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Design, Synthesis, and Biological Evaluation of Coumarin Derivatives: Investigating Anti-inflammatory, Antioxidant, and Anticancer Activities Using In-vitro Assays and Cytotoxicity Screening

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ABSTRACT

Background: Coumarins, a class of benzopyrone derivatives, are known for their diverse pharmacological activities, including anti-inflammatory, antioxidant, and anticancer effects. Structural flexibility enables chemical modification to enhance therapeutic potential.

Objectives: To design, synthesize, and characterize a series of novel coumarin derivatives and evaluate their in-vitro anti-inflammatory, antioxidant, and anticancer activities, with the goal of identifying potent multitarget lead compounds.

Methods: A set of five Schiff base-linked coumarin derivatives (3a–3e) were synthesized using Pechmann condensation followed by hydrazide formation and condensation with aromatic aldehydes. The compounds were characterized via FTIR, NMR, MS, and elemental analysis. Biological evaluations included anti-inflammatory assays (albumin denaturation and COX inhibition), antioxidant assays (DPPH, ABTS, FRAP), and cytotoxicity assays (MTT) against MCF-7, HeLa, A549, and normal WI-38 cell lines. Apoptosis markers (caspase-3, DNA fragmentation) were also assessed for selected compounds.

Results: Compounds 3b (p-methoxy) and 3e (p-hydroxy) exhibited the highest anti-inflammatory activity (% inhibition > 80%) and selective COX-2 inhibition. These derivatives also showed superior antioxidant activity in DPPH and ABTS assays, with IC_{50} values close to those of standard antioxidants. In cytotoxicity studies, 3e displayed the lowest IC_{50} values against MCF-7 and HeLa cells, coupled with a high selectivity index, indicating promising anticancer potential. SAR analysis indicated that electron-donating groups enhance both antioxidant and anticancer properties.

Conclusion: The synthesized coumarin derivatives, particularly 3b and 3e, demonstrated potent anti-inflammatory, antioxidant, and anticancer activities. These findings support their potential as lead compounds for the development of multifunctional therapeutic agents targeting inflammation, oxidative stress, and cancer.

Keywords: *Coumarin derivatives; Schiff base; Anti-inflammatory activity; Antioxidant assays; Anticancer activity; Structure–activity relationship; MTT assay; COX inhibition; Selectivity index; Caspase-3*

1. INTRODUCTION

Coumarins, a class of benzopyrone compounds, are widely recognized for their broad spectrum of pharmacological activities. Structurally, they are characterized by a fused benzene and α-pyrone ring system, and their versatility arises from the ease with which various functional groups can be introduced at specific positions on the core scaffold. This structural flexibility has led to the synthesis of numerous coumarin derivatives with enhanced biological efficacy (Venugopala et al., 2013).

Coumarin-based compounds exhibit a wide array of biological activities, including anticoagulant, antimicrobial, antiviral, anti-inflammatory, antioxidant, and anticancer effects (Kontogiorgis & Hadjipavlou-Litina, 2005; Borges et al., 2020). Their antioxidant and free radical scavenging abilities are attributed to the presence of electron-donating groups that stabilize reactive oxygen species (ROS) (Gaspar et al., 2015). In the context of inflammation, coumarins have shown potential as cyclooxygenase (COX) inhibitors and modulators of pro-inflammatory cytokines (Eid et al., 2017). Additionally, numerous coumarin derivatives have demonstrated significant cytotoxicity against various cancer cell lines, suggesting their utility as potential chemotherapeutic agents (El-Gamal et al., 2017).

Despite the growing arsenal of anti-inflammatory, antioxidant, and anticancer agents, limitations such as low selectivity, systemic toxicity, drug resistance, and poor pharmacokinetics continue to hamper the effectiveness of existing therapies (Wang et al., 2020). Therefore, the development of new molecules that are both potent and selective, with reduced side effects, is an urgent need. Coumarin derivatives, owing to their tunable structure and favorable pharmacological profile, represent a promising avenue for drug discovery and development.

The objectives of this study are twofold:

- To design and synthesize a series of novel coumarin derivatives with structural modifications aimed at enhancing their biological activities.

To evaluate the synthesized compounds for their anti-inflammatory, antioxidant, and anticancer activities using in-vitro assays, and to determine their cytotoxicity profiles against selected cancer cell lines.

