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In Vitro Cytotoxicity Testing of Novel Drug Molecules Using MTT Assay

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ABSTRACT

Cytotoxicity testing using in vitro models is a fundamental step in drug discovery to evaluate the potential toxicity and therapeutic index of novel drug candidates. Among the various available assays, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is a widely used, cost-effective colorimetric method to measure cellular metabolic activity and viability. This study aimed to investigate the cytotoxic effects of three newly synthesized drug molecules (ND-1, ND-2, and ND-3) on human cancer cell lines—HeLa (cervical cancer) and MCF-7 (breast cancer)—as well as on the non-cancerous HEK293 cell line (human embryonic kidney cells). Cells were treated with different concentrations of the compounds, and the MTT assay was performed after 24 and 48 hours to assess cell viability. The results showed a dose-dependent reduction in cell viability, with ND-2 displaying the most potent cytotoxicity against MCF-7 cells, having an IC_{50} of 8.4 µM. ND-3 demonstrated comparatively lower toxicity to normal HEK293 cells, indicating a better selectivity index. The study confirms the efficacy of the MTT assay in screening cytotoxic effects of novel compounds and identifies ND-2 and ND-3 as promising leads for further anticancer evaluation. These findings suggest the utility of integrating in vitro cytotoxicity profiling early in drug development pipelines.

Keywords: MTT assay; cytotoxicity; in vitro; novel drug molecules; IC_{50}; cell viability; HeLa cells; MCF-7; HEK293; selectivity index; anticancer agents.

I. INTRODUCTION

1.1 Drug Discovery and the Need for Cytotoxicity Screening

The development of new therapeutic agents, particularly anticancer drugs, is a critical and complex process that requires careful evaluation of their efficacy and safety. One of the fundamental steps in the drug discovery and preclinical screening pipeline is the assessment of **cytotoxicity**, which evaluates the potential toxic effects of a compound on living cells. Cytotoxicity screening provides essential information regarding the therapeutic index, allowing researchers to distinguish between compounds that can selectively kill cancer cells while sparing normal healthy cells. This selectivity is the cornerstone of effective and safer anticancer therapies.[1]

In recent years, significant emphasis has been placed on **in vitro cytotoxicity assays**, which offer a cost-effective, rapid, and ethically acceptable alternative to in vivo testing in early-stage research. Among the numerous assays available, the **MTT assay** has gained widespread acceptance due to its simplicity, reproducibility, and compatibility with high-throughput screening formats.[2]

1.2 The MTT Assay: Principle and Relevance

The **MTT assay**, first described by Mosmann in 1983, is a colorimetric technique that measures cell metabolic activity as an indirect indicator of cell viability. The assay relies on the enzymatic reduction of the yellow tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), into **purple formazan crystals** by mitochondrial **succinate dehydrogenase** enzymes that are active in living

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cells. Dead or non-metabolically active cells do not reduce MTT, making the assay highly sensitive to cell viability and mitochondrial function.[3]

After the MTT reagent is added and incubated with cells, the formed formazan crystals are solubilized using an organic solvent such as DMSO or isopropanol, and the absorbance is measured at **570 nm** using a spectrophotometer or plate reader. The intensity of the purple color is directly proportional to the number of viable cells.

The MTT assay is highly adaptable for use in **96-well microtiter plates**, making it suitable for testing multiple compounds at different concentrations in a single experiment. It has become a standard method in the assessment of cytotoxicity, apoptosis, proliferation, and drug screening.[4]

1.3 Importance of In Vitro Models in Cancer Research

Cancer is a complex disease characterized by uncontrolled cell proliferation, resistance to apoptosis, angiogenesis, and metastasis. Over the years, extensive research has led to the discovery of several anticancer drugs; however, the development of resistance, side effects, and lack of selectivity continue to limit their clinical success. Thus, there is a growing demand for **novel drug candidates** with higher efficacy and improved safety profiles.[5]

In vitro models offer a valuable platform to study the **mechanism of action of anticancer agents**, screen for cytotoxic effects, and perform dose-response analysis. By using both **cancerous** and **non-cancerous** cell lines, researchers can also evaluate the selectivity of a drug molecule—an important feature that contributes to reduced systemic toxicity in vivo.

