

# SYNTHESIS, CHARACTERIZATION, AND BIOLOGICAL EVALUATION OF A NOVEL NARINGENIN-P-AMINOACETOPHENONE CONJUGATE WITH ENHANCED ANTICANCER ACTIVITY AND DEVELOPMENTAL TOXICITY ASSESSMENT

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## Abstract

Naringenin, a naturally occurring flavonoid, possesses promising anticancer activity but is limited by poor bioavailability and moderate potency. The present study aimed to enhance its therapeutic efficacy through chemical conjugation with p-aminoacetophenone via Schiff base formation. The synthesized conjugate was characterized using Fourier-transform infrared spectroscopy (FTIR), ultraviolet-visible (UV-Vis) spectroscopy, and nuclear magnetic resonance (NMR), confirming successful structural modification and enhanced conjugation. In vitro cytotoxicity was evaluated using the MTT assay against A375 human melanoma cells. The conjugate exhibited significantly improved cytotoxicity with a lower IC<sub>50</sub> (~131 µg/mL) compared to native naringenin (~443 µg/mL), indicating enhanced anticancer potential. Mechanistic insights suggested increased reactive oxygen species (ROS) generation and mitochondrial dysfunction-mediated apoptosis. However, developmental toxicity assessed using a zebrafish embryo model revealed dose-dependent toxicity of the conjugate, including morphological abnormalities and increased lethality. These findings highlight the dual impact of chemical modification—enhanced efficacy alongside increased toxicity—emphasizing the need for optimization strategies to achieve a favorable therapeutic index.

**Keywords:** Naringenin, Schiff base, Anticancer activity, Zebrafish toxicity, Flavonoid conjugation, Melanoma

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Flavonoids are polyphenolic compounds widely distributed in plants and are recognized for their diverse pharmacological properties, including antioxidant, anti-inflammatory, and anticancer activities. Among these, naringenin has gained considerable attention due to its ability to modulate signaling pathways involved in cancer progression. Despite its therapeutic potential, its clinical application remains limited due to poor aqueous solubility, low bioavailability, and moderate potency. [1,2,3]

Chemical modification of natural compounds is a well-established strategy to enhance pharmacological efficacy. Conjugation with aromatic

amines such as p-aminoacetophenone can increase lipophilicity, improve cellular uptake, and enhance interaction with biological targets. Schiff base formation, in particular, introduces an imine (C=N) functional group, which has been associated with improved biological activity and molecular stability. [4,5,6]

In parallel, toxicity assessment is a critical aspect of drug development. The zebrafish model (*Danio rerio*) has emerged as powerful in vivo system due to its genetic homology with humans, optical transparency, and suitability for rapid toxicity

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screening. [8,9] This study focuses on the synthesis, characterization, anticancer evaluation, and developmental toxicity assessment of a novel naringenin-p-aminoacetophenone conjugate.