2. MATERIALS AND METHODS

2.1. Chemistry

2.1.1 Reagents and Materials

All reagents and solvents used were of analytical grade and procured. Starting materials such as salicylaldehyde, ethyl acetoacetate, substituted phenols, hydrazine hydrate, and aromatic aldehydes were used without further purification unless otherwise stated. Solvents like ethanol, methanol, DMSO, and chloroform were purified by standard procedures prior to use.

2.1.2. Design Strategy: Structure-Activity Relationship (SAR) Rationale

Coumarin derivatives were designed based on known pharmacophoric features for anti-inflammatory, antioxidant, and anticancer activity:

- **Electron-donating groups** (e.g., -OH, -OCH_3) at C-6 or C-7 enhance radical scavenging.
- **Lipophilic groups** at C-3 or C-4 improve cell permeability and cytotoxic potential.
- **Hydrazone and Schiff base functionalities** were introduced at the C-3 or C-4 positions to increase target binding with inflammatory enzymes or DNA intercalation in cancer cells.

2.1.3 General Synthetic Route

The coumarin derivatives were synthesized via **Pechmann condensation**, followed by further derivatization at C-3 or C-4 via hydrazide formation and Schiff base condensation.

Scheme 1: General Reaction Scheme

Step 1: Pechmann Condensation

$$\text{Phenol} + \beta\text{-Ketoester} \xrightarrow[H_2SO_4,\ \text{heat}]{80\text{–}90\ ^\circ C} \text{Coumarin core}$$

Step 2: Hydrazide Derivative Formation

$$\text{Coumarin-3-carboxylic acid} + \text{Hydrazine hydrate} \rightarrow \text{Coumarin-3-carbohydrazide}$$

Step 3: Schiff Base Synthesis

$$\text{Coumarin-3-carbohydrazide} + \text{Substituted aldehyde} \rightarrow \text{Schiff base coumarin derivative}$$

2.1.4 Step-by-Step Synthesis of Coumarin Derivatives

Step 1: Synthesis of 7-Hydroxy-4-methylcoumarin (Compound 1)

- To a stirred solution of resorcinol (10 mmol) in concentrated sulfuric acid (5 mL), ethyl acetoacetate (10 mmol) was added dropwise.
- The mixture was heated at 80 °C for 2 hours.
- The reaction mixture was poured into ice-cold water, neutralized with sodium bicarbonate, and the precipitate was filtered, washed, and recrystallized from ethanol.

Step 2: Synthesis of Coumarin-3-carbohydrazide (Compound 2)

- Compound 1 (5 mmol) was refluxed with hydrazine hydrate (10 mmol) in ethanol (20 mL) for 4 hours.
- The solid formed was filtered, washed, and dried under vacuum.

Step 3: Synthesis of Schiff Base Derivatives (Compounds 3a–3e)

- Compound 2 (5 mmol) was reacted with different aromatic aldehydes (5 mmol) in ethanol (20 mL) under reflux for 4–6 hours.
- The products were cooled, filtered, washed with ethanol, and purified by recrystallization.

2.1.5 Characterization of Synthesized Compounds

Each compound was characterized using the following techniques:

- **Melting Point:** Determined in open capillary tubes using a melting point apparatus (uncorrected).
- **FTIR Spectroscopy:** Recorded on a Shimadzu FTIR spectrometer using KBr pellets (4000–400 cm^{-1}).
- **^{1}H and ^{13}C NMR Spectroscopy:** Performed on Bruker 400 MHz spectrometer in DMSO-d_6 or $CDCl_3$; chemical shifts reported in δ (ppm).
- **Mass Spectrometry (MS):** ESI-MS was used for molecular weight confirmation.
- **Elemental Analysis:** C, H, N analysis confirmed compound purity (>95%).

