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Formulation and characterization of azacitidine nanoemulsion for the treatment of tumor suppressor genes activity in myeloid leukaemia cell line

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KEYWORDS

Pyrimidine, Azacitidine, Nano-emulsion, Test

ABSTRACT:

The goal of the current study is to define and developed an Azacitidine nanoemulsion. Azacitidine is a derivative of pyrimidine nucleoside that prevents DNA methyltransferase from working properly, hence affecting DNA methylation. Incorporated largely into RNA, it additionally functions as an antimetabolite of cytidine. The anti-cancer drug azacytidine has been utilized and absorption rate is very minimal. Thus, nano-emulsions have been formulated in order to address these problems. Nano-emulsion made up of distilled water, plysorbate 80, Azacitidine, various oils (including oleic acid), and co-surfactants Transcutol P. By using the high-speed homogenization method, various water-in-oil nano-emulsion are formulated. Thermodynamic stability testing, viscosity test, pH test, FTIR test, in-vitro drug release test, and stability testing were used to evaluate the nano-emulsion formulations.Methotrexate, a standard medication, and nanoemulsion were found to have the greatest % inhibitions, 89.09% and 85.37%, correspondingly.Given that it has been shown that AZT nanoemulsion may also function to block histone deacetylases, we propose that it may one day be employed as a therapeutic option in myeloid leukaemia cell lines.

Introduction

In order to create a small-scale medication delivery system, nanotechnology is crucial. For a medicine to act in a safe, successful, and productive manner, nanotechnology is required.The features of nanotechnology include enhanced durability and absorption, quantitative transfers of the medicine, controlled release of medication, as well as desired pharmacodynamic action.**[1]** Effective pharmacological components are released more effectively when using nanoemulsions**.[2]** Nanoemulsions are typically regarded as safe-level additives since they circumvent physiological and physical constraints on drug administration, such as particle size, production methods, thermodynamic durability, etc.**[3]**

Nanoemulsions were created to carry out various functions for targeted medication administration, extended blood circulation, examination,Target-specific binding, etc. **[4]** the aforementioned characteristics can be adjusted to aid in delivering the medicine or diagnostic agent to the targeted place of interest based on both the active & passive target selection strategies. The medicine may be effectively delivered for those suffering from cancer using this nanoemulsion. By encapsulating the medication in an enclosed structure at the centre of the nanoemulsion, which increases the bioavailability of the medication and minimizes any unintended effects on adjacent cells or tissues, the drug can be transferred to the specific spot more effectively in the context of therapy for cancer. **[5]**

The resistance to drugs is the main factor keeping cancer patients from being cured. The varied characteristics of the tumour, developmental kinetics, and tumour microenvironment are only a few of the many factors that influence treatment resistance in cancer. Combination

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treatment, immunotherapy checkpoint blockade, and the use of nanoformulations are all ways to combat cancer recurrence.**[6]** One in eight women will be identified as having one of the several kinds of breast tumours over their lifetimes. Breast tumours are a malignant development that starts in the tissues of the female breast. For instance, over seventy-five percent of breast cancers are ductal carcinomas, which start in the cells surrounding the ducts that transport milk to the nipple.**[7]** Although lobular malignancy, a different kind of breast cancer, starts within the milk-secreting ducts of the female breast it behaves similarly to carcinoma of the ducts in other ways. In contrast, other types of carcinoma of the breast can develop from cells found in the breast's surface, fat, blood vessels, and other tissues.**[8]**

The majority of cancers are non-skin malignancy in women as well as the second-leading cause of cancer-related fatalities in American women is breast cancer. Forty thousand breast cancer fatalities and 215 000 newly identified cases of the disease were both reported in 2004.**[9]** In the beginning of 2005, it was predicted that there would be more than 5,70000 fatalities in the US alone. According to one forecast, cancer accounts for one in four fatalities in the country and causes over one million new instances and 3,70000 mortality per year globally.**[10]**