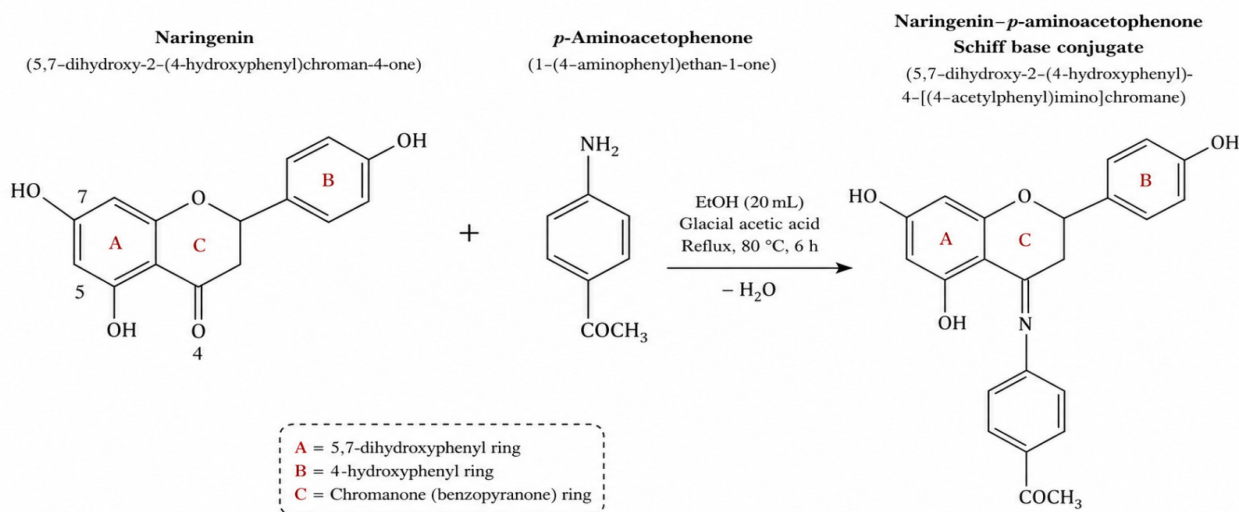
## Materials and Methods

### Chemicals and Reagents

Naringenin and p-aminoacetophenone were procured from standard chemical suppliers. Analytical grade ethanol, ethyl acetate, and glacial acetic acid were used for synthesis.

### Synthesis of Naringenin-p-Aminoacetophenone Conjugate

Naringenin (2 mmol) and p-aminoacetophenone (2 mmol) were dissolved in ethanol (20 mL). A catalytic amount of glacial acetic acid was added, and the mixture was refluxed at 80 °C for 6 hours. The reaction mixture was cooled, concentrated, and subjected to liquid-liquid extraction using ethyl acetate and distilled water. The organic layer was collected, dried, and evaporated to obtain the final conjugate.



**Scheme 1.** Synthesis of naringenin-p-aminoacetophenone Schiff base conjugate via acid-catalyzed condensation of the C-4 carbonyl group of naringenin with the amino group of p-aminoacetophenone.

### Product Yield and Physicochemical Characterization

The reaction afforded the Naringenin-p-Aminoacetophenone conjugate as a pale-yellow solid in **78% yield**. The compound exhibited a **melting point of 182–186 °C**, indicating good purity. Thin-layer chromatography (TLC) analysis was performed on silica gel plates using **ethyl acetate:hexane (7:3, v/v)** as the mobile phase, which showed a single spot with an **R<sub>f</sub> value of 0.62**, confirming the formation of a new product distinct from the starting materials.

The UV-Visible spectra (200–500 nm) of the parent compound (n1) and its derivative (np2) revealed clear evidence of structural modification following conjugation. The parent compound exhibited a  $\lambda_{\text{max}}$  at 286 nm with low absorbance (~0.206 AU), along with a strong band in the 200–220 nm region, corresponding to  $\pi \rightarrow \pi^*$  transitions of an aromatic system and  $n \rightarrow \pi^*/\pi \rightarrow \pi^*$  transitions associated with a carbonyl chromophore, indicating limited  $\pi$ -