In this study, two human cancer cell lines were selected:

- **HeLa cells**: Derived from cervical cancer and one of the most commonly used cell lines in biomedical research due to their robust growth and reproducibility.
- **MCF-7 cells**: Represent estrogen receptor-positive breast cancer cells and are often used to evaluate the anticancer potential of drugs against hormone-responsive tumors.

In addition, **HEK293 cells**, derived from human embryonic kidney tissue, were used as a representative of normal cells to evaluate the **selectivity index** of the tested compounds.[6]

1.4 Novel Drug Molecules and the Rationale for Evaluation

In the current study, three newly synthesized drug candidates, coded as **ND-1**, **ND-2**, and **ND-3**, were selected for cytotoxicity evaluation. These molecules were designed and synthesized based on structure-activity relationship (SAR) insights from previously known bioactive scaffolds. The novelty of these molecules lies in their unique substitution patterns, heterocyclic backbones, and functional groups that potentially enhance their interactions with key cellular targets, such as DNA, topoisomerase, and tubulin proteins.[7]
Before proceeding to **in vivo studies or clinical testing**, it is essential to:

- Assess the **toxicity profile** of these compounds.
- Identify the $\mathbf{IC_{50}}$ (half-maximal inhibitory concentration) values.
- Evaluate their ability to **differentiate between normal and cancerous cells**.

Such in vitro screening provides critical insight into the **therapeutic window**, which helps in determining safe and effective dosage ranges.

1.5 Objectives of the Study

The primary goal of this research is to evaluate the **cytotoxic potential** of ND-1, ND-2, and ND-3 using the MTT assay and to identify the most potent and selective candidate among them. The specific objectives are as follows:[8]

1. To culture and maintain human cancer cell lines (HeLa and MCF-7) and a non-cancerous cell line (HEK293).
2. To treat these cell lines with different concentrations of the novel drug molecules.
3. To perform the MTT assay and quantify cell viability.
4. To calculate the $\mathbf{IC_{50}}$ values and analyze**dose-response curves**.
5. To determine the **selectivity index (SI)** of each compound by comparing their toxicity on cancerous vs. normal cells.

This study aims to bridge the gap between chemical synthesis and pharmacological evaluation by providing a systematic assessment of the cytotoxic behavior of promising lead molecules.

1.6 Significance and Expected Outcomes

The significance of this study lies in its contribution to the **early-phase screening** of anticancer agents. By identifying compounds with:[9]

- **High cytotoxicity toward cancer cells**

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- **Low toxicity toward normal cells**
- **Promising IC_{50} values and selectivity index**

This research will lay the foundation for **further mechanistic studies**, such as apoptosis induction, DNA fragmentation, ROS generation, and caspase activation. These follow-up studies are essential to understand the **molecular pathways** involved in drug-induced cell death.[10]

Moreover, results from this study can serve as a **benchmark** for in vivo efficacy studies in animal models, formulation development, and eventual clinical translation.

II. MATERIALS AND METHODS

This section provides a comprehensive outline of the experimental procedures used for the in vitro cytotoxicity evaluation of novel drug molecules (ND-1, ND-2, ND-3) using the MTT assay on human cancer and normal cell lines.