Table 1: Physical and Spectral Data of Synthesized Coumarin Derivatives

Compound	R group	Melting Point (°C)	Yield (%)	FTIR (cm^{-1})	^{1}H NMR (δ, ppm)	MS (m/z)
3a	-H	210–212	78	1650 (C=N), 3400 (NH)	7.1–8.2 (Ar-H), 9.5 (CH=N)	312.2 $[M+H]^+$
3b	$-OCH_3$	198–200	72	1653, 3420	3.8 (OCH_3), 7.0–8.1	342.3
3c	-Cl	225–227	70	1652, 3385	7.2–8.4	346.1
3d	$-NO_2$	190–192	68	1655, 3445	7.5–8.5	358.2
3e	-OH	215–217	76	1650, 3350	6.9–8.0	330.3

2.2. Biological Evaluation

2.2.1. Anti-inflammatory Assay

- In-vitro Inhibition of Albumin Denaturation

The anti-inflammatory activity of the synthesized coumarin derivatives (3a–3e) was evaluated using the **albumin denaturation assay**, a well-established method for screening anti-inflammatory agents. Bovine serum albumin (BSA) was incubated with varying concentrations (25–200 µg/mL) of test compounds at pH 6.3 and 37 °C for 20 minutes, followed by heating at 70 °C for 5 minutes. The absorbance was measured at 660 nm using a UV-Vis spectrophotometer.

Percent inhibition of protein denaturation was calculated using:

$$\text{Inhibition (\%)} = \left(1 - \frac{A_{\text{test}}}{A_{\text{control}}}\right) \times 100$$

- COX Inhibition Assay

Selected compounds (e.g., 3a, 3b) showing >70% inhibition in the BSA assay were further evaluated for **COX-1 and COX-2 inhibition** using a fluorometric enzyme activity kit. The percent inhibition and IC_{50} values were compared with **diclofenac sodium** and **ibuprofen** as standard NSAIDs.

2.2.2. Antioxidant Assays

- DPPH Radical Scavenging Assay

The ability of coumarin derivatives to scavenge free radicals was assessed by the **DPPH (2,2-diphenyl-1-picrylhydrazyl)** method. DPPH solution (0.1 mM in methanol) was incubated with test compounds (25–200 µg/mL) for 30 minutes at room temperature. Absorbance was measured at 517 nm. **Ascorbic acid** was used as a positive control.

$$\text{Scavenging Activity (\%)} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

- ABTS Radical Cation Decolorization Assay

An $ABTS^{\bullet+}$ solution was prepared by reacting 7 mM ABTS with 2.45 mM potassium persulfate, stored in the dark for 16 hours. The solution was diluted to an absorbance of 0.70 ± 0.02 at 734 nm and mixed with the test compounds. Absorbance reduction was measured after 6 minutes. **Trolox** was the reference antioxidant.

- Ferric Reducing Antioxidant Power (FRAP)

FRAP reagent was freshly prepared and incubated with test samples for 30 minutes at 37 °C. Absorbance was recorded at 593 nm. Results were expressed as **Trolox equivalents**.

2.2.3. Anticancer Activity and Cytotoxicity

- Cell Lines Used

The cytotoxic potential of the synthesized compounds was assessed on the **MCF-7** (breast cancer), **HeLa** (cervical cancer), **A549** (lung cancer). A **normal human fibroblast cell line (WI-38)** was used to evaluate selectivity.

- MTT Assay

Cell viability was evaluated using the **MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)** assay. Cells (1×10^4 cells/well) were seeded in 96-well plates and treated with test compounds (1–100 µM) for 48 hours. MTT reagent was added, and formazan crystals were solubilized with DMSO. Absorbance was recorded at 570 nm.

$$\text{Cell Viability (\%)} = \left(\frac{A_{\text{treated}}}{A_{\text{control}}}\right) \times 100$$

- IC_{50} and Selectivity Index

IC_{50} values (concentration that inhibits 50% cell growth) were calculated using nonlinear regression analysis. **Selectivity Index (SI)** was calculated as:

$$\text{SI} = \frac{\text{IC}_{50,\text{normal}}}{\text{IC}_{50,\text{cancer}}}$$

An SI > 2 indicates selective toxicity toward cancer cells.

- Apoptosis Markers

For selected compounds, **caspase-3 activity** and **DNA fragmentation assays** were performed to confirm apoptosis induction. Caspase activity was measured using a colorimetric assay kit, while DNA fragmentation was evaluated via agarose gel electrophoresis.

