ORIGINAL ARTICLE

Azacitidine Nanoemulsion Formulation and Characterization for the Tumor Treatment Suppressor Genes Activity in Myeloid Leukemia Cell Lines

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Abstract

Aims: The present study aimed to develop and evaluate an Azacitidine (AZA) nanoemulsion to enhance absorption and therapeutic effectiveness. AZA, a pyrimidine nucleoside analogue, interferes with DNA methylation by inhibiting DNA methyltransferase and acts as a cytidine antimetabolite incorporated predominantly into RNA. Although clinically effective as an anticancer drug, its low absorption limits its utility. **Materials and Methods:** Nanoemulsions were formulated using distilled water, co-surfactants (Transcutol P, plysorbate 80), AZA, and selected oils such as oleic acid. Various water-in-oil nanoemulsion systems were prepared by high-speed homogenization. The formulations were evaluated for stability, Fourier transform infrared (FTIR) compatibility, pH, thermodynamic stability, viscosity, and *in vitro* drug release profiles. **Results and Discussion:** The optimized nanoemulsion displayed favorable physicochemical characteristics, including acceptable pH, viscosity, and thermodynamic stability. FTIR analysis confirmed the absence of major drug-excipient interactions. *In vitro* studies showed improved drug release compared to conventional formulations. The nanoemulsion demonstrated significant inhibitory activity (85.37%), approaching that of methotrexate (89.09%), suggesting its potential cytotoxic effect. **Conclusion:** The developed AZA nanoemulsion exhibited enhanced release and inhibitory activity, indicating improved therapeutic potential. These findings support its possible application as a treatment strategy for myeloid leukemia cell lines, with additional studies warranted to confirm its histone deacetylase inhibitory effects.

Key words: Anticancer, azacitidine, co-surfactants, leukemia, nanoemulsion, tumor

INTRODUCTION

The development of a modest medication distribution device requires nanotechnology. The safe, efficient, and effective operation of a drug depends on nanotechnology. Improved durability and incorporation, quantitative drug transfers, regulated drug release, and the intended pharmacodynamic activity are some of the characteristics of nanotechnology.[1] Employing nanoemulsions improves the release of beneficial pharmacological ingredients.[2] Since they get over physical and physiological restrictions on how medication is given, such as particle diameter, manufacturing processes, and thermodynamic durability nanoemulsions are often thought of as safe-level additions.[3]

Nanoemulsions were developed to perform a number of tasks, including target-specific binding, prolonged blood circulation, targeted drug delivery, and testing.[4] emanate from the pair of active and passive target selection procedures, the previously described features can be modified to help transport the medication or diagnostic substance to the intended location of interest. This nanoemulsion could potentially be used to deliver the medication to patients with cancer in an efficient manner. The drug's bioavailability is increased, and any potential side effects on nearby cells or tissues are reduced simply by enclosing it in a closed system in the heart of the

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nanoemulsion. The drug can be transferred to the specific spot more effectively in the context of therapy for cancer.[5]

The primary obstacle to the cure of patients is medication resistance. A few of the numerous elements that affect cancer treatment resistance include the tumor's diverse features, developmental dynamics, and tumor surroundings. Tumor regrowth can be prevented through immunotherapy, checkpoint inhibition, combination therapy, and the use of nanoformulations.[6] Over the course of their lives, one in eight women will experience being diagnosed with one of the several types of breast tumors. Malignant growths known as breast tumors originate in the tissues of the female mammary gland. As an illustration, ductal malignancies start in the cells beneath the passages that transport milk to the breast, account for more than 75% of all breast malignancies.[7] Although globular malignancy, a distinct kind of cancer of the breast, originates in the female mammary's milk-secreting ducts, it shares characteristics with ductal carcinoma. However, cells from the mammary's surface, fat, arteries, veins, and further tissues can give rise to various forms of breast cancer.[8]

For American women, carcinoma of the breast ranks as the subsequent most prevalent leading cause of cancer-related deaths, and the majority of malignancies in women are non-skin malignancies. In the year 2004, 215,000 new instances of cancer of the breast were reported, although the disease claimed 40 thousand lives.[9] It was estimated at the start of 2005 that over 5,70,000 people would die in the United States alone. A recent study states that one out of every four individuals in the country dies from cancer, which causes more than 1 million new cases along with 3,70,000 mortality globally each year.[10]

As a substitute for the DNA methylation transferase inhibitor 20-deoxycitidine, azacitidine (AZA) was first developed at the beginning of the 1960s.[11] AZA is the drug of choice and recommendation for addressing breast cancer associated with myelodysplastic syndrome (MDS).[12,13] AZA is cytidine's synthetic pyrimidine nucleoside analogue. The anti-cancer effects of AZA are often mediated through a variety of mechanisms, including direct cytotoxicity of abnormal hematologic cells in the marrow of the patient, incorporation into DNA and RNA, and inhibition of DNA methyltransferase, which leads DNA to become hypomethylated.[14] Through hypomethylation, genes essential for divisions and proliferative mechanisms can resume their normal functions. Generally, AZA has little effect on cells that are not replicating. The relative importance of cytotoxicity and hypomethylation in DNA is uncertain.[15] AZA is authorized to treat progressive myelomonocytic malignancies as well as myelodysplastic disorders. Acute myeloblastic leukemia, gastrointestinal cancer, carcinoma of the breast, cancer of the ovaries, cancer of the skin, as well as additional cancers, are also treated with it.[16] In clinical trials, AZA is also used with a number of anti-cancer drugs, such as amsacrine, cytarabine, vincristine, prednisone, and vinblastine.[17]