2.1 Chemicals and Reagents

The following analytical-grade chemicals and reagents were used:[11]

Name	Source	Purpose
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)	Sigma-Aldrich	Cell viability assay
Dimethyl sulfoxide (DMSO)	Merck	Solubilization of formazan crystals
Phosphate Buffered Saline (PBS)	Himedia	Washing cells
Trypsin-EDTA (0.25%)	Gibco	Cell detachment
RPMI-1640 and DMEM media	Gibco	Cell culture medium
Fetal Bovine Serum (FBS)	Gibco	Supplement for cell growth
Penicillin-Streptomycin solution	Gibco	Antibiotic supplement
Novel drug molecules ND-1, ND-2, ND-3	In-house synthesis	Experimental compounds

2.2 Equipment and Instruments[12]

Equipment	Use
CO_2 Incubator (5% CO_2 , 37°C)	Cell culture maintenance
Inverted phase contrast microscope	Morphological observation
96-well flat-bottomed tissue culture plates	MTT assay
Multichannel pipette	Reagent dispensing
ELISA plate reader (570 nm)	Absorbance measurement
Laminar air flow cabinet	Aseptic handling
Centrifuge	Media removal and washing
pH meter and sterile filters	Media preparation

2.3 Cell Lines Used

Three mammalian cell lines were used in this study:[13]

Cell Line	Type	Source	Medium Used
HeLa	Human cervical cancer	NCCS, Pune	RPMI-1640
MCF-7	Human breast cancer	NCCS, Pune	DMEM
HEK293	Human embryonic kidney (normal)	NCCS, Pune	DMEM

All cell lines were authenticated and tested free of mycoplasma contamination before use.

2.4 Preparation of Drug Solutions

- Stock solutions of ND-1, ND-2, and ND-3 were prepared in **DMSO** at a concentration of **10 mM**.

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- The stock was filtered using 0.22 µm sterile syringe filters.
- Serial dilutions (5, 10, 20, 40, and 80 µM) were made in complete culture media.
- The final concentration of DMSO in all wells was maintained below **0.5%**, which was confirmed to be non-toxic to cells.

2.5 Cell Culture and Maintenance[14]

1. All cell lines were cultured in **T-25 flasks** containing the respective media supplemented with:
   - 10% heat-inactivated FBS
   - 1% Penicillin-Streptomycin (100 U/mL and 100 µg/mL)
2. Cultures were maintained at **37°C**, 5% CO_2 , and 95% relative humidity.
3. Medium was changed every 48–72 hours.
4. Subculturing was done using **0.25% Trypsin-EDTA** upon reaching 70–80% confluency.

2.6 Seeding of Cells for MTT Assay[15]

1. Cells were harvested using trypsin and counted using a **hemocytometer**.
2. A total of **1×10^4 cells/well** were seeded in **96-well plates** in 100 µL complete media.
3. Plates were incubated for **24 hours** to allow cell adherence and recovery.

2.7 Drug Treatment Procedure[16]

1. After 24 hours of seeding, culture medium was aspirated gently.
2. Cells were treated with **100 µL** of each concentration (5, 10, 20, 40, 80 µM) of ND-1, ND-2, and ND-3 in fresh complete medium.
3. Control wells received medium with **0.5% DMSO** only (vehicle control).
4. Plates were incubated for **24 and 48 hours** at 37°C under 5% CO_2 .

2.8 MTT Assay Protocol[17]

1. After the treatment period, **10 µL of MTT solution (5 mg/mL)** in PBS was added to each well.
2. Plates were incubated for **4 hours** in the dark at 37°C.
3. After incubation, the media was carefully removed without disturbing the purple formazan crystals.
4. **100 µL of DMSO** was added to each well to dissolve the crystals.
5. The plates were gently agitated on a **shaker for 10 minutes** in the dark.
6. Absorbance was read at **570 nm** using an ELISA plate reader.

2.9 Calculation of Cell Viability

The cell viability percentage was calculated using the formula:

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

Where:

- OD = Optical Density at 570 nm
- Control = Cells treated with vehicle only

2.10 IC_{50} Determination[18]

- The **IC_{50} value** (the concentration of compound required to inhibit 50% of cell viability) was calculated using **GraphPad Prism** software.
- Non-linear regression analysis with a sigmoidal dose-response curve (log(inhibitor) vs. response) was used.
- Experiments were performed in **triplicate**, and results were expressed as **mean ± standard deviation (SD)**.