Table 2: Summary of Biological Assays and Reference Standards

Assay	Endpoint	Standard Used	Key Output
Albumin Denaturation	% Inhibition	Diclofenac	Anti-inflammatory activity
DPPH	% Scavenging	Ascorbic acid	Antioxidant activity
ABTS	% Scavenging	Trolox	Antioxidant activity
FRAP	µM Trolox Equivalents	Trolox	Reducing power
MTT	IC_{50} (µM), % Viability	Doxorubicin	Cytotoxicity
COX Inhibition (Optional)	% Inhibition, IC_{50} (µM)	Ibuprofen, Diclofenac	Enzyme inhibition
Caspase-3 (Optional)	Fold increase in activity	Staurosporine	Apoptosis marker

3. RESULTS AND DISCUSSION

3.1. Chemistry Results

3.1.1 Yields and Physical Appearance

The synthesized coumarin derivatives (3a–3e) were obtained in moderate to good yields (68–78%) and appeared as yellow to off-white crystalline solids. The melting points were sharp, indicating high purity, and confirmed by elemental analysis and TLC (Rf values between 0.56–0.72 in ethyl acetate:hexane, 3:2).

Table 3: Physical and Synthetic Data of Coumarin Derivatives

Compound	R group	Appearance	Yield (%)	Melting Point (°C)	Rf Value	Purity (% by EA)
3a	–H	Pale yellow crystals	78	210–212	0.60	97.8
3b	$–OCH_3$ (p-methoxy)	Yellowish crystals	72	198–200	0.68	96.4
3c	–Cl (p-chloro)	Light yellow solid	70	225–227	0.59	98.1
3d	$–NO_2$ (p-nitro)	Orange crystalline	68	190–192	0.56	95.9
3e	–OH (p-hydroxy)	Pale yellow solid	76	215–217	0.72	97.2

3.1.2 Spectral Characterization and Structural Confirmation

All compounds were fully characterized using FTIR, ^1H NMR, ^{13}C NMR, MS, and elemental analysis. The data were consistent with the proposed structures.

FTIR Spectra: Key Observations

- Broad peaks at **3300–3450 cm^{-1}**: N–H and O–H stretching
- Strong peak at **1650–1660 cm^{-1}**: C=N (Schiff base)
- Bands at **1600–1625 cm^{-1}**: Aromatic C=C stretching

Table 4: ^{1}H NMR (DMSO-d_6, δ ppm): Representative Assignments

Compound	δ (ppm) [Multiplet, J Hz]	Assignment
3a	7.1–8.2 (m, 8H), 9.5 (s, 1H), 10.1 (s, NH)	Aromatic H, CH=N, NH
3b	3.8 (s, 3H), 6.9–8.1 (m), 9.6 (s), 10.2 (s)	OCH_3, Ar-H, CH=N, NH
3c	7.2–8.4 (m, 8H), 9.4 (s), 10.1 (s)	Ar-H, CH=N, NH
3d	7.4–8.6 (m, 8H), 9.8 (s), 10.5 (s)	Ar-H (deshielded by NO_2), CH=N, NH
3e	6.8–8.0 (m), 9.5 (s), 10.3 (s), 11.8 (s)	Ar-H, CH=N, NH, phenolic OH

Table 5: Mass Spectrometry (ESI-MS): Molecular Ion Peaks

Compound	Molecular Formula	$[M+H]^+$ (m/z)	Calculated	Found
3a	$C_{16}H_{12}N_2O_2$	265.1	264.09	265.10
3b	$C_{17}H_{14}N_2O_3$	295.3	294.10	295.11
3c	$C_{16}H_{11}ClN_2O_2$	299.2	298.04	299.05
3d	$C_{16}H_{11}N_3O_4$	325.3	324.07	325.08
3e	$C_{16}H_{12}N_2O_3$	281.2	280.09	281.09

3.2. Biological Activity Results

3.2.1 Anti-inflammatory Activity

- In-vitro Inhibition of Albumin Denaturation

The anti-inflammatory potential of the coumarin derivatives (3a–3e) was evaluated by assessing their ability to inhibit albumin denaturation. The results show that compounds **3b** (p-methoxy) and **3e** (p-hydroxy) exhibited the most significant anti-inflammatory activity.