MATERIALS AND MATHODS

Drug and chemicals

We bought AZA from the market. The scientific-grade ingredients were acquired through JK Chemicals, New Delhi, for Polysarbate 80, Loba Chemical Pvt. Limited for Oleic acid, as well as Gattefosse (Saint-Priest) for Transcutol. We acquired the human malignancy cell lines KG-1, along with U937, directly from the National Centre for Cell Science and Research in Pune, which is in India. We also bought an MTT Proliferation evaluation kit through the city of Pune, India.

Method for the preparation of the AZA nanoemulsion formulation

With the help of a high-speed homogenization process, nanoemulsions are developed. A surfactant (polysorbate 80), a drug (AZA), and oil (Oleic acid) make up the homogenous organic solution in this. Water, as well as trancutol P, a co-surfactant, combined to produce the homogeneous aqueous phase. The phase with water was introduced while the stage with organic material was being continually homogenized. Following that, an o/w emulsion formed. The stirring was kept up to help the system reach equilibrium.[18]

Evaluation concerning the AZA nanoemulsion composition

Visual examination

As show in Figure 1 above, the formulation of a nanoemulsion is displayed.

Droplet size, polydispersity index (PDI), and zeta potential

There have been reports of light dispersing at a pleasant temperature as well as occurring at an angle of 90°. A micropipette and a washable polystyrene cuvette were used to apply a small quantity of the nanoemulsion formulation (1–1.5 mL). Three evaluations of the standard droplet dimension were conducted. Diluting the nanoemulsion does not change the size of the globules. The mean PDI along with particle dimensions were measured using the

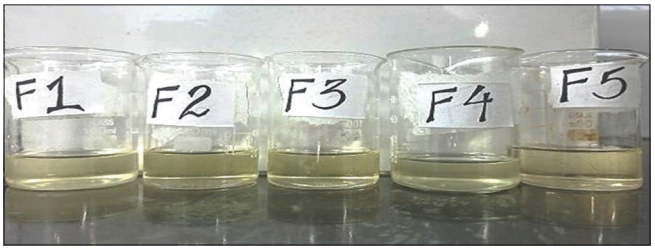

Figure 1: Visual assessment of the formulation of the nanoemulsion

Malvern Nanosizer (Malvern Instruments, USA) at room temperature.[19,20]

Determination of refractive index

The first step was calibrating the Atago Refractometer Rx 5000i using milli-q fluid at 20–25°C. The refractive indices of the additional compositions were determined using the same procedure after just The instrument's research phase was filled with just a tiny quantity of a drug-loaded nanoemulsion, the container's lid was shut, the temperature at which it ran was adjusted to 20–25° the form of Celsius, as well as the readings that showed up on the screen had been noted.[21]

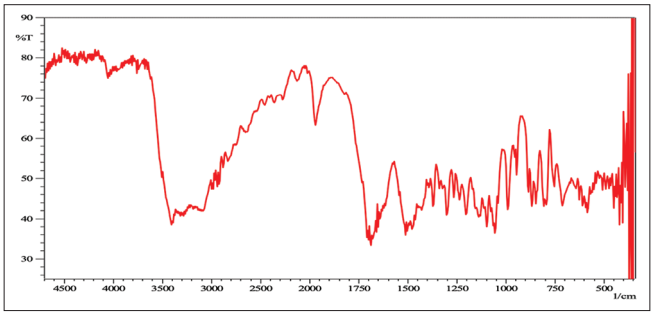

Figure 2: Azacitidine infrared spectrum

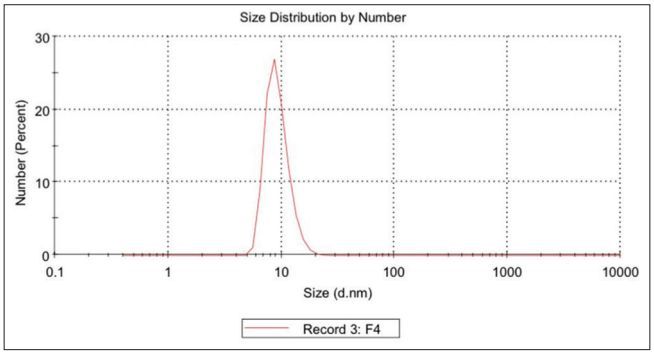

Figure 3: The distribution and size of droplets in the nanoemulsion formulation (F4)