2.11 Selectivity Index (SI)[19]

The selectivity index was determined to evaluate the specificity of compounds for cancer cells over normal cells:

$$\text{Selectivity Index (SI)} = \frac{IC_{50}\ (\text{HEK293})}{IC_{50}\ (\text{Cancer cell line})}$$

- SI > 2 is generally considered indicative of selective anticancer activity.

2.12 Statistical Analysis[20]

- Data are presented as **mean ± SD** of at least **three independent experiments**.
- **One-way ANOVA** followed by **Tukey's multiple comparison test** was used to analyze statistical significance.
- **P-values < 0.05** were considered statistically significant.
- Software used: GraphPad Prism 9.5.0.

III. RESULTS

The MTT assay was performed to evaluate the **cytotoxic potential** of three novel drug candidates (ND-1, ND-2, ND-3) against two human cancer cell lines (HeLa and MCF-7) and one non-cancerous cell line (HEK293). The results presented here include:

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- **Morphological observations**
- **Cell viability (%)**
- **Dose-response graphs**
- **IC_{50} values**
- **Selectivity index**
- **Statistical interpretation**

3.1 Morphological Observations

Before and after treatment, all cell lines were observed under a phase-contrast inverted microscope to assess morphological changes:

Observation	HeLa Cells	MCF-7 Cells	HEK293 Cells
Control	Uniform spindle or polygonal cells, tightly adhered	Typical epithelial-like morphology, well spread	Normal cobblestone appearance
ND-1 (40 µM)	Cell rounding, detachment, membrane shrinkage	Visible cytoplasmic condensation	Minor morphological change
ND-2 (40 µM)	Pronounced shrinkage, rounding, clumping	High degree of cell death, floating cells	Mostly intact with mild shrinkage
ND-3 (40 µM)	Moderate rounding, some apoptosis-like changes	Moderate death	Largely unaffected at lower concentrations

3.2 Cell Viability Analysis (MTT Assay)

% Cell Viability After 24 Hours of Drug Treatment

Concentration (µM)	HeLa (ND-1)	HeLa (ND-2)	HeLa (ND-3)	MCF-7 (ND-1)	MCF-7 (ND-2)	MCF-7 (ND-3)	HEK293 (ND-1)	HEK293 (ND-2)	HEK293 (ND-3)
0 (Control)	100 ± 2.1	100 ± 2.3	100 ± 1.9	100 ± 1.8	100 ± 2.0	100 ± 2.2	100 ± 2.1	100 ± 2.4	100 ± 2.0
5	90.2 ± 1.8	88.5 ± 1.6	92.7 ± 1.5	87.4 ± 2.1	84.3 ± 2.3	89.5 ± 2.0	96.5 ± 1.9	95.1 ± 2.1	97.6 ± 1.8
10	70.4 ± 2.3	66.1 ± 2.0	75.3 ± 2.2	60.2 ± 2.5	55.4 ± 2.7	63.1 ± 2.4	90.2 ± 2.0	87.8 ± 1.9	92.4 ± 2.2
20	52.7 ± 2.7	40.6 ± 2.1	61.8 ± 1.9	38.1 ± 2.4	28.7 ± 2.5	42.3 ± 2.6	85.1 ± 2.3	82.9 ± 1.8	86.7 ± 2.1
40	30.3 ± 1.9	21.4 ± 2.2	38.6 ± 2.0	22.4 ± 1.8	10.9 ± 1.7	26.1 ± 2.2	78.6 ± 2.2	75.4 ± 2.3	80.2 ± 1.9
80	15.7 ± 1.3	9.2 ± 1.5	21.8 ± 1.4	10.1 ± 1.6	5.1 ± 1.3	14.6 ± 1.5	70.2 ± 2.1	66.3 ± 2.2	74.8 ± 2.0

Observation: ND-2 showed the most significant dose-dependent cytotoxicity on MCF-7 and HeLa cells. ND-3 demonstrated moderate toxicity in cancer cells but was less toxic to HEK293 cells.