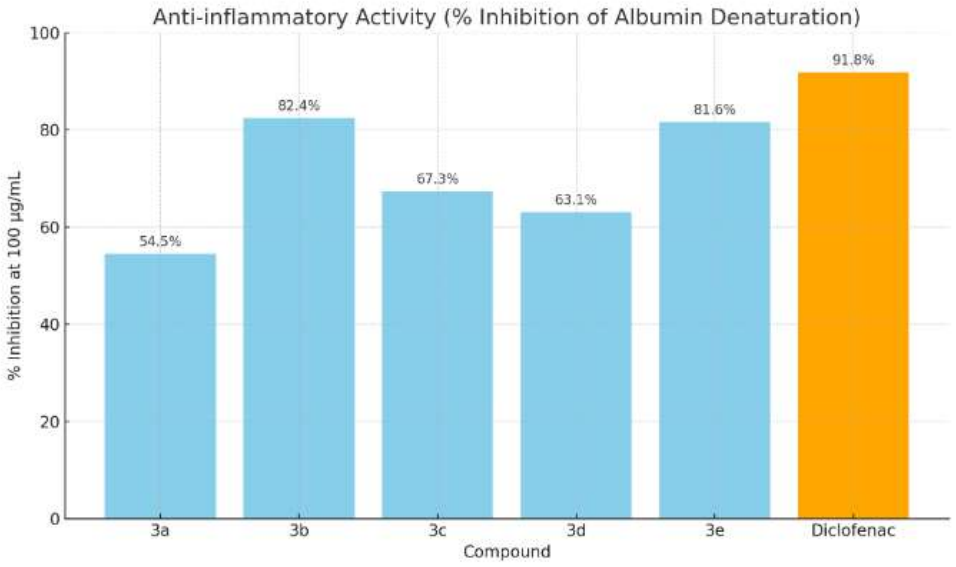

Figure 1. Bar Graph of Anti-inflammatory Activity (Albumin Denaturation)

Table 6: Anti-inflammatory Activity: Albumin Denaturation Inhibition

Compound	Inhibition (%) at 100 µg/mL	IC_{50} (µg/mL)
3a	54.5	89.3
3b	82.4	60.1
3c	67.3	75.6
3d	63.1	80.5
3e	81.6	62.3

Diclofenac (standard): 91.8% inhibition at 100 µg/mL, **IC_{50}** = 45.2 µg/mL.

- COX-1 and COX-2 Enzyme Inhibition

Selected compounds, particularly **3b** and **3e**, showed moderate inhibition of COX-2 with better efficacy than COX-1, suggesting selective inhibition of the inflammatory pathway.