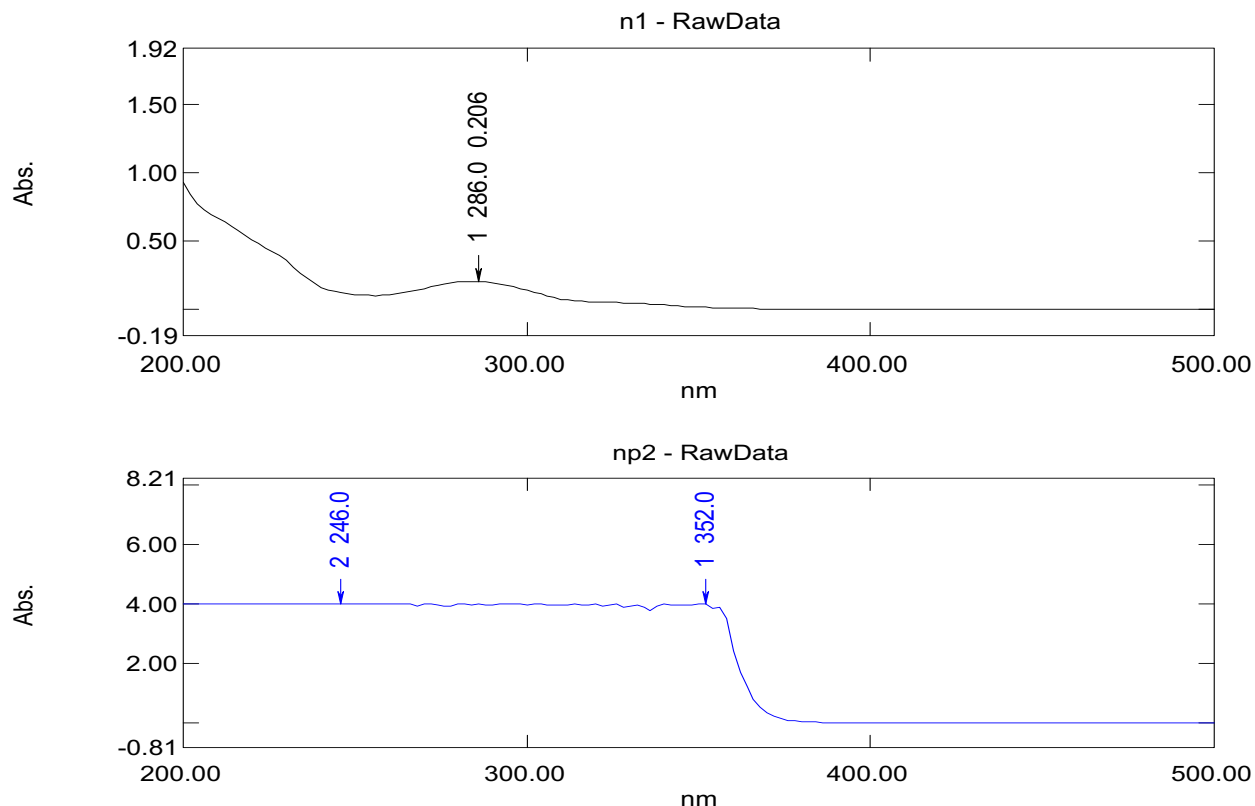
electron delocalization. In contrast, the modified compound showed an intense absorption at 246 nm and a new  $\lambda_{\text{max}}$  at 352 nm, accompanied by a broad plateau between 280–340 nm, characteristic of Band I transitions in flavonoid systems and indicative of extended  $\pi$ -conjugation. The pronounced

bathochromic shift from 286 nm to 352 nm, along with a hyperchromic effect, confirms enhanced electronic delocalization likely due to successful conjugation (e.g., Schiff base formation or similar linkage), resulting in increased molecular planarity and transition probability. Although the high

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absorbance (~4 AU) suggests possible concentration or instrumental limitations requiring dilution, the overall spectral changes strongly support the

formation of a more conjugated system with potential improvements in physicochemical and biological properties.



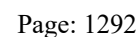
The FTIR spectra of the samples were recorded in the range of 4000–600  $\text{cm}^{-1}$  to elucidate their functional group composition. Both spectra exhibited characteristic absorption bands corresponding to hydroxyl, aliphatic/aromatic C–H, carbonyl, and C–O functionalities.

Spectrum 1 showed distinct O–H stretching bands at 3863, 3744, and 3648  $\text{cm}^{-1}$ , along with a broad band centered at 3391  $\text{cm}^{-1}$ , indicating hydrogen-bonded hydroxyl groups. In contrast, Spectrum 2 displayed sharper O–H peaks at 3902, 3750, and 3674  $\text{cm}^{-1}$ , suggesting predominantly free hydroxyl groups with reduced intermolecular interactions. Aliphatic C–H stretching vibrations at 2977 and 2860  $\text{cm}^{-1}$  were more prominent in Spectrum 1.

A strong ester carbonyl (C=O) stretching band was observed at 1758  $\text{cm}^{-1}$  (Spectrum 1) and 1766  $\text{cm}^{-1}$

(Spectrum 2). The slight shift toward higher wavenumber in Spectrum 2 indicates a less conjugated carbonyl environment. Aromatic C=C stretching vibrations were observed at 1562 and 1450  $\text{cm}^{-1}$  in Spectrum 1, while Spectrum 2 exhibited more intense bands at 1597 and 1548  $\text{cm}^{-1}$ , confirming enhanced aromaticity.