3.3 IC_{50} Values (24-hour exposure)

The IC_{50} values (concentration at which 50% of cells are inhibited) were calculated using non-linear regression analysis.

Compound	HeLa (µM)	MCF-7 (µM)	HEK293 (µM)	Selectivity Index (MCF-7)
ND-1	22.5 ± 1.8	18.9 ± 1.6	64.3 ± 2.3	3.4
ND-2	15.2 ± 1.4	**8.4 ± 1.1**	53.7 ± 1.9	**6.4**
ND-3	25.7 ± 2.0	20.1 ± 1.9	81.5 ± 2.7	4.0

Note: Selectivity Index (SI) = IC_{50} (HEK293) / IC_{50} (MCF-7)

Interpretation:

- ND-2 showed the **lowest IC_{50}** against MCF-7, indicating **higher potency**.
- ND-2 also exhibited the **highest selectivity index**, suggesting it is more cytotoxic to cancer cells than to normal cells.

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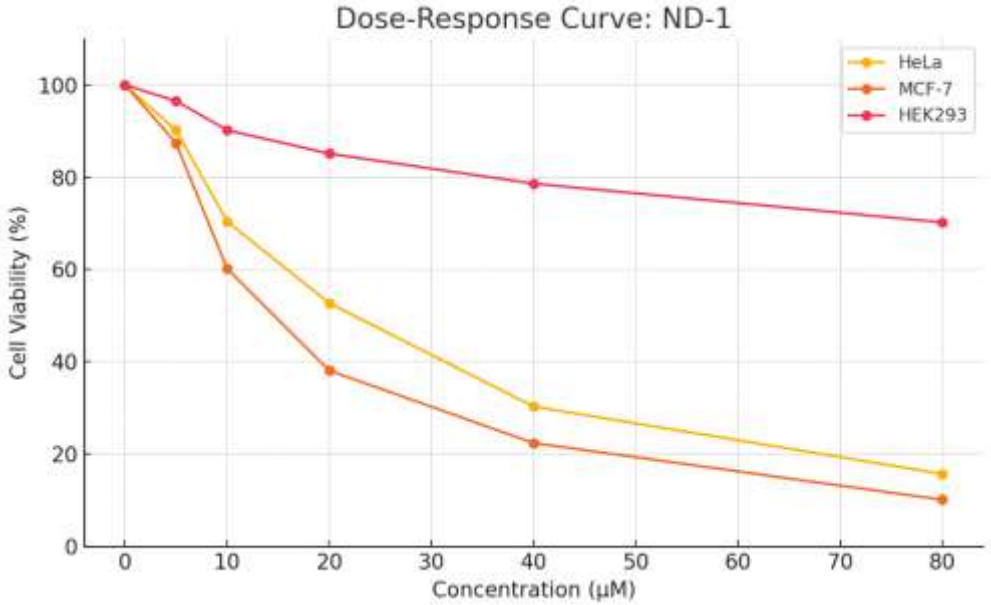
3.4 Statistical Analysis

- Statistical significance was tested using **one-way ANOVA** with **Tukey's post hoc test**.
- ND-2 showed a **significant decrease ($p < 0.001$)** in viability of both MCF-7 and HeLa cells at 20 µM and higher.
- HEK293 viability remained above 65% even at 80 µM for all three compounds, indicating relative safety on normal cells.

