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# Phytochemical Analysis and In Vitro Anticancer Evaluation of Neferine: A Herbal Drug Study

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**ABSTRACT:**

This study aimed to isolate Neferine from Nelumbo nucifera seeds and evaluate its cytotoxic effects on cancer cell lines, particularly SK-N-MC and HepG2 cells. Neferine was successfully isolated using a combination of hot methanol extraction, solvent partitioning, and flash column chromatography. The purity of the isolated compound was confirmed through chromatographic analysis. Cytotoxicity assessment using the MTT assay revealed dose-dependent decreases in cell viability for both Neferine and the reference standard cisplatin against SK-N-MC cells, with Neferine demonstrating significant cytotoxic effects even at lower concentrations. Cell cycle analysis and flow cytometry showed alterations in cell cycle distribution and fluorescence characteristics upon treatment with Neferine, comparable to those induced by Cisplatin, suggesting potential cell cycle arrest or delay at the G2/M checkpoint. Apoptosis investigation using Annexin V FITC/PI analysis further supported the cytotoxic effects of Neferine, with increased levels of apoptosis or necrosis observed in Neferine-treated HepG2 cells. These findings highlight the promising therapeutic potential of Neferine as an anti-cancer agent, warranting further mechanistic studies and clinical investigations.

**Keywords:** Neferine, Cytotoxicity, SK-N-MC Cells, Apoptosis, HepG2 cells

## 1. Introduction

Cancer remains a significant global health challenge, with its prevalence and impact continuing to rise. Conventional cancer treatments often come with severe side effects and limited efficacy, necessitating the exploration of alternative therapeutic strategies derived from natural sources [1]. Nelumbo nucifera, commonly known as the sacred lotus, has been a subject of considerable interest due to its rich pharmacological properties, including anticancer potential. Among its bioactive compounds, neferine has emerged as a promising candidate for cancer therapy, exhibiting diverse pharmacological effects, including antioxidant, anti-inflammatory, and anticancer properties [2, 3].

Neferine, a bisbenzylisoquinoline alkaloid abundantly found in Nelumbo nucifera, has garnered attention for its potential anticancer activity against various cancer types [4]. As a natural compound, neferine offers several advantages, including low toxicity and high bioavailability, making it an attractive candidate for further investigation as a cancer therapeutic agent [5-8]. Understanding the mechanisms underlying the anticancer effects of neferine is crucial for its development as a viable treatment option.

Cell cycle analysis involves the quantification and characterization of cells at different stages of the cell cycle, including G1, S, G2, and M phases [9, 10]. Alterations in cell cycle distribution induced by neferine can elucidate its mechanisms of action, including cell cycle arrest, apoptosis induction, and modulation of cell cycle regulatory proteins [11]. By elucidating the precise effects of neferine on the cell cycle, researchers can gain valuable insights into its anticancer mechanisms and therapeutic potential.

Isolation of neferine from Nelumbo nucifera involves extraction and purification techniques to obtain a highly concentrated and pure form of the compound. Once isolated, neferine can be subjected to comprehensive in vitro and in vivo studies to evaluate its anticancer efficacy. One of the fundamental approaches in assessing the anticancer activity of neferine is through cell cycle analysis, which provides insights into the compound's effects on cell cycle progression and cell proliferation.

In this study, we aim to evaluate the anticancer activity of neferine isolated from *Nelumbo nucifera* using comprehensive cell cycle analysis. Through a series of in vitro experiments, we will investigate the effects of neferine on cell cycle progression, apoptosis induction, and cell viability in cancer cell lines. Our findings aim to contribute to the growing body of evidence supporting the therapeutic potential of neferine as a novel anticancer agent derived from natural sources, thereby paving the way for its further development and clinical translation in cancer therapy.

## 2. Materials and Methods

Nelumbo nucifera seeds were collected from local ponds in the region of Ranga Reddy district, Telangana, India. Collected specimen of Nelumbo nucifera seeds were authenticated by Government Degree College, Kukatpally. All Solvents, Chemical reagents utilized in the extraction process were of LR grade and procured from Sd Fine Chem Ltd, Hyderabad, India.

### 1.1. Isolation of Neferine from Nelumbo Nucifera

The procedure involved the extraction of embryos from Nelumbo nucifera seeds, weighing 1.3 kg, utilizing hot methanol (MeOH). Following this extraction, the MeOH extracts were concentrated under vacuum, yielding a residual mass of 0.728 kg. Subsequently, this residue underwent exhaustive extraction using a 3% aqueous tartaric acid solution. The resulting acidic aqueous solutions were then adjusted to a basic pH using 28% ammonium hydroxide (NH4OH) and subsequently extracted with chloroform (CHCl3). Concentration of the CHCl3 layers led to the formation of a residue weighing 8.21 g. This residue was further subjected to

flash column chromatography (CC) utilizing Wakogel FC40 as the stationary phase. The elution process involved a solvent mixture of chloroform (CHCl3), methanol (MeOH), and ammonium hydroxide (NH4OH) in the proportions of 95:4.5:0.5 [12, 13].