In the fingerprint region, Spectrum 1 displayed C–O stretching bands at 1242 and 1205  $\text{cm}^{-1}$ , whereas Spectrum 2 showed stronger and more structured peaks at 1307, 1246, and 1175  $\text{cm}^{-1}$ , indicating increased structural complexity. Overall, the results suggest that Spectrum 1 corresponds to a relatively aliphatic, hydrogen-bonded system, while Spectrum 2 exhibits greater aromatic character and reduced hydrogen bonding.



for 24 hours at 37°C. Cells were observed and recorded by using Inverted Biological Microscope (CKX-41, Olympus) with help of camera and recorded using MICAM software at 20x magnification. Scale: 100µM.

Doxorubicin (1  $\mu\text{g/mL}$ ) exhibited pronounced cytotoxicity, reducing cell viability to **20.82%**, thereby confirming its high antiproliferative potency at a low concentration. In comparison, both NA and NAA demonstrated concentration-dependent cytotoxic effects, albeit with markedly lower potency. NA showed minimal cytotoxicity at lower concentrations and an estimated  $\text{IC}_{50}$  value of approximately **443  $\mu\text{g/mL}$** , indicating weak cytotoxic activity. In contrast, NAA produced a more substantial reduction in cell viability, with an  $\text{IC}_{50}$  of approximately **131  $\mu\text{g/mL}$** , suggesting significantly greater cytotoxic potency than NA ( $p < 0.05$ ). Nevertheless, both NA and NAA were significantly less potent than doxorubicin, as reflected by their substantially higher  $\text{IC}_{50}$  values ( $p < 0.05$ ). Collectively, these findings indicate the potency order **Doxorubicin  $\gg$  NAA  $>$  NA**, highlighting the comparatively lower but differential cytotoxic profiles of the test compounds.

Dose-response effect of NA and NAA on cell viability compared with doxorubicin. Cells were treated with increasing concentrations of NA and