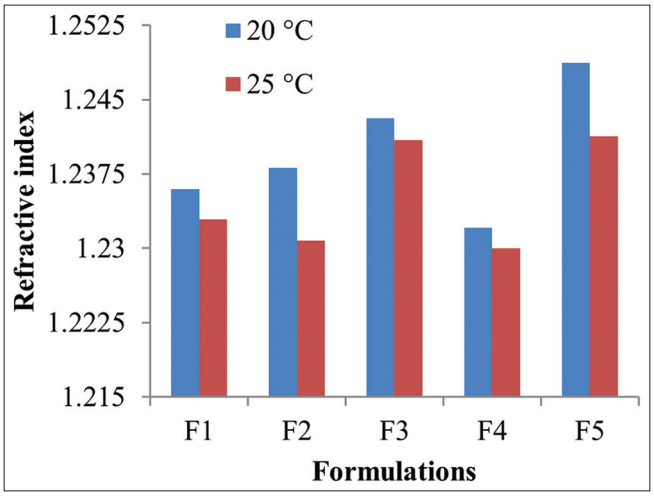

Figure 4: The formulation of the nanoemulsion's refraction index between 20°C and 25°C

Viscosity

The rheological evaluation of the nano-emulsion formulations was conducted employing a Brookfield Viscometer equipped with 61 stages. 5 equal quantities (about 100 mL) containing the nanoemulsion composition were contained in five separate 150 mL beaker containers with the labels F1, F2, F3, F4, and F5. At predetermined shearing frequencies (10, 20, 40, and 50) and a steady temperature of 20–25°C, the measurement instrumentation was activated to record the rheological characteristics. To prevent mistakes, three copies of every single nanoemulsion formulation's measurements were recorded.[22]

pH

This characteristic, which can be determined by a pH test device such as the Mettler Toledo type, is crucial for preventing skin reactions. The acid-base instrument was calibrated prior to pH readings being recorded using an appropriate buffer solution. This was carried out in order to ascertain the formulation's pH level.[23]

Measurement of electrical conductivity

A particular kind of 611E computerized sensitivity detector was used to evaluate the formulation's electrical

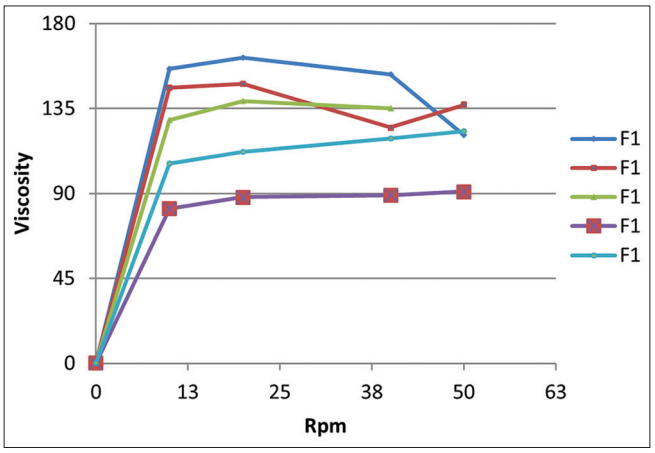

Figure 5: Viscosity of various nanoemulsion formulation

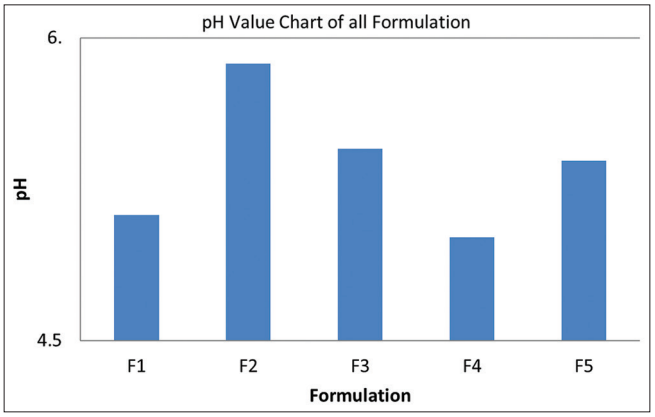

Figure 6: The pH values of the different formulations of nanoemulsions

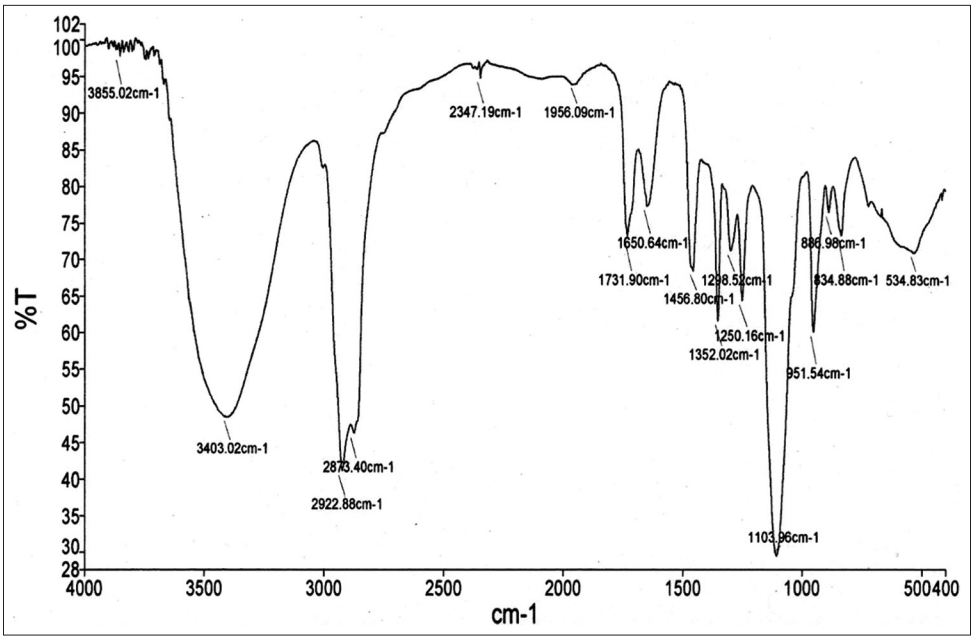

Figure 7: The nanoemulsion formulation's Fourier transform infrared spectrum (F4)