### 1.2. MTT Assay [14]

#### 1.2.1. Preparation of test solutions

For MTT assay, serial two-fold dilutions (6.25 – 100 µg) were prepared from this assay.

#### 1.2.2. Cell lines and culture medium

SK-N-MC cell line was procured from NCCS, stock cell was cultured in DMEM medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) in a humidified atmosphere of 5% $CO_2$ at 37ºC until confluent.

#### 1.2.3. Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x $10^5$ cells/mL using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100 µL of the diluted cell suspension (1 x $10^4$ cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µL of different concentrations of test samples were added on to the partial monolayer in microtiter plates. The plate was then incubated at 37ºC for 24 h in 5% $CO_2$ atmosphere. After incubation the test solutions in the wells were discarded and 20 µL of MTT (2 mg/1 mL of MTT in PBS) was added to each well. The plate was incubated for 4 h at 37ºC in 5% $CO_2$ atmosphere. The supernatant was removed and 100 µL of DMSO was added and the plate was gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm. The percentage of viability was calculated using the following formula, % viability = Sample abs/Control abs x 100

### 1.3. Cell Cycle Analysis by Flow Cytometry [15]

#### 1.3.1. Study Design

This study employed to investigate the effects of Neferine on the cell cycle progression of HepG2 cells. The experimental conditions included treatment with reference standard Cisplatin, test sample neferine and a control condition with untreated HepG2 cells.

#### 1.3.2. Cell Culture

HepG2 cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM)] supplemented fetal bovine serum, penicillin-streptomycin. Cells were maintained in a humidified incubator at 37°C with $CO_2$ concentration, 5% $CO_2$] until reaching approximately 70% confluence.

#### 1.3.3. Treatment Conditions

HepG2 cells were divided into three experimental groups based on the treatment conditions:

Cisplatin (Reference Standard): HepG2 cells were treated with Cisplatin at a concentration of 6.25, 12.5, 25, 50, and 100 µg/ml for 24 hours.

Control: Untreated HepG2 cells served as the control group and were cultured under identical conditions without the addition of any treatment agent.

Neferine (Test Sample): HepG2 cells were treated with Cisplatin at a concentration of concentration of 6.25, 12.5, 25, 50, and 100 µg/ml for 24 hours.

#### *1.3.4. Cell Harvesting and Fixation*

Following the treatment period, cells were harvested using trypsinization and collected in centrifuge tubes. The harvested cells were washed with phosphate-buffered saline (PBS) and fixed with ice-cold 70% ethanol for a minimum of 1 hour at -20°C to ensure complete fixation.

#### *1.3.5. DNA Staining and Flow Cytometry Analysis*

Fixed cells were permeabilized with PBS containing 0.1% Triton X-100 and stained with a DNA fluorescent dye, such as propidium iodide (PI), to label cellular DNA. Flow cytometry

analysis was performed using a [specify flow cytometer model] equipped with appropriate filters for detecting PI fluorescence.

#### *1.3.6. Data Analysis*

Flow cytometry data were analyzed using (BioRad ZE5). Cell cycle distribution was determined based on the DNA content revealed by PI staining, allowing for quantification of cells in different phases of the cell cycle (G1, S, G2/M). Statistical analysis, including ANOVA, was conducted to compare cell cycle distribution between treatment groups.

### 1.4. Apoptosis Investigation

The assessment of apoptosis induced by Neferine (NEF) and Cisplatin (DOX) in HepG2 cells was conducted using Annexin V FITC/PI analysis. HepG2 cells were cultured in appropriate media supplemented with fetal bovine serum and antibiotics and then treated with NEF or DOX at the desired concentrations for a specified duration. Untreated cells served as controls. Following treatment, cells were harvested using trypsinization and fixed in ice-cold 70% ethanol to ensure complete fixation. The fixed cells were then permeabilized with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 to allow Annexin V FITC/PI staining. Annexin V FITC/PI staining solution was added to the permeabilized cells, and the stained cells were incubated in the dark for 15-30 minutes at room temperature. Flow cytometry analysis was performed using a flow cytometer equipped with appropriate filters for detecting FITC and PI fluorescence. Gating parameters were set to exclude debris and doublets, and data were acquired for each sample to record the fluorescence intensity of the stained cells. The proportion of apoptotic cells, including early apoptotic (Annexin V FITC-positive, PI-negative) and late apoptotic or necrotic (Annexin V FITC-positive, PI-positive) cells, was quantified using flow cytometry data analysis software. Results were interpreted based on the comparison of apoptotic cell populations between treatment groups and the control group. All procedures were conducted aseptically, and proper handling and disposal of NEF, DOX, and other hazardous materials were ensured in accordance with institutional guidelines. Validation of staining and gating parameters using appropriate controls was performed to ensure the reliability and reproducibility of the results [16, 17].