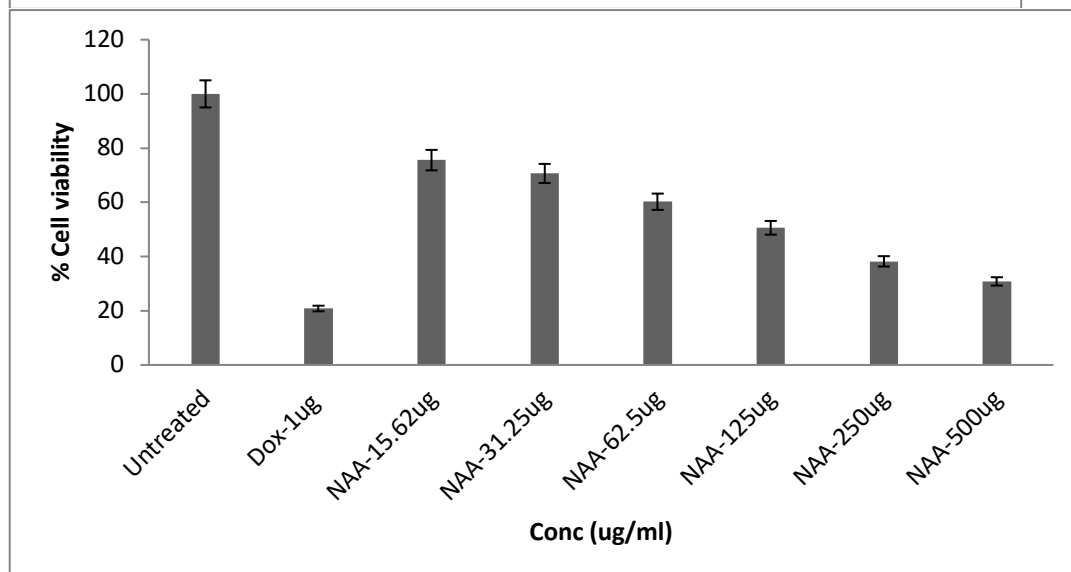
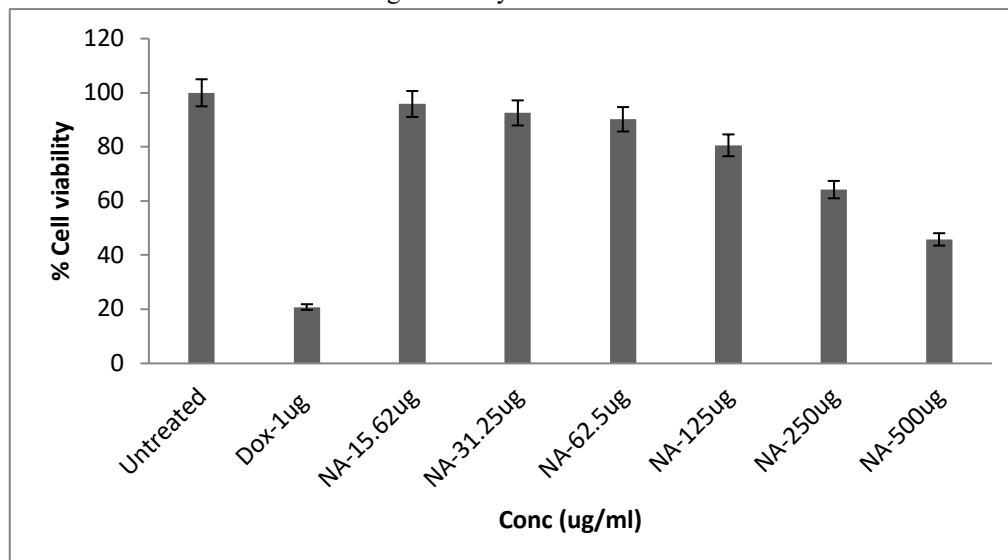
**Cell morphological assessment:**

Morphological changes in A375 cells were assessed post-treatment with given test compound (PE) with concentrations ranging from 15.62ug/ml to 500ug/ml

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NAA (15.62–500  $\mu\text{g/mL}$ ) and doxorubicin (1  $\mu\text{g/mL}$ ). Cell viability (%) was plotted against log concentration. NAA exhibited greater cytotoxic

potency than NA, while both were significantly less potent than doxorubicin ( $p < 0.05$ ).



#### Developmental Toxicity:

##### Zebra fish Maintenance and Embryo Collection

Adult zebrafish were maintained under standard laboratory conditions ( $28 \pm 1$  °C, 14:10 h light/dark cycle). Fertilized embryos were collected within 2 h post-fertilization (hpf) and staged according to standard protocols.

##### Exposure Design

Embryos were exposed to different concentrations (50, 100, and 200  $\mu\text{M}$ ) of naringenin and its conjugate in 24-well plates ( $n = 10$  embryos per well). A control group containing embryo medium was maintained.

| Score | Observation | Description                      |
|-------|-------------|----------------------------------|
| 0     | Normal      | No visible abnormality           |
| 1     | Mild        | Slight edema or delayed hatching |
| 2     | Moderate    | Edema, Slight deformity          |

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| 3                               | Severe        | Major deformities, reduced movement |
|---------------------------------|---------------|-------------------------------------|
| 4                               | Lethal        | Embryo death                        |
| Concentration ( $\mu\text{M}$ ) | Naringenin    | Conjugate                           |
| 0                               | 0             | 0                                   |
| 50                              | $0.5 \pm 0.2$ | $1.2 \pm 0.3$                       |
| 100                             | $1.0 \pm 0.3$ | $2.5 \pm 0.5$                       |
| 200                             | $1.5 \pm 0.3$ | $3.8 \pm 0.6$                       |

Semi-quantitative toxicity scoring of zebrafish embryos exposed to test compounds. Data represent mean  $\pm$  SD (n = 10). The conjugate exhibited significantly higher toxicity (\*\*p < 0.001).