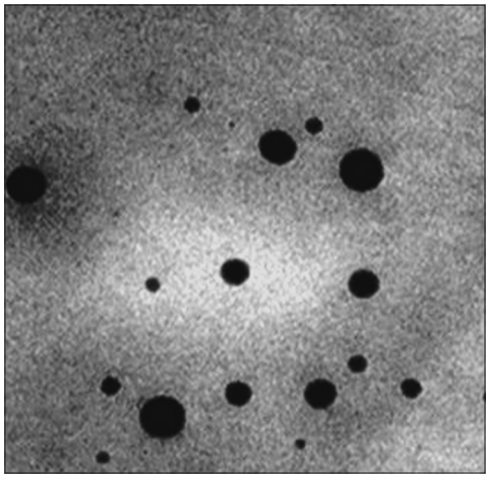

Figure 8: Transmission electron microscopy of nanoemulsion formulation F4

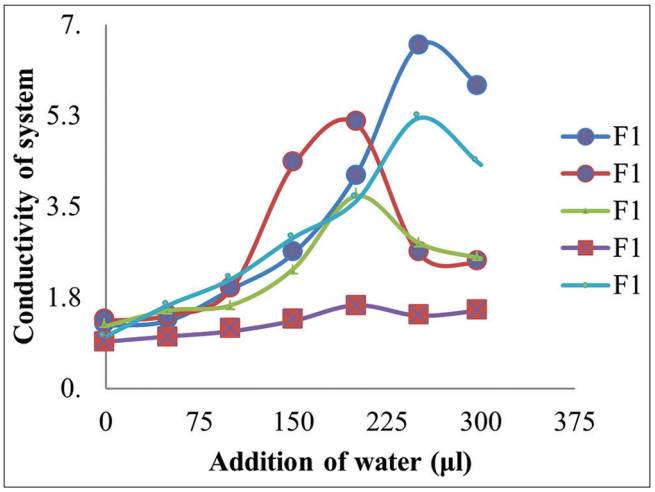

Figure 9: The electrical conductivity chart for formulations of nanoemulsions

characteristics. Initially, a KCl solution at around 25°C was used to measure the conductometer's cell characteristic. The electrodes made from platinum were then submerged in a single milliliter of the resulting solution, which had been taken out of a vessel containing an average of 10 mL in capacity being utilized. After that, the conductometer displayed the electrical conductance significance, which stayed constant until an ongoing measurement appeared on the device's readout. The electrical conductivity of each composition's dispersion system was measured after all the components had been properly mixed and each of them had been gradually diluted with the water-based solution in a glass beaker.[24]

Fourier-transform infrared (FTIR) evaluation

It is necessary to look at the stability and potency of AZA in conjunction with the nanoemulsion's constituents. To measure a liquid sample, potassium bromide material is packed as a disc with a nanoemulsion sample in a specific cuvette. After setting the wavelength range of the length under examination to 400–4,000 cm^{-1}, evaluated, and utilized to ascertain the compatibility of the samples.[25]

Transmission electron microscopy (TEM)

The outermost characteristics of the finished mixture were investigated using TEM microscopy. Following the preparation of the AZN-loaded composition, a small amount was distributed onto an aluminium grid with a carbon screen 400 as well as allowed to air dry for a day at the appropriate temperature. The sample was then imaged using an electron microscope with transmission, operating at a 30 kV acceleration voltage.[26]

Thermodynamic stability study

In order to run through six heating and cooling cycles, the refrigerator's temperature was maintained between 4°C and 45°C for 48 h. To determine if the different stages had separated out, the different parts of the sustainable nanoemulsion at 4 as well as 45°C were centrifuged for 30 min at around 3500 rotations/min.[27]

In-vitro drug release study

0.9 L of freshly prepared dissolved medium and an example II dissolution apparatus. The *in vitro* release of drugs of each of the developed NS compositions was performed using dialysis treatment the membranes with had pore sizes of 2.4 nm and a molecular weight range of ranging around 8000–14000 kDa. A pH 7.2 buffered solution of phosphate had been employed as a distributing medium. Ten hours of the research were carried out with stirring at 50 revolutions/min at about 37°C. The package contained 2½ mg of the nanoemulsion encapsulating AZN. To prevent leaks, the bag must be tightly closed on both of its sides. To maintain the sink circumstances, each of the 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360, as well as 600 min, a sample of 5 mL of freshly made solution was injected, and 5 mL of releasing media were retrieved for the NS. The following stage involved filtering these specimens using a filter composed of a statistically assessed AZA nanoemulsion around 241 nm and needles with a 0.45 m length.[28-30]