## 3. Results and Discussion

### a. Isolation of Neferine

During the elution process, a solvent mixture of chloroform ($CHCl_3$), methanol (MeOH), and ammonium hydroxide ($NH_4OH$) in the proportions of 95:4.5:0.5 was utilized. This chromatographic separation resulted in the isolation of Fraction I, which weighed 1.33 g. Importantly, analysis revealed that Fraction I was nearly pure neferine, indicating the successful purification of the target compound through the described extraction and chromatographic processes.

The successful isolation of Neferine from Nelumbo nucifera seeds underscores the effectiveness of the extraction and chromatographic techniques employed in this study. The utilization of hot methanol extraction followed by solvent partitioning with aqueous tartaric acid and subsequent basic-$CHCl_3$ extraction allowed for the isolation of neferine in relatively high yield. The choice of flash column chromatography with Wakogel FC40 as the stationary phase further facilitated the purification process, resulting in the isolation of Fraction I, which was nearly pure neferine. The purity of Fraction I suggests that the described extraction and chromatographic procedures effectively removed impurities and contaminants from the crude extract, resulting in the isolation of a highly purified compound.

**b. MTT Assay**

The results of MTT assay presented in Table 1 demonstrate the percentage viability and IC50 values of Neferine compared to cisplatin, a reference standard, against the SK-N-MC cell line. The control group, with a concentration of 0 µg/ml for both Neferine and cisplatin, exhibited 100% viability, indicating no cytotoxic effects on the SK-N-MC cells.

In the Neferine-treated groups, a dose-dependent decrease in cell viability was observed as the concentration of Neferine increased. At the highest concentration tested (100 µg/ml), Neferine resulted in a viability of 27.93%, indicating a significant cytotoxic effect on the SK-N-MC cells. The IC50 value for Neferine was calculated to be 18.48 µg/ml, indicating the concentration required to inhibit cell viability by 50%.

Similarly, treatment with cisplatin also led to a dose-dependent decrease in cell viability. The IC50 value for cisplatin was notably lower (7.53 µg/ml) compared to Neferine, indicating its higher potency in inhibiting cell viability. At the highest concentration tested (100 µg/ml), cisplatin resulted in a viability of 17.07%.

The results demonstrate that both Neferine and cisplatin exhibit cytotoxic effects on the SK-N-MC cell line in a dose-dependent manner. Cisplatin showed higher potency, with a lower IC50 value compared to Neferine.

However, Neferine still exhibited significant cytotoxicity at higher concentrations, with a considerable decrease in cell viability observed even at lower doses. These findings suggest that Neferine holds promise as an alternative or adjunctive treatment option for neuroblastoma.

**c. Cell Cycle Analysis and Flow Cytometry**

The analysis reveals significant differences in cell cycle distribution and fluorescence characteristics between the treated and control groups. The Results of Cell cycle analysis and flow cytometry were enumerated in Table 2 and the cell distribution of each treatment groups were displayed in Figure 1. Cisplatin-treated group, with a cell count of 17,120 and a volume of 4.1 µL, displays a significantly higher mean FL2-A (78,087.04) compared to the control group. Across different phases, the Cisplatin-treated group exhibits a substantial proportion of cells in the M4 phase (69.79%), indicating potential cell cycle arrest or delay at the G2/M checkpoint induced by Cisplatin treatment.

In contrast, the control group, comprising 15,323 cells and a volume of 10.9 µL, shows a lower mean FL2-A (8,464.18) and a higher proportion of cells in the M2 phase (85.07%). The Neferine-treated group, with a cell count of 23,266 and a volume of 15.8 µL, demonstrates a higher mean FL2-A (46,588.85) compared to the control group.

Similar to the Cisplatin-treated group, the Neferine-treated group exhibits a significant proportion of cells in the M4 phase (57.93%), suggesting potential cell cycle alterations induced by NEF treatment. Contrastingly, the control group displays lower mean FL2-A (8,464.18) and a higher proportion of cells in the M2 phase (85.07%).