Azacitidine (AZA) was initially created in the early 1960s as an equivalent of the DNA methylation transferase inhibitor 20-deoxycitidine.**[11]** Overwhelmingly favoured and suggested medication for treating breast cancer with the condition myelodysplastic syndrome (MDS) is AZA.**[12,13]** An artificial pyrimidine nucleoside analogue of cytidine is called azacitidine. Azacitidine tends to have many methods by which it exerts its anti-cancer effects, such as immediate cytotoxicity on aberrant hematologic cells in the bone marrow, as well as by incorporating into DNA as well as RNA and regulation of DNA methyltransferase, which results in hypomethylation level of DNA.**[14]** Genes necessary for division and proliferative processes may regain their usual functioning by hypomethylation. Cells that are not reproducing are mostly unaffected by azacitidine. It is unknown how significant cytotoxicity vs hypomethylation in DNA is in comparison. **[15**] Myelodysplastic disorders and chronic myelomonocytic malignancies are authorized conditions for azacitidine treatment. Acute myeloblasticleukemia, cancer of the ovary, breast cancer, cancer of the intestines, melanoma, as well as additional cancers is also treated with it.**[16**] Azacitidine is additionally combined with various anti-cancer medications in clinical studies, including vincristine, prednisone, vinblastine, cytarabine, and amsacrine.**[17**]

Material and Methods

Drug and Chemials

Azacitidine was obtained as a gift sample (Intas pharmaceuticals Ltd, Ahmedabad, Gujarat). Polysarbate 80 was purchased from JK Chemicals Delhi, Oleic acid was purchased from Loba Chemie Pvt. Ltd and Transcutol were purchased from the Gattefosse (Saint Priest, Cedex, France) and other chemicals were analytical grades.The human leukemia cell lines U937 and KG-1 were obtained from the National Centre for Cell Science Pune., India. MTT Proliferation test kit was also purchased from Pune India.

Method for the preparation of the Azacitidine Nanoemulsion formulation

With the help of a high-speed homogenization process, nanoemulsions are developed. A surfactant (polysorbate 80), a drug (azacitidine), and oil (Oleic acid) make up the homogenous organic solution in this.Together, water and the co-surfactant trancutol P, formulated the homogenous aqueous phase.While the organic phase was continuously homogenized, the phase containing water was added.After that, an o/w emulsion developed. To allow the system attain equilibrium, the stirring was continued.

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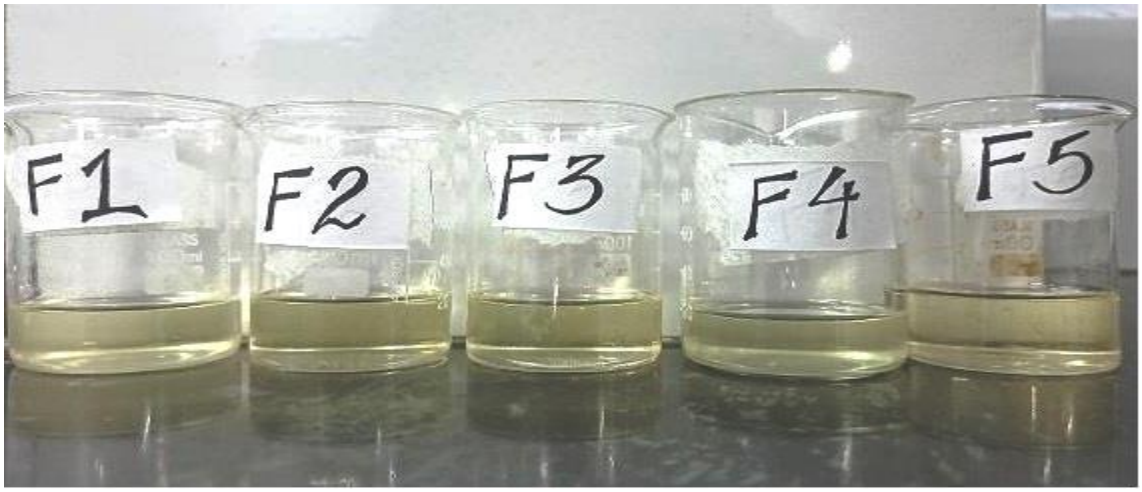

Figure 1: Visual evaluation of Nanoemulsion formulation

Characterization of the Azacitidine Nanoemulsion formulation

Visual Inspection

Nanoemulsion formulation is shown in the below figure 1.