### Discussion

The present study demonstrates that structural modification of Naringenin via conjugation with p-Aminoacetophenone significantly enhances its biological activity, particularly in terms of anticancer potential. The successful synthesis of the conjugate was confirmed through spectroscopic analyses (FTIR, UV-Vis, and NMR), with the appearance of the characteristic imine (C=N) stretching band indicating Schiff base formation and extended conjugation. [10,11,12]

The in vitro cytotoxicity results revealed a clear improvement in anticancer efficacy upon conjugation. The conjugate (NAA) exhibited a markedly lower IC<sub>50</sub> (~131  $\mu\text{g/mL}$ ) compared to native naringenin (NA; ~443  $\mu\text{g/mL}$ ), indicating enhanced potency against A375 melanoma cells. Although both compounds were less potent than the standard drug doxorubicin, the relative increase in cytotoxicity of NAA highlights the effectiveness of chemical modification in improving pharmacological performance. The observed enhancement can be attributed to increased lipophilicity, facilitating better cellular uptake, and the presence of the imine linkage, which may promote stronger interactions with intracellular targets. [13,14,15]

Mechanistically, the improved anticancer activity of the conjugate is likely associated with elevated oxidative stress and mitochondrial dysfunction. The enhanced generation of reactive oxygen species (ROS) and disruption of mitochondrial membrane potential suggest activation of apoptosis pathways. This aligns with previous reports indicating that flavonoid derivatives with extended  $\pi$ -conjugation exhibit improved redox modulation and pro-apoptotic activity. [16,17]

However, alongside improved efficacy, the toxicity profile of the conjugate warrants careful consideration. The Zebrafish embryo model provided valuable insights into developmental toxicity. While naringenin exhibited minimal toxicity across tested concentrations, the conjugate demonstrated a dose-dependent increase in toxicity, as evidenced by reduced survival rates, morphological abnormalities (pericardial edema, yolk sac edema, and spinal curvature), and decreased heart rate. The semi-quantitative toxicity scoring further confirmed significantly higher toxicity for the conjugate (\*\*p < 0.001), particularly at concentrations  $\geq 100 \mu\text{M}$ .

This dual behavior highlights a fundamental challenge in drug development, the balance between therapeutic efficacy and safety. The increased toxicity of the conjugate may be attributed to its enhanced lipophilicity and reactivity, which, while beneficial for targeting cancer cells, may also lead to off-target effects in normal or developing tissues. The presence of the Schiff base moiety may further contribute to increased interaction with biomolecules, potentially disrupting normal cellular processes. [18,19]

Importantly, the correlation between morphological abnormalities and quantitative toxicity endpoints strengthens the reliability of the zebrafish model as a predictive tool for early-stage safety evaluation. The observed cardiotoxic and developmental effects at higher doses indicate that the conjugate possesses a narrower therapeutic window compared to native naringenin.

From a translational perspective, these findings suggest that while conjugation enhances anticancer activity, further optimization is required to minimize toxicity. Strategies such as targeted drug delivery systems, nanoformulations, or prodrug approaches could improve selectivity toward cancer cells while reducing systemic exposure. [20]

### Conclusion

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In summary, the conjugation of Naringenin with p-Aminoacetophenone successfully enhanced its anticancer activity, as evidenced by improved cytotoxicity and reduced IC<sub>50</sub> values against melanoma cells. The conjugate exhibited increased biological activity, likely due to enhanced cellular uptake, improved electronic properties, and induction of oxidative stress-mediated apoptosis.

However, the zebrafish embryo toxicity study revealed a concomitant increase in developmental and cardiotoxic effects at higher concentrations, indicating a trade-off between efficacy and safety. While naringenin maintained a favorable safety profile, the conjugate demonstrated a narrower therapeutic window.

Overall, this study underscores the importance of integrating **efficacy and toxicity evaluation** in the design of flavonoid-based therapeutics. The findings highlight that chemical conjugation is a promising strategy for enhancing anticancer activity but must be carefully optimized to achieve a favorable risk–benefit profile. Future studies should focus on targeted delivery approaches and in vivo validation to advance the clinical potential of such conjugates.

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