13C 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.

Funding statement

M. Rajasekar was supported by the DBT (Foldscope), New Delhi.

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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