Droplet size, PDI and zeta Potential

At a comfortable temperature and an incidence angle of ninety degrees, light has been seen to disperse. Using a washable polystyrene cuvette and a micropipette, a small amount of nanoemulsion formulation (1–1.5 mL) was added.We evaluated the average droplet size three times. No difference in globule size is seen when nanoemulsion is diluted. At the temperature of the room, the Malvern Nanosizer (Malvern Instruments, USA) was used for determining the mean polydispersity index (PD index) and the dimension of the particles.**[19,20]**

Determination of refractive index

First, milli-q fluid at 20 to 25 degrees Celsius was used to calibrate the Atago Refractometer Rx 5000i.After placing a small amount of a drug-loaded nanoemulsion in the instrument's study stage, closing the lid, adjusting the operating temperature to 20 to 25 degrees Celsius, and recording the measurements that appeared on the showing, the same process was used to calculate the refractive indices of the other compositions.**[21]**

Viscosity

The formulation of the nano-emulsion was rheologically evaluated using a Brookfield Viscometer with a 61 spindles. Five distinct 150 millilitre beakers, each labelled F1, F2, F3, F4, and F5, each had five identical volumes (100 millilitres) of the nano-emulsion composition.The measuring device was turned on to keep track of the rheological characteristics at preset shearing speeds (10, 20, 40, as well as 50) and a preset ambient temperature of 25 C. To prevent mistake, three copies of every single nano-emulsion formulation's measurements were recorded.**[22]**

pH

This characteristic, which may be measured using a pH meter like the Mettler Toledo model, is crucial for preventing skin irritation.Before recording pH values using a reference buffer solution, the acid-base meter was calibrated. It was done in order to determine the pH level of the formulation.**[23]**

Measurement of electrical conductivity

For the purpose of evaluating the formulation's electrical properties, a computerized conductivity meter type 611E

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JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

was employed.Theconductometer's cell constant was first determined by using a KCl solution at an approximate temperature of 25 °C. After that, 1.0 ml of the resulting solution was taken from a 10 ml-capacity the vessel and used for immersing the platinum electrodes, and the conductometer then displayed the electrical conductance significance, which was maintained until a continuous reading had been shown by the instrument's display.Following correct mixing of all the ingredients and gentle dilution of each composition with the water solution into a glass beaker, the electrical conductance of the dispersing system of each composition was measured.**[24]**

FTIR Evaluation

The potency and stability of azacitidine with the elements of the nanoemulsion must be investigated. When measuring a liquid sample, a special cuvette is used to pack potassium bromide material with a nanoemulsion sample as a disc. The range of wavelengths of the examined length is set to (400 to 4000 cm^{-1}), and the spectrum information is then registered, investigated, and used to determine whether or not the samples are compatible.**[25]**

TEM (Transmission electron microscopy)

Examining the surface characteristics of the finished composition included using TEM microscopy. After creating the AZN-loaded formulation, a small amount of the sample was pipetted upon a copper grid with a carbon mesh 400 and left to air dry for one day at a comfortable temperature before being imaged through a transmission electron microscope, which was running at an acceleration voltage of 30 kV.**[26]**

Thermodynamic stability study

The refrigerator's temperatures is regulated around 4 and 45 degrees Celsius, and it is kept at each setting for 48 hours to complete six heating and cooling cycles. The long-lasting nano-emulsion compositions at 4 and 45 degrees Celsius were centrifuged for thirty minutes at 3500 revolutions per minute to check for separation of the phases.**[27]**

In-Vitro drug release study

Utilizing a type II dissolution device, 0.9 L of newly made dissolving media. Every of the created NS formulations' in vitro drug release was carried out employing a dialysis membranes, which has a pore size of 2.4 nm and a variety of molecular weights of roughly 8000–14000 kDa.As a releasing media, a buffered phosphate solution with pH 7.2 was employed**.** The experiment was conducted for 10 hours at 37°1°C and 50 rpm stirring. The 2.5 milligrams of AZN-containing nanoemulsion was placed in the bag. To prevent leaks, the bag must have tightly closed on both of its sides.In order to maintain the sink circumstances, a sample of 5 ml of newly prepared solution was injected and 5 millilitre of release media was extracted every 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360 and 600 minutes for the NS.The following stage involved filtering these samples via the use of a syringe filter with a 0.45 m aperture and an azacitidine nanoemulsion statistically assessment at the wavelength of 241 nm.**[28-30].**

Stability study for nanoemulsion

Stability study

The durability of the produced nanoemulsion was evaluated by subjecting them to different demanding conditions, such as mechanical stress along with elevated temperatures.The developed compositions' many properties were evaluated, including:

Mechanical stress study

Following varying centrifugation periods of every formulation at 2,000 revolutions per minute, the amount of the distinct portions of the nanoemulsion had been determined.