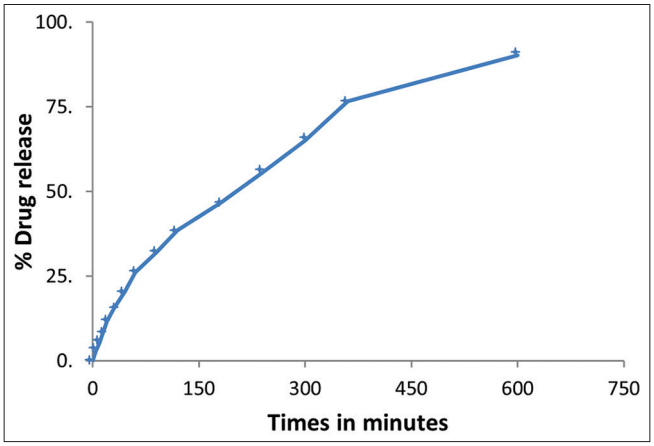

Figure 10: The F4 formulation's in-vitro drug release

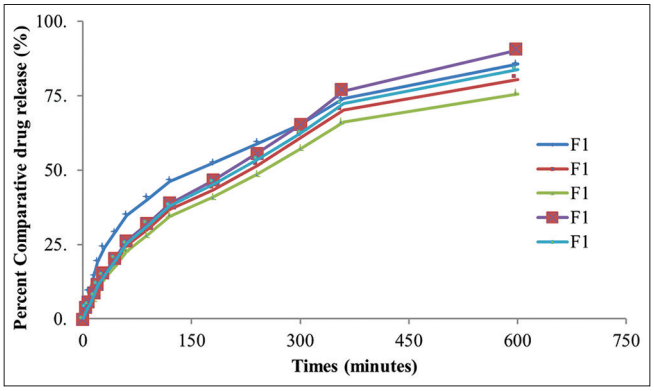

Figure 11: Comparative in vitro drug release for different nanoformulations (F1-F5)

Stability study

By exposing them to various demanding circumstances, including mechanical stress and extremely high temperatures, the resulting nanoemulsion's durability was assessed. Numerous features of the created compositions were assessed, such as:

Mechanical stress study

The quantity of the different parts of the nanoemulsion was established after each formulation was centrifuged for a different amount of time at 2,000 rpm.

Accelerated temperature study

By maintaining the compositions at different accelerated temperatures, the division of phases of the materials has been examined to evaluate them. Three different sets of similar compositions were kept for 30 days at three different temperatures: room temperature (25°C), fridge temperature (4°C), and higher temperature (40°C) in sealed, scaled containers. The physical capacity of each formulation to differentiate between the phases was assessed after 1, 10, 20, and 30 days.[31]