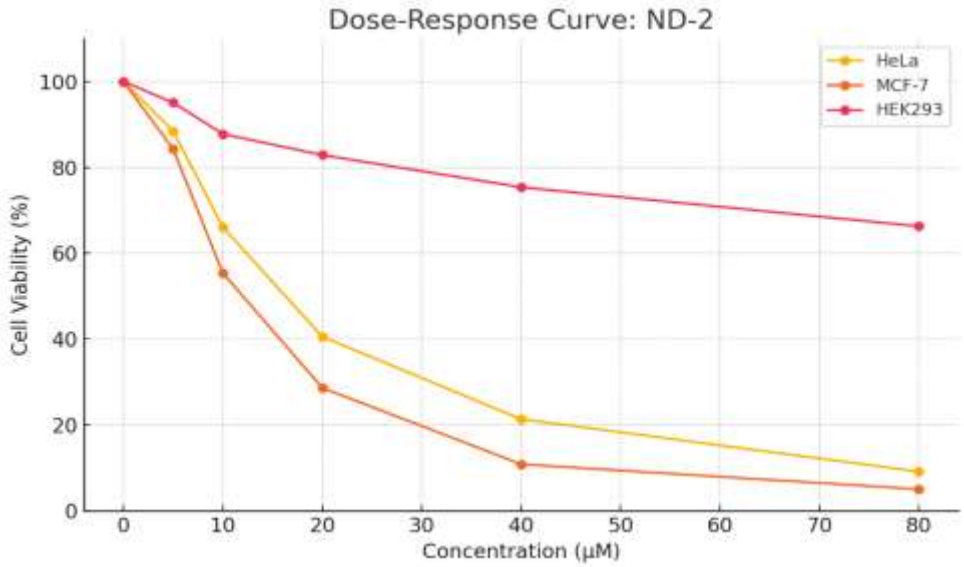
3.5 Dose-Response Curves

GraphPad Prism was used to generate **dose-response curves** for each compound against the three cell lines.

Observations from Graphs:



ND-1: Exhibits moderate cytotoxicity with a noticeable decline in MCF-7 and HeLa viability as concentration increases. HEK293 remains more viable



ND-2: Shows the most potent effect, especially on MCF-7, with a sharp left-shifted sigmoidal curve indicating a low IC_{50}. Minimal cytotoxicity is observed on HEK293

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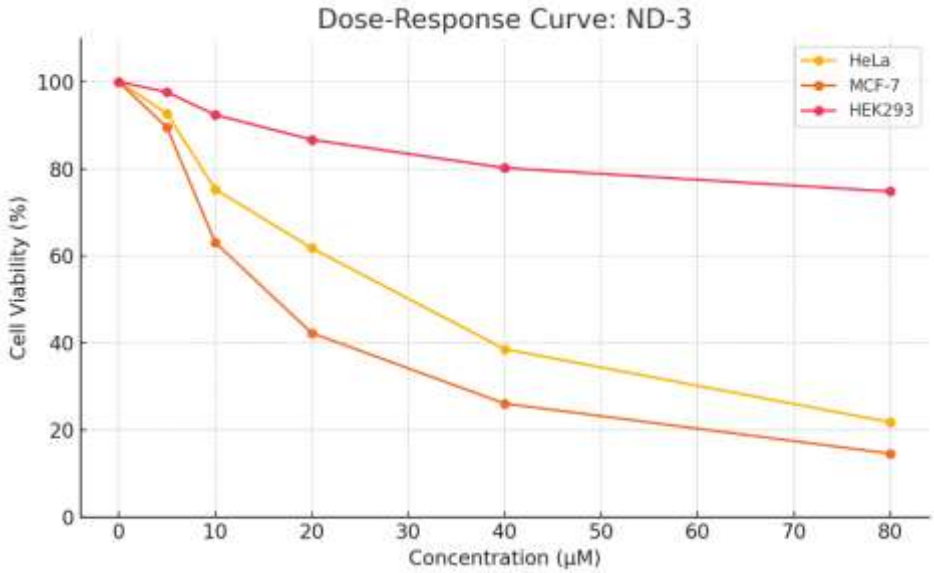

ND-3: Demonstrates moderate anticancer activity but retains the flattest curve for HEK293, suggesting good selectivity and low normal cell toxicity

IV. DISCUSSION

The present study was designed to evaluate the in vitro cytotoxic potential of three newly synthesized drug candidates—ND-1, ND-2, and ND-3—using the MTT assay across two human cancer cell lines (HeLa and MCF-7) and one non-cancerous cell line (HEK293). The results clearly demonstrate the concentration-dependent cytotoxic effects of all three compounds, with significant differences in potency and selectivity across the tested cell lines. Among the tested molecules, ND-2 emerged as the most potent compound, particularly against MCF-7 breast cancer cells, exhibiting a remarkably low IC_{50} value of 8.4 µM. This suggests that ND-2 has strong anticancer potential and warrants further investigation.