Both Neferine and Cisplatin treatments lead to significant alterations in cell cycle distribution and fluorescence characteristics compared to their respective control groups. Notably, both treatments induce a higher proportion of cells in the M4 phase, indicating potential cell cycle arrest or delay at the G2/M checkpoint. While Neferine shows comparable effects to Cisplatin in altering cell cycle dynamics, the specific mechanisms and therapeutic implications may differ and warrant further investigation.

Moreover, examination of fluorescence intensity reveals a higher mean fluorescence intensity in the NEF-treated group compared to the control group, indicating potential alterations in DNA content, cell size, or fluorescence labelling efficiency induced by NEF treatment. This observation aligns with the findings in the Cisplatin treated group, where an elevated mean fluorescence intensity is also noted.

Contrary to both NEF and Cisplatin treated groups, the control group demonstrates a different pattern of cell cycle distribution and fluorescence characteristics. With a lower proportion of cells in the M4 phase compared to the NEF and Cisplatin treated groups, the control group suggests a distinct effect on cell cycle progression induced by NEF and Cisplatin treatments.
Furthermore, the control group exhibits a lower mean fluorescence intensity compared to both NEF and Cisplatin treated groups, indicating differences in DNA content, cell size, or fluorescence labelling efficiency between the treated and control groups.
The observed alterations induced by Neferine treatment underscore its potential as an anti-cancer agent, comparable to the reference standard Cisplatin. The significant increase in cells in the G2/M phase and the higher proportion of apoptotic or dead cells in the NEF-treated group highlight its cytotoxic effects on HepG2 cells. These findings emphasize the promising therapeutic potential of Neferine in cancer treatment and warrant further investigation into its underlying mechanisms of action and clinical applications.
The results demonstrate that Neferine treatment induces substantial alterations in cell cycle dynamics and fluorescence characteristics, comparable to the effects elicited by the reference standard Cisplatin, thereby highlighting its potential as a novel therapeutic agent for cancer treatment.

**d. Apoptosis Study**

The apoptosis data presents the results of Annexin V FITC/PI analysis for three different experimental conditions: HepG2 control cells, HepG2 cells treated with Cisplatin, and HepG2 cells treated with Neferine (NEF). Notable results from the analysis include distinct variations in the distribution of cells across different quadrants (Q) and alterations in the mean fluorescence intensities (Figure 2) and coefficients of variation (CVs) for Annexin V FITC and Propidium Iodide-A.
In the control group, comprising 29,572 cells with a volume of 148.3 µL, a majority of the cells (90.50%) were observed in the Q2-LL quadrant, indicating viable cells. Conversely, the Cisplatin-treated group showed a more balanced distribution across all quadrants, with significant proportions of cells in Q2-UR (23.14%) and Q2-LR (26.98%), representing late apoptotic or necrotic cells. Meanwhile, in the NEF-treated group, a similar distribution pattern was observed, with a majority of cells in Q2-LL (55.40%), albeit with notable proportions in Q2-UR (16.32%) and Q2-LR (25.60%).
Furthermore, the mean fluorescence intensities for Annexin V FITC and Propidium Iodide-A varied significantly between the experimental groups. Notably, the NEF-treated group exhibited higher mean fluorescence intensities for both Annexin V FITC and Propidium Iodide-A compared to the control group, suggesting increased levels of apoptosis or necrosis in NEF-treated cells.
The observed differences in Annexin V FITC/PI staining patterns between the control, Cisplatin-treated, and NEF-treated groups suggest distinct effects on apoptosis induction. Cisplatin treatment appears to induce a higher proportion of late apoptotic or necrotic cells, as evidenced by the increased population in Q2-UR and Q2-LR. Similarly, NEF treatment also leads to alterations in cell viability, with an increased proportion of cells in late apoptotic or necrotic states compared to the control group.
Neferine treatment demonstrates a significant impact on apoptosis induction in HepG2 cells, as indicated by the increased levels of Annexin V FITC and Propidium Iodide-A staining. These results suggest that NEF treatment promotes apoptosis or necrosis in HepG2 cells, highlighting its potential as a therapeutic agent for cancer treatment. Further investigations are necessary to elucidate the underlying mechanisms of NEF-induced apoptosis and its potential clinical applications.

## 4. Conclusion

In conclusion, the isolation of Neferine from Nelumbo nucifera seeds was achieved through a series of extraction and chromatographic techniques, resulting in the production of nearly pure Neferine. Cytotoxicity evaluation demonstrated the efficacy of Neferine against SK-N-MC cells, with significant decreases in cell viability observed in a dose-dependent manner. Cell cycle analysis and flow cytometry revealed alterations in cell cycle dynamics and fluorescence characteristics induced by Neferine treatment, suggesting potential mechanisms of action related to cell cycle regulation. Furthermore, apoptosis investigation indicated the ability of Neferine to induce apoptosis or necrosis in HepG2 cells, supporting its potential as a therapeutic agent for cancer treatment. These findings provide valuable insights into the pharmacological properties of Neferine and underscore its potential as a novel anti-cancer agent deserving further exploration in preclinical and clinical studies.