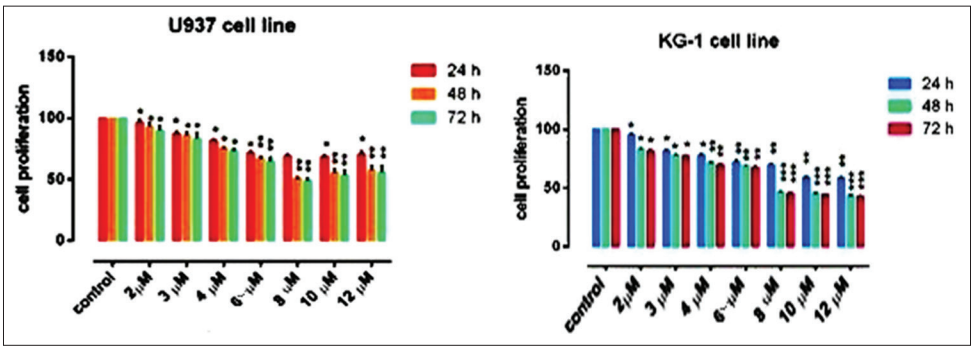

Figure 12: Cell proliferation

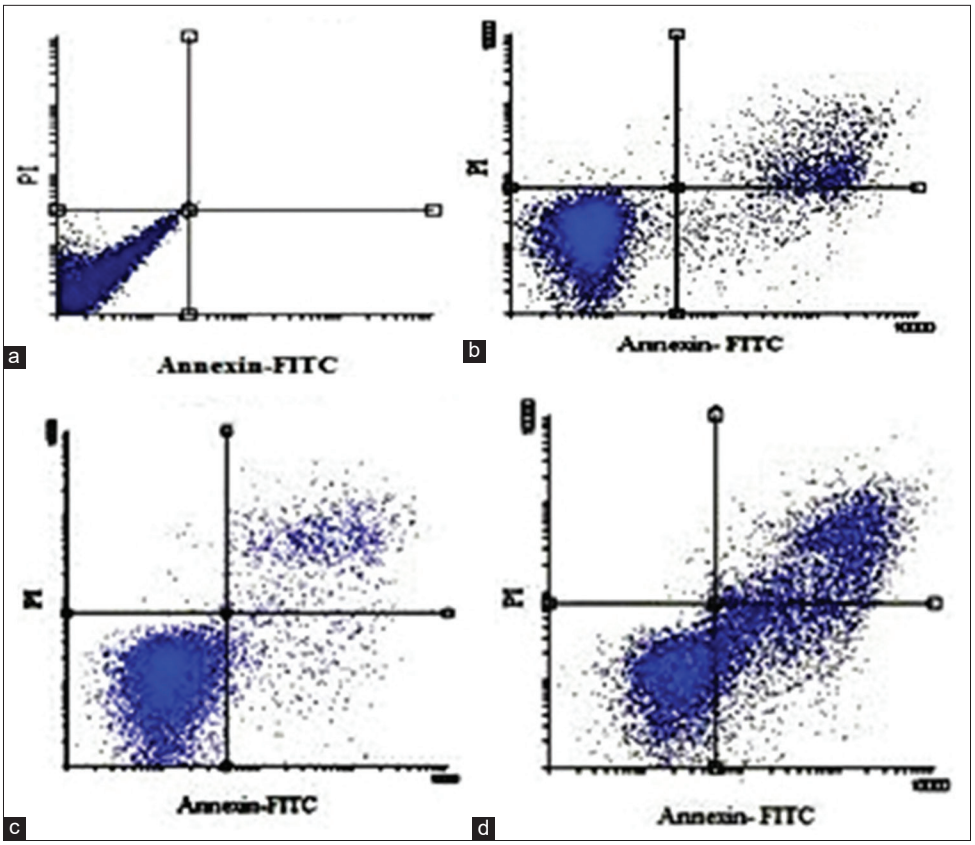

Figure 13: (a) Control: U937, (b) AZT Nanoemulsion: U937, (c) Control: KG-1, and (d) AZT Nanoemulsion: KG-1, showing morphological variations under microscopy indicative of cytotoxic response and structural alteration compared to untreated controls.

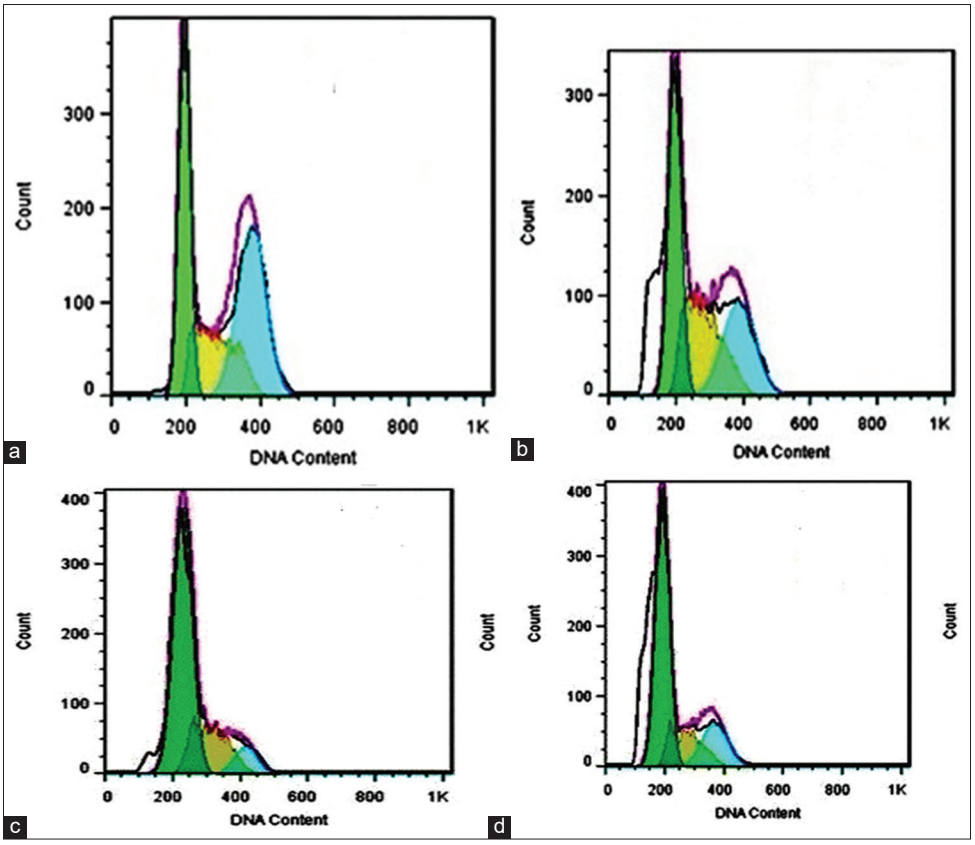

Figure 14: (a) KG-1 Control, (b) KG-1 AZT Nanoemulsion, (c) U937 Control, and (d) U937 AZT Nanoemulsion, respectively, illustrating cellular morphological differences indicating cytotoxic and structural alterations induced by AZT nanoemulsion treatment compared to control cells.