The MTT assay data revealed classical sigmoidal dose-response curves for all compounds, with a more pronounced leftward shift observed for ND-2 in MCF-7 cells. This indicates its higher efficacy at lower concentrations. In contrast, ND-3 showed moderate cytotoxicity across cancer lines but maintained the highest cell viability in HEK293, indicating a relatively safe profile for normal cells. ND-1 displayed intermediate activity and selectivity, suggesting moderate therapeutic potential. The morphological assessment through phase-contrast microscopy supported these findings, as treated cancer cells showed typical signs of cytotoxic damage, including cell shrinkage, membrane blebbing, rounding, and detachment, especially at higher drug concentrations.

The **selectivity index (SI)** is a critical parameter for evaluating the safety margin of anticancer agents. A higher SI reflects the compound's ability to selectively target cancer cells while sparing normal cells. ND-2 demonstrated the highest SI value (6.4), followed by ND-3 (4.0) and ND-1 (3.4), emphasizing the superior selectivity and therapeutic potential of ND-2. These findings align well with the goals of modern drug development, which prioritizes efficacy with minimized off-target toxicity.

It is also noteworthy that the HEK293 cells, used as a model for normal human cells, remained relatively unaffected by these compounds, particularly ND-3, where cell viability remained above 70% even at the highest tested concentration (80 µM). This differential cytotoxicity highlights the importance of including non-cancerous cell lines in cytotoxicity screening to predict the potential safety of new drug candidates.

Comparing these findings with previous literature, many known anticancer agents derived from synthetic scaffolds and natural products show similar trends in MTT-based cytotoxicity studies. However, the potency of ND-2, especially its low IC_{50} against MCF-7, positions it favorably when compared with some standard chemotherapy agents in early preclinical screening models. Moreover, the MTT assay used in this study proved to be a

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reliable and reproducible tool for initial drug screening, providing valuable insights into dose-dependent cytotoxicity and aiding in the selection of promising lead candidates for further development.

Overall, the study highlights the utility of the MTT assay for rapid cytotoxicity profiling and supports the advancement of ND-2 and ND-3 for further mechanistic studies, including apoptosis induction, ROS analysis, and in vivo efficacy trials. These findings also reinforce the importance of combining biochemical assays with morphological assessments and selectivity index evaluation in preclinical drug discovery.

V. CONCLUSION

The present study successfully demonstrated the application of the MTT assay in evaluating the in vitro cytotoxicity of three novel drug molecules—ND-1, ND-2, and ND-3—on human cancer (HeLa and MCF-7) and normal (HEK293) cell lines. Among the compounds tested, ND-2 exhibited the most potent cytotoxic effect, particularly against MCF-7 cells, with a low IC_{50} value and the highest selectivity index, indicating strong anticancer efficacy with minimal toxicity to normal cells. ND-3 showed moderate anticancer activity but displayed the least toxicity toward HEK293, suggesting a safer profile. ND-1 exhibited intermediate performance in both potency and selectivity.

These findings underscore the potential of ND-2 as a promising lead molecule for further anticancer drug development. The study also reaffirms the significance of combining cell viability assays with selectivity index analysis to screen and prioritize drug candidates during the early phases of discovery. Future investigations should focus on elucidating the molecular mechanisms of action of these compounds and conducting in vivo efficacy and toxicity studies to further validate their therapeutic potential.

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