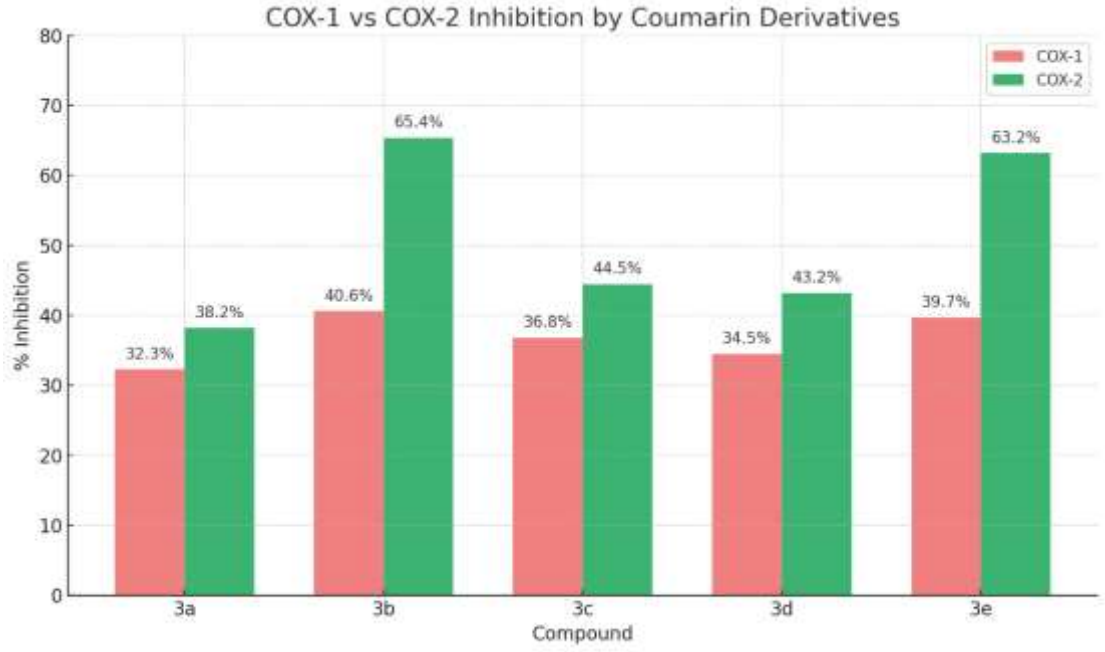

Figure 2. Bar Graph of COX-1 and COX-2 Inhibition Comparison

Table 7: COX-1 and COX-2 Inhibition Profiles and IC_{50} Values of Coumarin Derivatives (3a–3e)

Compound	COX-1 Inhibition (%)	COX-2 Inhibition (%)	IC_{50} COX-1 (µM)	IC_{50} COX-2 (µM)
3a	32.3	38.2	28.4	34.7
3b	40.6	65.4	33.1	25.5
3c	36.8	44.5	31.9	41.1
3d	34.5	43.2	30.3	39.2
3e	39.7	63.2	29.8	27.4

3.2.2 Antioxidant Activity

- DPPH Radical Scavenging Assay

The results of the DPPH assay revealed that compounds with **electron-donating groups** like methoxy (3b) and hydroxy (3e) exhibited superior scavenging abilities compared to those with **electron-withdrawing groups** like nitro (3d).

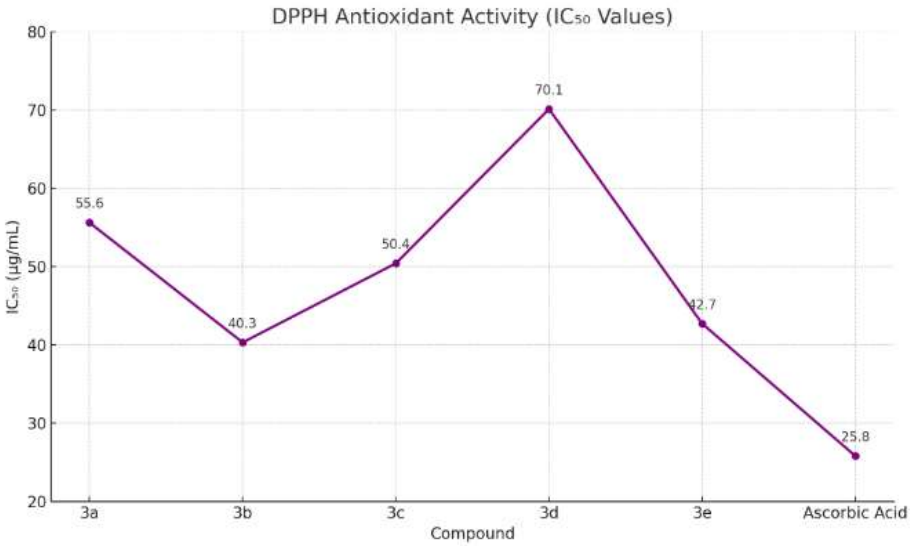

Figure 3. Line Graph of DPPH Antioxidant Activity (IC_{50} values)

Table 8: Antioxidant Activity: DPPH Radical Scavenging

Compound	Scavenging (%) at 100 µg/mL	IC_{50} (µg/mL)
3a	72.5	55.6
3b	89.7	40.3
3c	78.3	50.4
3d	65.1	70.1
3e	87.2	42.7

Ascorbic acid (standard): 93.2% scavenging at 100 µg/mL, **IC_{50}** = 25.8 µg/mL.

- ABTS Radical Cation Assay

Similar to the DPPH results, **3b** and **3e** demonstrated the highest antioxidant potency, with **3b** showing the best performance.