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**Sravani Batchu**: Conceptualization, Data curation and Writing, **Dr.Bhavya E**: Reviewing , **C. Ronald Darwin**: Supervision.

## 5. References

1. Debela DT, Muzazu SG, Heraro KD, Ndalama MT, Mesele BW, Haile DC, Kitui SK, Manyazewal T. New approaches and procedures for cancer treatment: Current perspectives. SAGE Open Med. 2021 Aug 12;9:20503121211034366. doi: 10.1177/20503121211034366. PMID: 34408877; PMCID: PMC8366192.
2. Bishayee A, Patel PA, Sharma P, Thoutireddy S, Das N. Lotus (Nelumbo nucifera Gaertn.) and Its Bioactive Phytocompounds: A Tribute to Cancer Prevention and Intervention. Cancers (Basel). 2022 Jan 21;14(3):529. doi: 10.3390/cancers14030529. Erratum in: Cancers (Basel). 2022 Apr 24;14(9):2116. doi: 10.3390/cancers14092116. PMID: 35158798; PMCID: PMC8833568.
3. Menéndez-Perdomo IM, Facchini PJ. Benzylisoquinoline Alkaloids Biosynthesis in Sacred Lotus. Molecules. 2018 Nov 6;23(11):2899. doi: 10.3390/molecules23112899. PMID: 30404216; PMCID: PMC6278464.
4. Bharathi Priya L, Huang CY, Hu RM, Balasubramanian B, Baskaran R. An updated review on pharmacological properties of neferine-A bisbenzylisoquinoline alkaloid from Nelumbo nucifera. J Food Biochem. 2021 Dec;45(12):e13986. doi: 10.1111/jfbc.13986. Epub 2021 Nov 14. PMID: 34779018.
5. Kadioglu O, Law BYK, Mok SWF, Xu SW, Efferth T, Wong VKW. Mode of Action Analyses of Neferine, a Bisbenzylisoquinoline Alkaloid of Lotus (Nelumbo nucifera) against Multidrug-Resistant Tumor Cells. Frontiers in Pharmacology [Internet]. 2017 May 5;8. Available from: https://doi.org/10.3389/fphar.2017.00238
6. Talib WH, Alsayed AR, Barakat M, Abu-Taha MI, Mahmod AI. Targeting Drug Chemo-Resistance in Cancer Using Natural Products. Biomedicines. 2021 Sep 29;9(10):1353. doi: 10.3390/biomedicines9101353. PMID: 34680470; PMCID: PMC8533186.
7. Asma ST, Acaroz U, Imre K, Morar A, Shah SRA, Hussain SZ, Arslan-Acaroz D, Demirbas H, Hajrulai-Musliu Z, Istanbullugil FR, Soleimanzadeh A, Morozov D, Zhu

K, Herman V, Ayad A, Athanassiou C, Ince S. Natural Products/Bioactive Compounds as a Source of Anticancer Drugs. Cancers (Basel). 2022 Dec 15;14(24):6203. doi: 10.3390/cancers14246203. PMID: 36551687; PMCID: PMC9777303.