Accelerated temperature study

The phase separation of the compositions has been investigated in order to assess them by keeping them at varied accelerated temps.Three separate sets of the comparable compositions and maintained for 30 days in sealed, scaled containers at three distinct temperatures: ambient temperature (25°C), refrigerator temperature (4°C), as well as higher temperature (40°C).After 1, 10,

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JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

20, & 30 days, the ability of each formulations to distinguish the phases was evaluated physically.**[31]**

In-Vitro Anticancer studies

Cell Lines

The human leukemia cell lines U937 and KG-1 were obtained from the National Centre for Cell SciencePune. These cell lines were grown in full RPMI-1640 media with 10% and 20% heat-activated FBS for U937 and KG-1 cell lines, correspondingly. In an incubator set to 37°C and 5% CO2, all cells were grown in a humid environment**.[32]**

Prolifiration Assay

In a 96-well plate, the cell lines U937 and KG-1 were planted (at a starting density of 5x103 cells per well). For 24, 48, and 72 hours, AZT Nanoemulsion was applied to cells in a 5% CO2 environment at 37 degrees Celsius. The MTT test was used to determine how quickly cells proliferated. Results were shown as a growth rate, with 100% denoting control cells that had only received 0.1% DMSO as treatment**.[33]**

Apoptosis Assay

Result and Discussion

Characterization of Nanoemulsion formulation

Determination of drug by FTIR

Cells of the U937 and KG-1 lines were planted at a density of 3 105 cells per well and cultured for 48 hours in the presence and absence of AZT Nanoemulsion. According to manufacturer procedure, a fluorescein-conjugated Annexin V (Annexin V-FITC) staining experiment was carried out to determine the proportion of apoptosis induced by treated compounds. Using a BD flow cytometer as well as the flowjo (Tree Star Inc., version 9.6.3, USA) application, the proportion of Annexin V+/PI- cells that underwent apoptosis was calculated.

DNA Cell Cycle Analysis

AZT Nanoemulsion was applied to the cells in the quantities indicated for 48 hours, after which the cells were fixed in 70% ethanol and dyed with PI. BD's flow cytometer was used to examine the results of the analysis of the cells. Hypodiploid sub-G0/G1 DNA proportion allows for the prediction of the apoptotic cell fraction**.[34]**

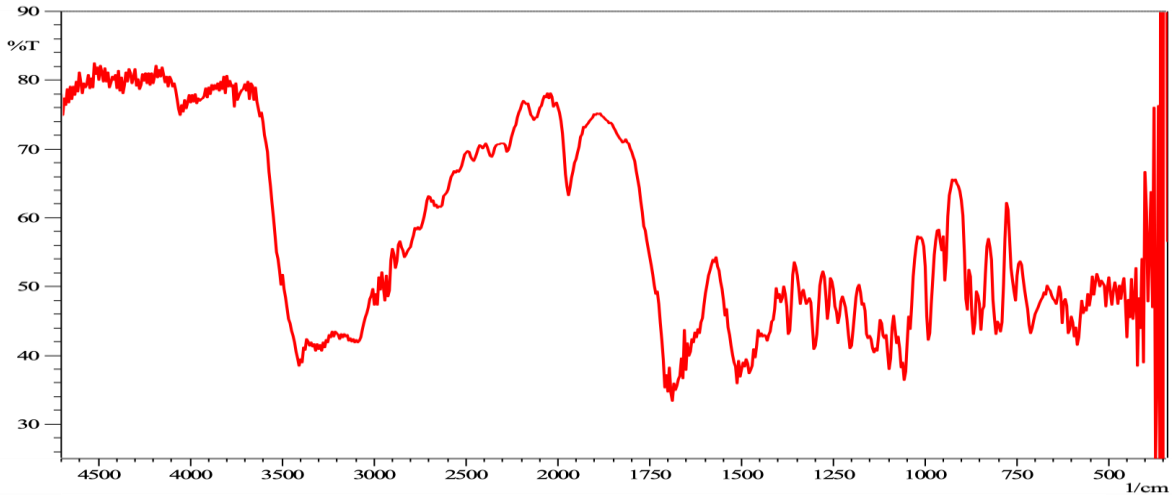

Figure 2: IR Spectrum of azacitindine.