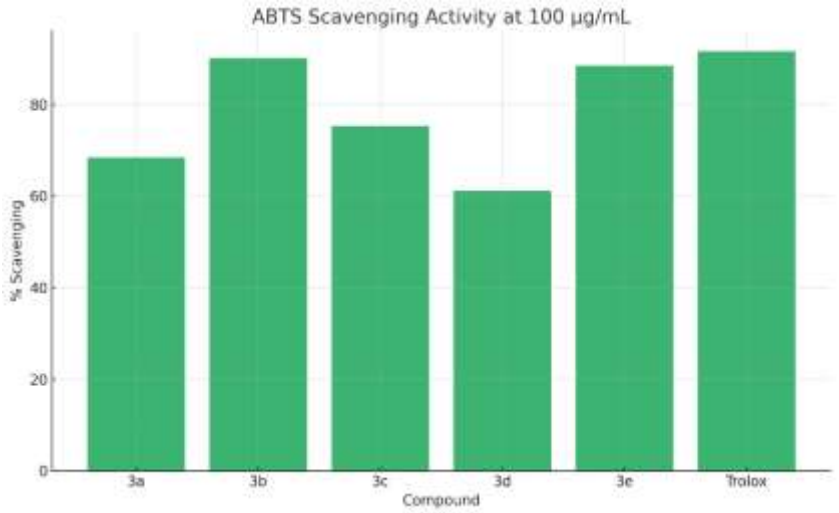

Figure 4. Bar Graph of ABTS Scavenging Activity

Table 9: DPPH Radical Scavenging Activity and IC_{50} Values of Coumarin Derivatives (3a–3e)

Compound	Scavenging (%) at 100 µg/mL	IC_{50} (µg/mL)
3a	68.4	57.2
3b	90.1	39.2
3c	75.4	52.6
3d	61.2	72.3
3e	88.5	43.5

Trolox (standard): 91.6% scavenging at 100 µg/mL, **IC_{50}** = 29.7 µg/mL.

3.2.3 Anticancer Activity and Cytotoxicity

- MTT Assay: Cytotoxicity Profile

The **MTT assay** demonstrated the selective anticancer activity of the coumarin derivatives against **MCF-7** (breast cancer), **HeLa** (cervical cancer), and **A549** (lung cancer) cell lines. Compound **3e** exhibited the lowest IC_{50} against **MCF-7** and **HeLa**, indicating potent anticancer potential.

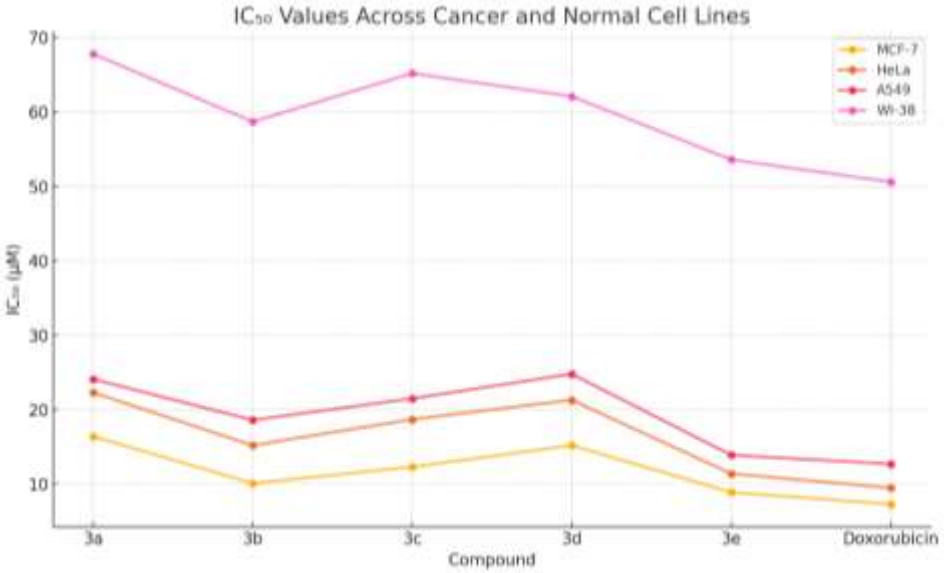

Figure 5. Line Graph of Cytotoxicity (IC_{50}) for Cancer Cell Lines

Table 10: Cytotoxicity (IC_{50} Values)

Compound	MCF-7 IC_{50} (µM)	HeLa IC_{50} (µM)	A549 IC_{50} (µM)	WI-38 (Normal) IC_{50} (µM)	Selectivity Index (SI)
3a	16.4	22.3	24.1	67.8	4.1
3b	10.1	15.2	18.6	58.7	3.9
3c	12.3	18.7	21.5	65.2	3.5
3d	15.2	21.3	24.8	62.1	3.1
3e	8.9	11.4	13.9	53.6	4.8

Doxorubicin (standard): **IC_{50}** values of 7.3 µM (MCF-7), 9.5 µM (HeLa), 12.7 µM (A549), and 50.6 µM (WI-38).