8. Manogaran P, Beeraka NM, Padma VV. The Cytoprotective and Anti-cancer Potential of Bisbenzylisoquinoline Alkaloids from Nelumbo nucifera. Curr Top Med Chem. 2019;19(32):2940-2957. doi: 10.2174/1568026619666191116160908. PMID: 31738137.
9. Ligasová A, Frydrych I, Koberna K. Basic Methods of Cell Cycle Analysis. Int J Mol Sci. 2023 Feb 12;24(4):3674. doi: 10.3390/ijms24043674. PMID: 36835083; PMCID: PMC9963451.
10. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer. 2009 Mar;9(3):153-66. doi: 10.1038/nrc2602. PMID: 19238148.
11. Law BYK, Michelangeli F, Qu YQ, Xu SW, Han Y, Mok SWF, Dias IRSR, Javed MU, Chan WK, Xue WW, Yao XJ, Zeng W, Zhang H, Wang JR, Liu L, Wong VKW. Neferine induces autophagy-dependent cell death in apoptosis-resistant cancers via ryanodine receptor and Ca2+-dependent mechanism. Sci Rep. 2019 Dec 27;9(1):20034. doi: 10.1038/s41598-019-56675-6. PMID: 31882989; PMCID: PMC6934498.
12. Chen Y, Fan G, Wu H, Wu Y and Mitchell A 2007 Separation, identification and rapid determination of liensine, isoliensinine and neferine from embryo of the seed of Nelumbo nucifera Gaertn. by liquid chromatography coupled to diode array detector and tandem mass spectrometry Journal of Pharmaceutical and Biomedical Analysis 43 99-104.
13. Liu S, Liang Y-Z, Luo J, Cao D-S, Li X-Z, Lei P and Yuan D-L 2008 Optimization of Liensinine, Isoliensinine and Neferine Extraction from the Embryo of the Seed of Nelumbo nucifera GAERTN Separation Science and Technology 43 3637-51.
14. Riss TL, Moravec RA, Niles AL, et al. Cell Viability Assays. 2013 May 1 [Updated 2016 Jul 1]. In: Markossian S, Grossman A, Arkin M, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK144065/
15. Kim KH, Sederstrom JM. Assaying Cell Cycle Status Using Flow Cytometry. Curr Protoc Mol Biol. 2015 Jul 1;111:28.6.1-28.6.11. doi: 10.1002/0471142727.mb2806s111. PMID: 26131851; PMCID: PMC4516267.
16. Kntayya SB, Ibrahim MD, Mohd Ain N, Iori R, Ioannides C, Abdull Razis AF. Induction of Apoptosis and Cytotoxicity by Isothiocyanate Sulforaphene in Human Hepatocarcinoma HepG2 Cells. Nutrients. 2018 Jun 4;10(6):718. doi: 10.3390/nu10060718. PMID: 29866995; PMCID: PMC6024841.
17. Paramasivan P, Kumar JD, Baskaran R, Weng CF, Padma VV. Reversal of doxorubicin resistance in lung cancer cells by neferine is explained by nuclear factor erythroid-derived 2-like 2 mediated lung resistance protein down regulation. Cancer Drug Resist. 2020 Apr 17;3(3):647-665. doi: 10.20517/cdr.2019.115. PMID: 35582448; PMCID: PMC8992493.

**Tables**

Table 1: **% Viability and IC50 values of Neferine and cisplatin against** SK-N-MC cell line

| Sample | **Concentration** | **% Viability** | | **IC50 (µg)** |
|---|---|---|---|---|
| | | **Mean** | **SD** | |
| Control | 0 | 100 | | |
| Neferine | 6.25 | 61.4527781 | 0.373936893 | 18.48 |
| | 12.5 | 52.87740939 | 0.2215552 | |
| | 25 | 50.59892716 | 0.943608509 | |
| | 50 | 38.30319096 | 0.41022485 | |
| | 100 | 27.93379199 | 0.34100406 | |
| CISPLATIN | 6.25 | 54.35773651 | 0.548999543 | 7.53 |
| | 12.5 | 42.38559914 | 0.293069666 | |
| | 25 | 31.46000191 | 1.26008364 | |
| | 50 | 26.6665505 | 0.842727655 | |
| | 100 | 17.07392451 | 0.588201455 | |

Table 2: **Summary of Results of Cell Cycle analysis and Flow cytometry**

| HepG2 vs Cisplatin (Reference standard) | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Count** | **Volume (µL)** | **% of This Plot** | **% of All** | **Mean FL2-A** | **CV FL2-A** | **Median FL2-A** |
| All | 17,120 | 4.1 | 100.00% | 100.00% | 78,087.04 | 87.58% | 78,939.50 |
| M1 (12.0 / 167.0) | 398 | 4.1 | 2.32% | 2.32 % | 89.36 | 48.59% | 89 |
| M2 (167.0 / 4,653.0) | 3,188 | 4.1 | 18.62% | 18.62 % | 1,625.89 | 73.20% | 1,310.50 |
| M3 (4,273.0 / 25,632.0) | 1,293 | 4.1 | 7.55% | 7.55 % | 10,881.28 | 54.39% | 8,927.00 |
| M4 (30,400.0 / 3,045,667.0) | 11,948 | 4.1 | 69.79% | 69.79 % | 1,09,937.62 | 52.44% | 98,477.50 |
| HepG2 vs Control | | | | | | | |
| | **Count** | **Volume (µL)** | **% of This Plot** | **% of All** | **Mean FL2-A** | **CV FL2-A** | **Median FL2-A** |
| All | 15,323 | 10.9 | 100.00% | 100.00% | 8,464.18 | 383.96 % | 1,972.00 |
| M1 (12.0 / 167.0) | 298 | 10.9 | 1.94% | 1.94 % | 102.07 | 39.93% | 109 |
| M2 (167.0 / 4,653.0) | 13,036 | 10.9 | 85.07% | 85.07 % | 1,941.46 | 52.35% | 1,820.00 |
| M3 (4,273.0 / 25,632.0) | 1,482 | 10.9 | 9.67% | 9.67 % | 6,754.52 | 47.52% | 5,640.00 |
| M4 (30,400.0 / 3,045,667.0) | 721 | 10.9 | 4.71% | 4.71 % | 1,32,218.81 | 59.95% | 1,16,914.00 |
| HepG2 vs **Neferine** (Test Sample) | | | | | | | |
| | **Count** | **Volume (µL)** | **% of This Plot** | **% of All** | **Mean FL2-A** | **CV FL2-A** | **Median FL2-A** |
| All | 23,2 | 15.8 | 100.00% | 100.0 | 46,588.8 | 119.60 | 41,458.50 |