Journal of Chemical Health Risks
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JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

Droplet size, PDI and zeta Potential

We measured globule size and polydispersity index using a Malvern zeta-sizer from the Nano series. Figures 3 provide a thorough graphical depiction of the results, which were summarized in table 3.Globule diameters ranged from 13.89 nanometers to 132.0nanometers, and every combination was created with a similar size distribution.

Table 3: Droplet size and distribution of F1-F5 nanoformulations.

Formulation Code	Droplet size (nm)	PDI
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

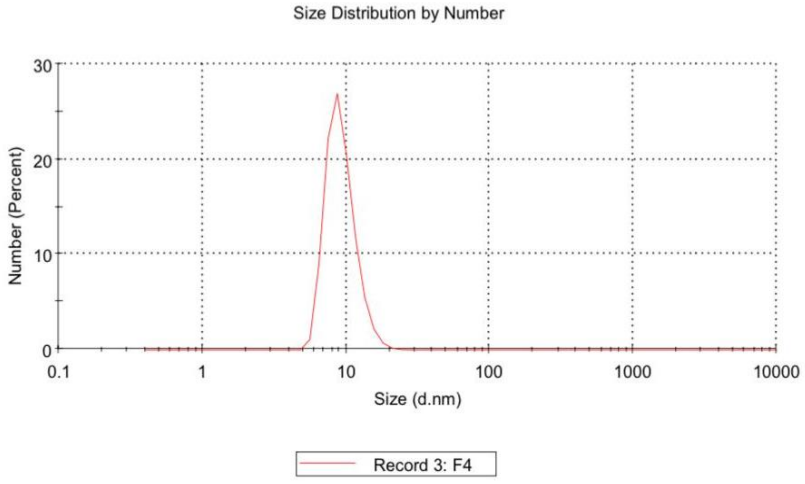

Figure 3: Droplet size and distribution of nanoemulsion formulation (F4)

Determination of refractive index

The refractive indices of all nano-emulsion compositions ranged from 1.2321 to 1.2487 at a temperature of 20 degrees Celsius alongside 1.23 to 1.2413 at a temperature of 25 degrees Celsius, according to the results.

Journal of Chemical Health Risks
www.jchr.org
JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

Table 4: Refractive index of the nano-emulsion 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

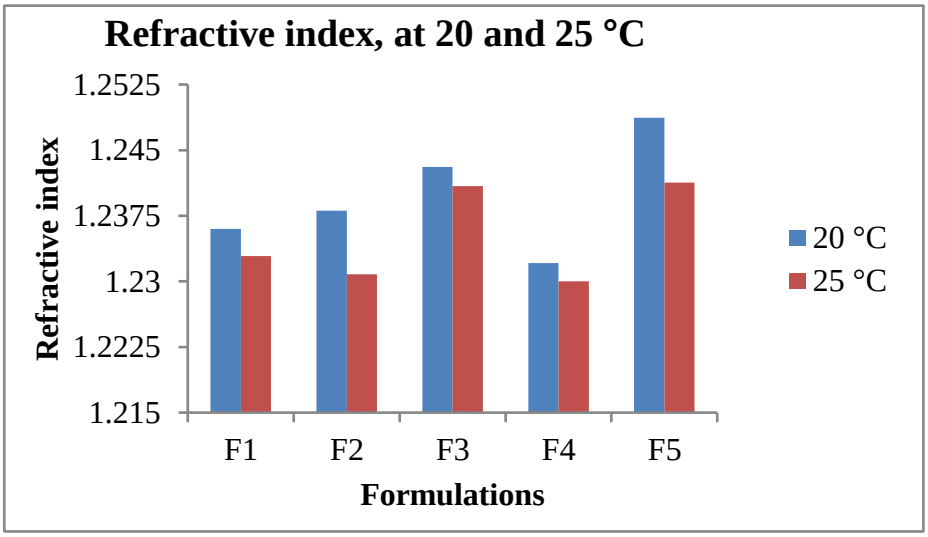

Figure 4: Refractive index at 20°C and 25°C of the nano-emulsion formulation

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www.jchr.org
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Viscosity