In vitro anti-cancer study

Cell lines

The National Centre for Cell Research in Maharashtra provided us with the individual human leukemia cell varieties KG-1 and U937. The KG-1, along with U937 cell lines, were propagated in full RPMI-1640 media supplemented with 10% and 20% heat-activated fetal bovine serum, correspondingly. Each cell was grown in a 37°C, moist condition containing 5% carbon dioxide (CO_2).[32]

Prolifiration assay

In an incubator with 96 wells, the KG-1, along with U937 cell populations, were first grown at an average density of 5×10^3 cells/well. AZT Nanoemulsion was administered to cells at 37°C with 5% CO_2 for 24, 48, and 72 h. The MTT test was used to evaluate the rate of cell division. The growth rate was used to demonstrate the data, with 100% of the cells acting as standards having only received 0.1% of the total dimethyl sulfoxide treatment.[33]

Apoptosis assay

Following 48 h of cultivation, in addition to or without AZT Nanoemulsion that cells belonging to the KG-1 and U937 lines were planted at an estimated population size of 3×10^5 individual cells/well. Following the manufacturer's procedure, a screening test was performed to determine the proportion of apoptosis caused by the treated compounds. The proportion of Annexin V+/PI-cells that had undergone apoptosis was ascertained using a BD flow cytometer utilizing the Flowjo software.

DNA cell cycle analysis

After applying the specified amounts of AZT Nanoemulsion to the cells for 48 h, the cells were submerged in 70% ethanol and colored with PI. Utilizing BD's flow cytometer, the outcomes of the cell analysis were examined. The proportion of hypodiploid sub-G0/G1 DNA can be calculated and used for determining the apoptotic cell portion.[34]

RESULTS AND DISCUSSION

FTIR Study

The nanoemulsion formulation is seen in the following FTIR spectrum [Figures 2 and 7]. It indicated that there is no significant interaction between drug and polymer because IR spectrum of nanoemulsion formulation showed prominent characteristic peaks as showed in IR spectrum of pure drug.

Droplet size, PDI, and zeta potential

Applying a Malvern zeta-sizer belonging to the Nano series, we determined the globule size along with PDI respectively. Table 1 provids a summary of those findings, which are shown in detail in Figure 3. These results were summarized in Table 1 and are presented thoroughly in Figure 3.

Calculating the refractive index

The results showed that all of the nanoemulsion formulations had refractive indices between 1.2321 and 1.2487 at 20°C and 1.23 and 1.2413 at 25°C [Figure 4 and Table 2].

Viscosity

The graph shows each formulation's the consistency [Figure 5].

pH

The figure provided a graphic representation of the data table, which showed the results for the nanoemulsion formulation's pH level varied between 5.01 and 5.87 [Figure 6 and Table 3].

FTIR evaluation

The nanoemulsion formulation is seen in the following FTIR images.

TEM

TEM was used to examine the microstructure of the AZN-loaded nanoemulsion. Particle size, surface structure, along fundamental carrier system structure are just a few of the aspects that may be efficiently analyzed with TEM. According to the TEM images, the droplets that formed were spherical and within the nanoscale size range. The images from the TEM photos displayed the AZN-loaded nanoemulsion droplets as black spheres, as can be seen in the Figure 8.

Thermodynamic stability study

Neither centrifugation nor a heating-cooling cycle was used on any of the resultant nanoemulsion formulae, and neither process resulted in phase segregation. Table 4 contains all of the information.

Electrical conductivity study

This study explains the creation of various ministructures along with their transition from continuous oil phases to continuous water at various stages. A bi-continuous technique was developed to demonstrate the electrical characteristics of the nanoemulsion formulations that required the nanoemulsion when the water phase was incorporated for a maximum of 250 µL [Figure 9 and Table 5].

In vitro drug release study

The equipment and process paragraph's recommendations were followed in order to investigate the release. To comprehend the behavior of drug dispersion from a chosen composition, *in vitro* dissolving investigations were conducted. According to the results of the 10-h distribution experiment, F4 had the greatest coefficient of absorption among the five varieties.

Table 1: F1-F5 nanoformulations' droplet size and dispersion

Formulation code	Droplet size (nm)	Polydispersity index
F1	97.7	0.563
F2	132.0	0.438
F3	27.02	0.369
F4	13.89	0.241
F5	31.12	0.341

Table 2: Refractive index of the nanoemulsion formulation

S. No.	Batch	20°C	25°C
1	F1	1.236±0.01	1.2329±0.05
2	F2	1.2381±0.05	1.2308±0.05
3	F3	1.2431±0.09	1.2409±0.07
4	F4	1.2321±0.03	1.230±0.06
5	F5	1.2487±0.01	1.2413±0.05

Table 3: pH of the various nanoemulsion formulations

S. No.	Formulation code	pH-value
1.	F1	5.87
2.	F2	5.12
3.	F3	5.41
4.	F4	5.01
5.	F5	5.46

Table 4: Study of thermodynamic stability

Formulations code	Centrifugation system	Heating-cooling cycle
F1	Not phase separation	Constant
F2	Not phase separation	Constant
F3	Not phase separation	Constant
F4	Not phase separation	Constant
F5	Not phase separation	Constant

Table 5: Conductivity of electricity for every version of the nanoemulsion

Addition of water (µL)	F1	F2	F3	F4	F5
0	1.2	1.3	1.2	0.9	1
50	1.3	1.4	1.5	1	1.6
100	1.9	1.9	1.6	1.1	2.1
150	2.6	4.3	2.3	1.3	2.9
200	4.1	5.1	3.7	1.6	3.6
250	6.6	2.6	2.8	1.4	5.2
300	5.8	2.4	2.5	1.5	4.3

There were also signs of prolonged, increasing drug release and drug dependency [Figures 10 and 11].