| | 66 | | | 0% | 5 | % | |
|---|---|---|---|---|---|---|---|
| M1 (12.0 / 167.0) | 2,373 | 15.8 | 10.20% | 10.20 % | 95.71 | 44.04% | 97 |
| M2 (167.0 / 4,653.0) | 5,845 | 15.8 | 25.12% | 25.12 % | 990.41 | 103.80 % | 529 |
| M3 (4,273.0 / 25,632.0) | 859 | 15.8 | 3.69% | 3.69 % | 10,479.34 | 62.12% | 7,670.00 |
| M4 (30,400.0 / 3,045,667.0) | 13,477 | 15.8 | 57.93% | 57.93 % | 78,644.53 | 68.38% | 64,119.00 |

**Figures**

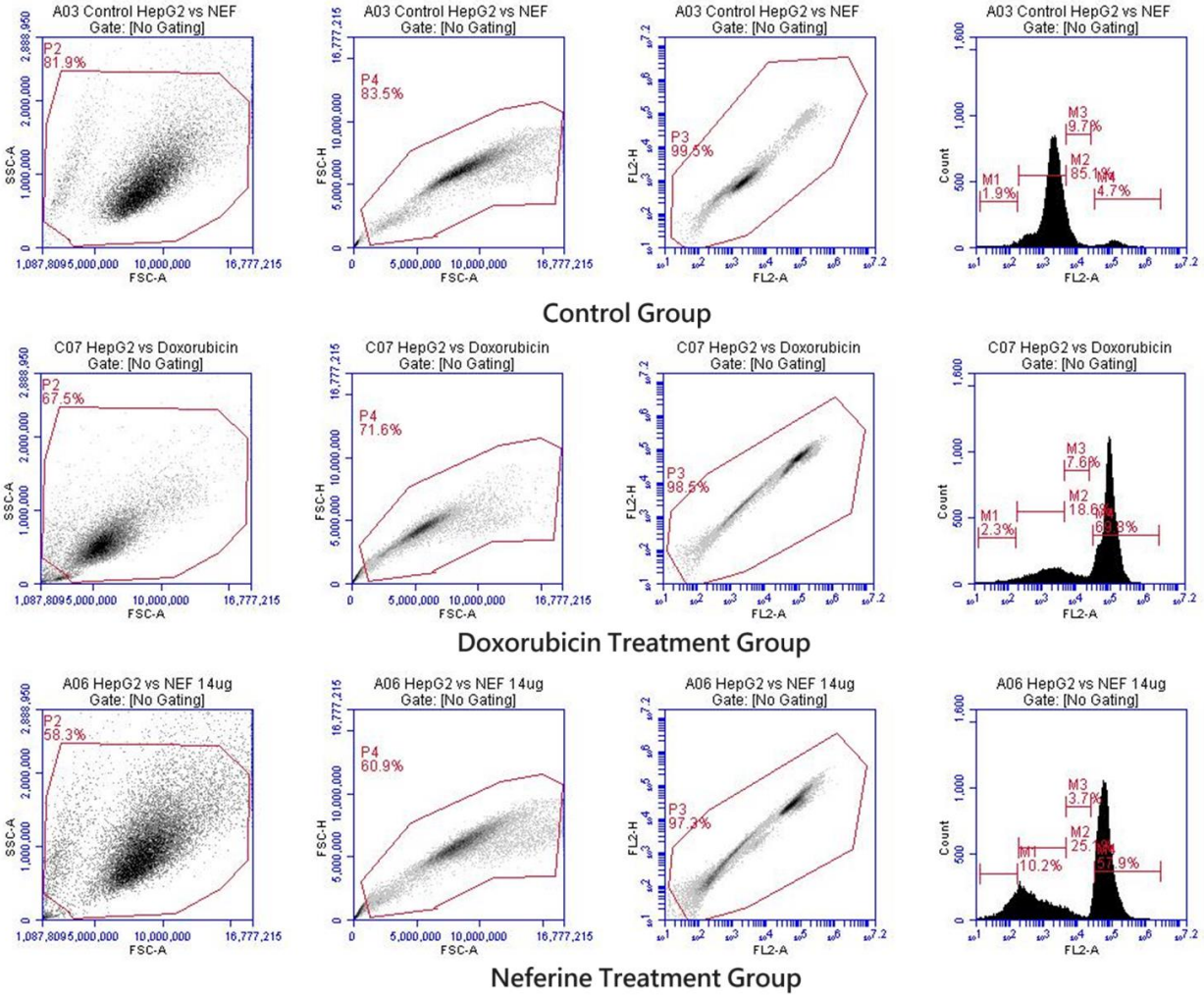


Figure 1: Cell Distribution of Control, Cisplatin and Neferine Treated groups in Hep G2 Cells