Viscosity of all formulation shown in the below graph:

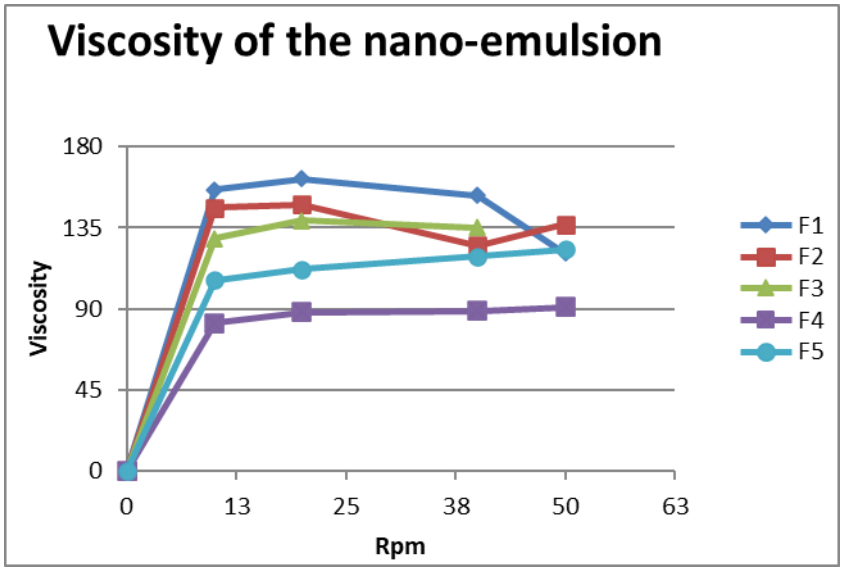

Figure 5: Viscosity of the nanoemulsion formulation

pH

The data table showed that the nano-emulsion formulation's pH fell between 5.01 as well as 5.87, and the image illustrated this information graphically.

Table 6: 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

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JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

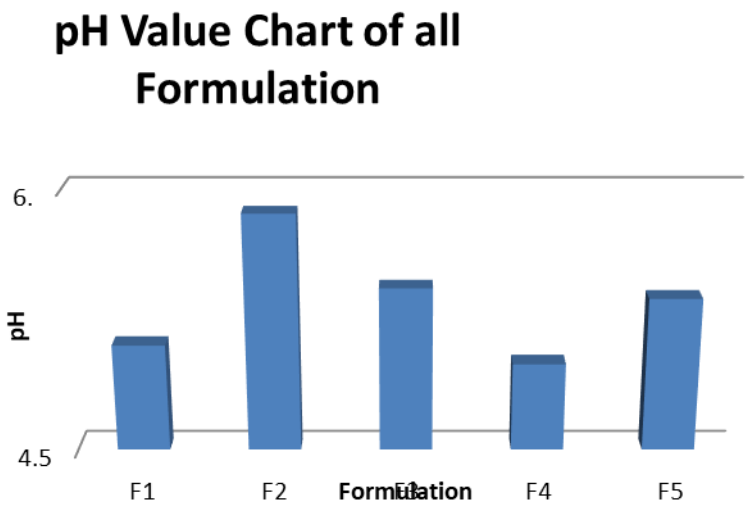

Figure 6: pH of the various nanoemulsion formulations

FTIR Evaluation

The following FTIR images depict the nanoemulsion formulation:

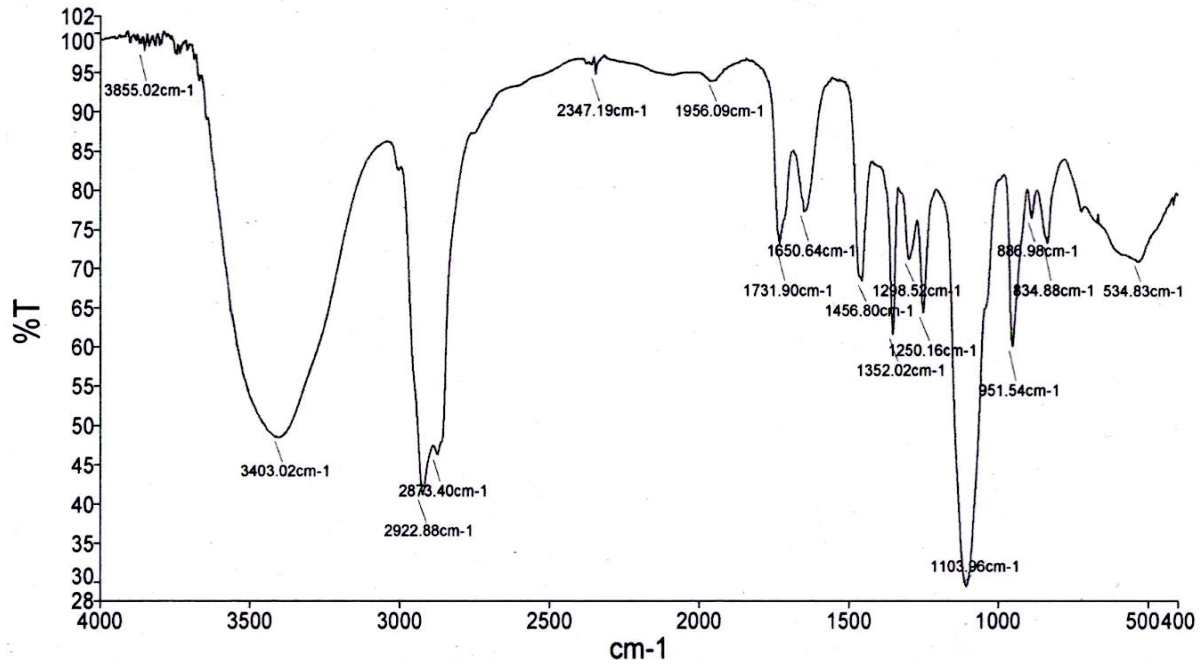

Figure 7: FTIR spectrum of the nanoemulsion formulation (F4).

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JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

TEM (Transmission electron microscopy)

The AZN loaded nanoemulsion's morphology was studied using TEM. Through TEM, it is possible to analyze a variety of features, including particle size, structure (Surface), and underlying carrier system structure, effectively.The TEM pictures showed that the droplets were spherical and that they were in the nanoscale range in terms of size.As seen in the pictures, the droplets of the AZN-loaded nanoemulsion showed as black spheres in the transmission electron microscope images.

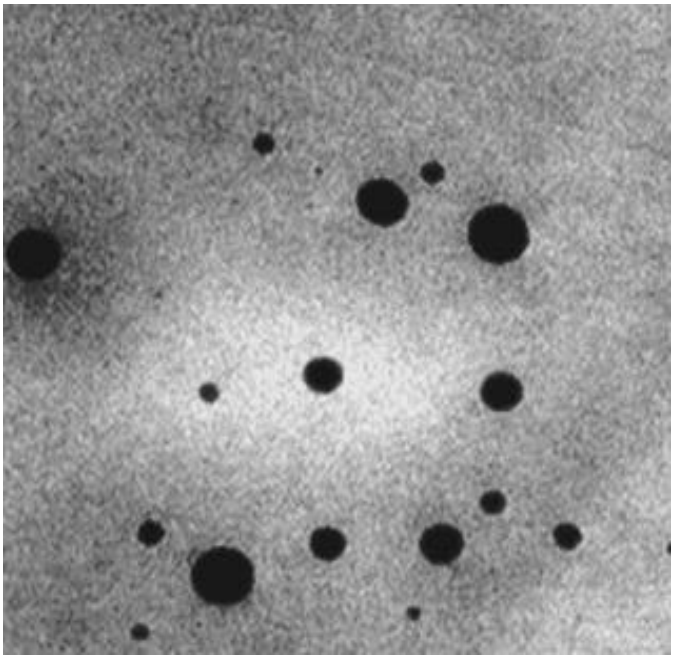

Figure 8: TEM of Nanoemulsion formulation F4.

Thermodynamic stability study

None of the resulting nano-emulsion formulas underwent a heating-cooling cycle or a centrifugation, and neither procedure produced any phase segregation. All of the data are in Table 7.