In vitro anticancer studies

Cell proliferation assessment with the MTT experiment

The MTT assay was used to determine the cell proliferation of U937 along with KG-1 following treatment with the recommended concentration of the medication AZT Nanoemulsion (3–15 µM) for a duration of 24 h, 48 h, respectively 72 h. We observed that the effects of substances changed with dosage and time. As seen in Figure 12, there was no appreciable variation between the periods of 48 h and 72 h. We evaluated the synergistic effects of the selected AZT Nanoemulsion dosages (8 µM for KG-1 as well as 6 µM for

Table 6: Mechanical stress technique stability research

Centrifugation time (minutes)	Phase separation upon centrifugation				
	F1	F2	F3	F4	F5
10	---	---	---	---	---
20	---	---	---	---	---
30	---	---	---	---	----
40	---	---	---	---	---
50	---	---	---	---	---
60	---	---	---	---	---

Significant no Changes (—-)

Table 7: Stability study

Temperature/Days	Phase separation (%)				
	F1	F2	F3	F4	F5
5±1°C					
1	---	---	---	---	---
10	---	---	---	---	---
20	---	---	---	---	---
30	---	---	---	---	---
25±1°C					
1	---	---	---	---	---
10	---	---	---	---	---
20	0.5	1.0	0.5	0.5	0.5
30	1.0	1.5	1.5	2.0	1.5
40±1°C					
1	1.0	2.5	2.5	2.0	2.0
10					
20			Breaking		
30					

(---): No significant change

U937). Our study found that, in comparison to the control group, the KG-1, along with the U937 cell lines, showed a substantial decrease in cell growth when exposed to AZT nanoemulsion.

Apoptosis assay

To investigate how the recommended medications affected apoptosis, we used Annexin V FITC/PI labelling in a flow cytometry assay. Figure 13 shows that in the two different U937 and KG-1 cell lines, treatment increased the total amount of early cells regarded as apoptotic (Annexin +/PI-) and decreased the fraction of necrosis. The AZT Nanoemulsion also showed a substantial spike in apoptotic cells (65% in KG-1 and 75% in U937). Similarly, exposure to AZT nanoemulsion significantly reduced the proliferation of the KG-1 and U937 lineages of cells.

Cell cycle assay

KG-1 and U937 cells' DNA was examined at different phases of the biological process in order to better comprehend how the cell process develops. Following treatment with U937, cells exposed to AZT Nanoemulsion for 28 h significantly increased the number of cells that were in the G0/G1 phase in the current study. According to our findings, AZT Nanoemulsion enhanced the hypodiploid partially G0/G1 DNA percentage in KG-1 cells during dose-dependent ways, this was 1.97–9.1%, and for U937 cells, it was 8.32–16.85%), suggesting apoptosis [Figure 14].

Stability study

Mechanical stress study

Using centrifugation under mechanical stress—2000 rotations/min. The long-term effectiveness of the different nanoemulsion compositions was assessed under certain conditions. According to the research, the compositions did not alter following centrifugation at 2000 revolutions/min for around 60 min [Table 6].

Stability study

In particular, the preparations had been preserved at 4 ± 1°C, 25 ± 1°C, and 40 ± 1°C for 1, 10, 20, and 30 days, respectively. As part of higher temperature research to assess the compositions' endurance at different temperatures, to determine the extent of any alteration in nature or division of phases [Table 7].

CONCLUSION

The present study's findings indicate that a high-speed homogenization procedure was successfully applied to produce an AZA Nano emulsion composition for solubility enhancement. These days, the usage of nanoemulsions as a carrier system for medication delivery is growing in popularity. A promising carrier system including transcutol P (co-surfactant), oleic acid, and polysorbate 80 was used to include AZA. Transcutol P and polysobate 80 complement each other effectively and help solubilize the drug during the theanoemulsion production process. There was 100% drug content, according to one analysis. According to zeta potential measurements, the final formulation had highly stable globules with a size range of 13.89 nm. The final composition exhibited a 90.30% drug distribution efficiency and remarkable stability for the tiny emulsion composition, based on *in vitro* medication release data.

In this *in vitro* work, we examined the consequences of AZT Nanoemulsion on cancer cells in order to show for the inaugural time its simultaneous anti-oncogenesis in hematopoietic myeloid tumors. AZT Nanoemulsion's impact on the proliferation of cells was initially investigated using the leukemia caused by myeloid cells, cellular variants U-937, as well as KG-1. According to earlier research, AZA repressed KG-1 along with U-937 cells in a manner that varied according to the duration and concentration. Finally, the AZA Nanoemulsion offers significant advantages in growth suppression compared to the use of either medicine, just like solid tumors do. In the study on apoptosis, we discovered that AZT nanoemulsion treatment markedly increased apoptosis levels. Furthermore, as it has been demonstrated that AZT nanoemulsion can further inhibit histone deacetylases, we propose whether it may 1 day be applied as a therapeutic alternative for leukemia with myeloid precursor cell varieties.

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