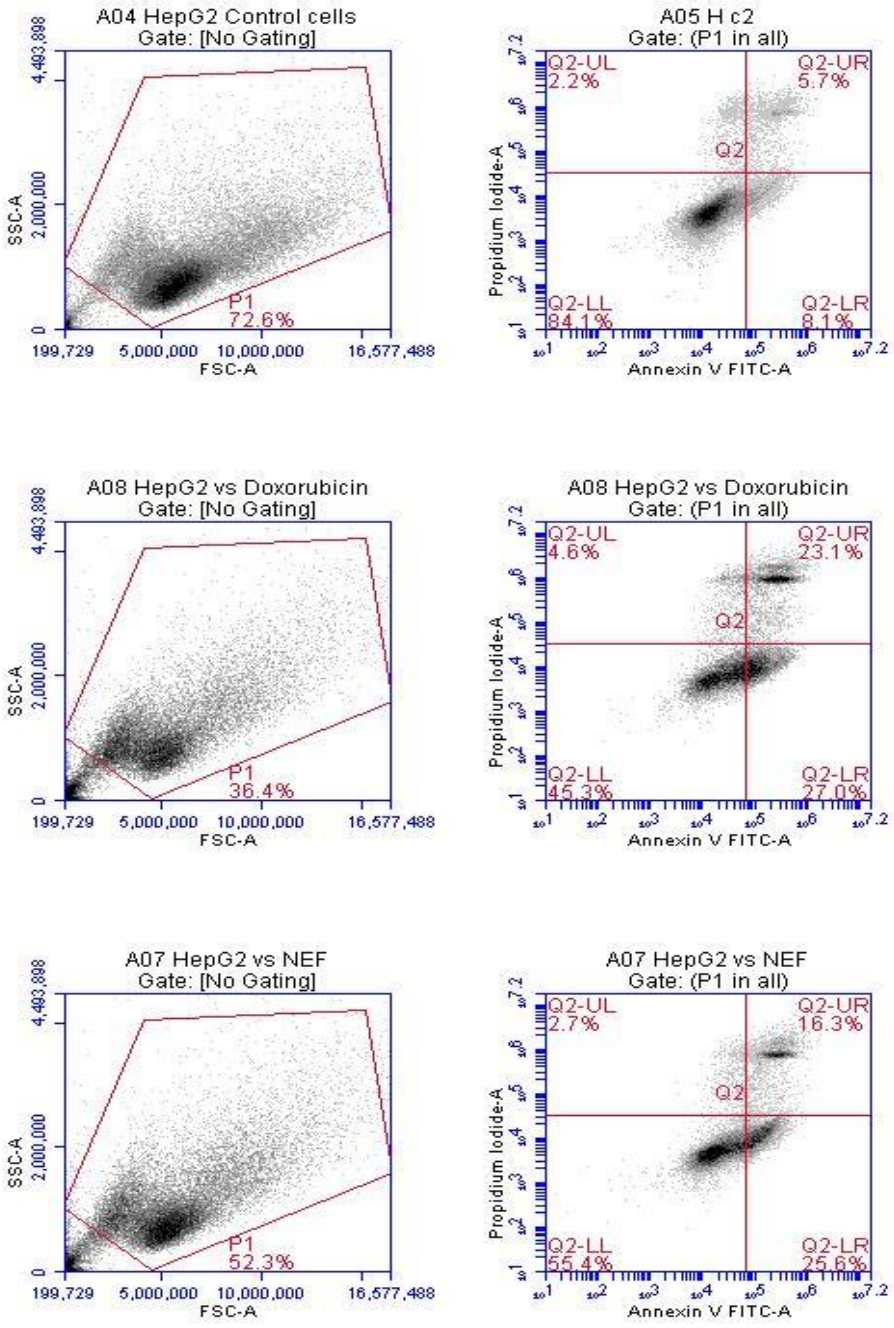


Figure 2: Cell distribution and fluorescence intensities in Hep G2 cells in control, Cisplatin and Neferine treatment groups.