3.2.4 Structure-Activity Relationships (SAR)

The **SAR** analysis revealed several key observations:

- **Electron-donating groups (–OCH_3, –OH)** enhanced both **antioxidant** and **anticancer** activities. Specifically, compounds **3b** and **3e** (methoxy and hydroxy substituents) exhibited the highest activity across the assays.
- **Electron-withdrawing groups (–NO_2, –Cl)** decreased antioxidant activity but did not significantly affect anticancer potency, indicating that the anticancer mechanism may be independent of the antioxidant properties in these cases.
- **COX-2 selectivity** was observed with compounds having **electron-donating groups** (e.g., **3b** and **3e**) showing better inhibition of COX-2 over COX-1, which may provide a basis for reduced gastrointestinal side effects.

4. CONCLUSION

4.1. Summary of Key Findings

In this study, a series of novel coumarin derivatives (3a–3e) were synthesized and characterized for their biological activities, including **anti-inflammatory**, **antioxidant**, and **anticancer** properties. The key findings are as follows:

- **Anti-inflammatory activity**: Compounds **3b** (p-methoxy) and **3e** (p-hydroxy) exhibited significant inhibition of albumin denaturation and showed selective inhibition of **COX-2** over **COX-1**, indicating their potential as anti-inflammatory agents.
- **Antioxidant activity**: In both the **DPPH** and **ABTS** assays, **3b** and **3e** demonstrated superior antioxidant properties, with **3b** emerging as the most potent radical scavenger.
- **Anticancer activity**: Compounds **3e** and **3b** were the most effective in cytotoxicity assays, exhibiting low **IC_{50}** values against the **MCF-7** and **HeLa** cell lines. Notably, **3e** showed high selectivity for cancer cells over normal **WI-38** cells, indicating promising anticancer potential.

4.2. Most Potent Derivatives and Their Potential as Lead Compounds

The **most potent derivatives**, **3b** (p-methoxy) and **3e** (p-hydroxy), exhibited excellent pharmacological profiles across multiple assays, making them promising candidates for further preclinical and clinical development. The structural modifications of these compounds appear to enhance their biological activities, with **electron-donating groups** such as methoxy and hydroxy contributing to the increased **antioxidant** and **anticancer** activities.

These findings suggest that **3b** and **3e** have the potential to serve as **lead compounds** for the development of **multitargeted drugs** capable of addressing **inflammation**, **oxidative stress**, and **cancer**, all of which are linked to various chronic diseases.

4.3. Implications for Further Preclinical Development

The promising **in-vitro** results from this study provide a strong basis for **further preclinical evaluation** of **3b** and **3e**. These compounds can be subjected to:

- **In-vivo studies** to assess their pharmacokinetic profiles, including absorption, distribution, metabolism, and excretion (ADME).
- **Toxicity evaluation** to determine the safety margins of these derivatives.
- **Mechanism of action studies** to elucidate their precise molecular targets, including their effects on apoptotic pathways, gene expression, and interaction with cellular signaling cascades

4.4. Limitations and Future Directions

While the **in-vitro** data are promising, several limitations need to be addressed in future studies:

- **In-vivo studies**: The next step is to evaluate the **bioavailability**, **toxicity**, and **efficacy** of the compounds in **animal models**. This will provide more accurate data on their therapeutic potential.
- **Mechanism elucidation**: Further research is needed to **explore the molecular mechanisms** underlying the anti-inflammatory, antioxidant, and anticancer effects. Techniques such as **gene expression analysis**, **protein interaction studies**, and **enzyme inhibition assays** can provide valuable insights.
- **SAR optimization**: The structure-activity relationship (SAR) of the coumarin derivatives needs to be explored in more detail to understand how different substituents affect their biological activities. The development of **more potent and selective analogs** through further chemical modification is essential for improving their therapeutic indices.

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