Table 7: Thermodynamic stability study

Formulations code	Centrifugation system	Heating cooling cycle
F1	No phase separation	Constant
F2	No phase separation	Constant
F3	No phase separation	Constant
F4	No phase separation	Constant
F5	No phase separation	Constant

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Electrical conductivity study

This research explains the development of numerous ministructures and their reconfiguration from an oil continuous to a water continuous phases.A bi-continuous approach was produced, displaying the electrical characteristics of the preparations for nano-emulsions without subtype nano-emulsion, while an aqueous phase was introduced up to 250µl.

Table 8: Electrical conductivity for all nano-emulsion formulation

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

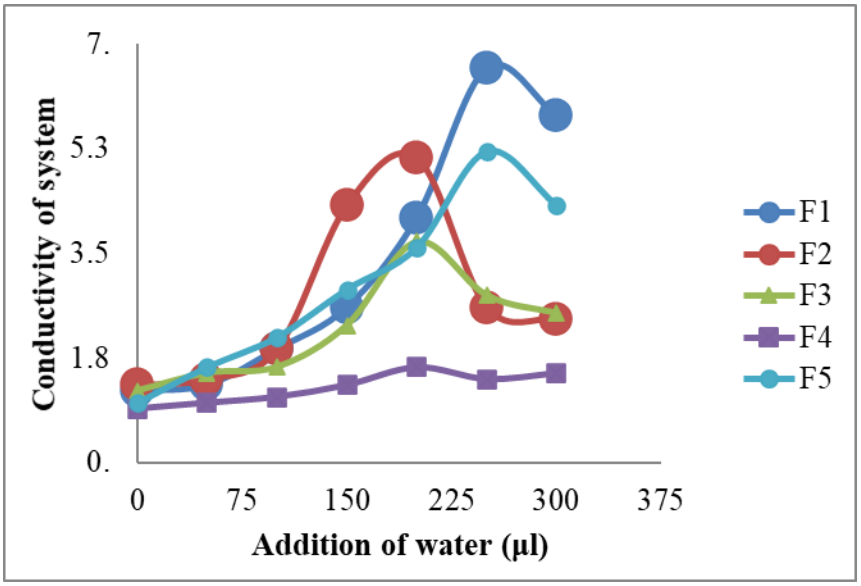

Figure 9: Electrical conductivity chart of Nano-emulsion formulations.

Journal of Chemical Health Risks
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JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

***In-Vitro* drug release study**

Following the instructions provided in paragraph of the equipment and procedure, the investigation on release was carried out.*In-vitro* dissolving studies were performed to understand the medication distribution behaviour from a selected composition.The ten hours release experiment showed that F4 had the greatest absorbance value out of the 5 formulations. Drug dependence and extended, progressive drug release were also present.

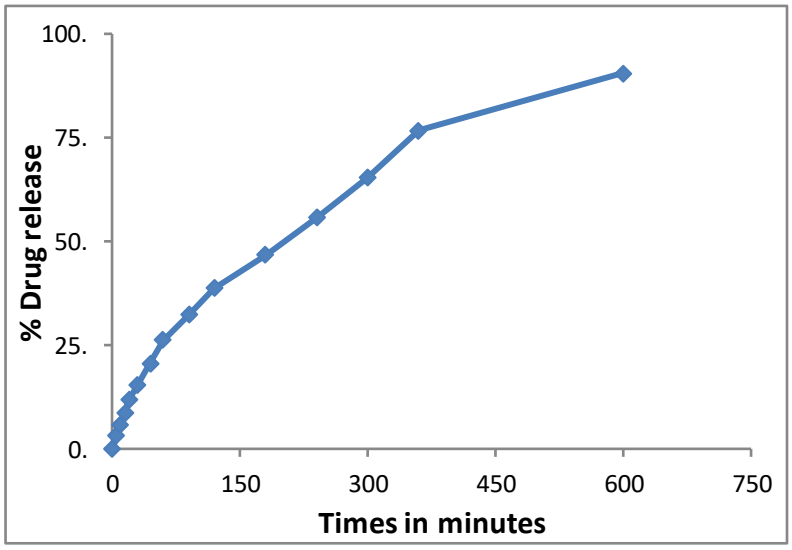

Figure 10: In-Vitro drug release for the formulation of F4

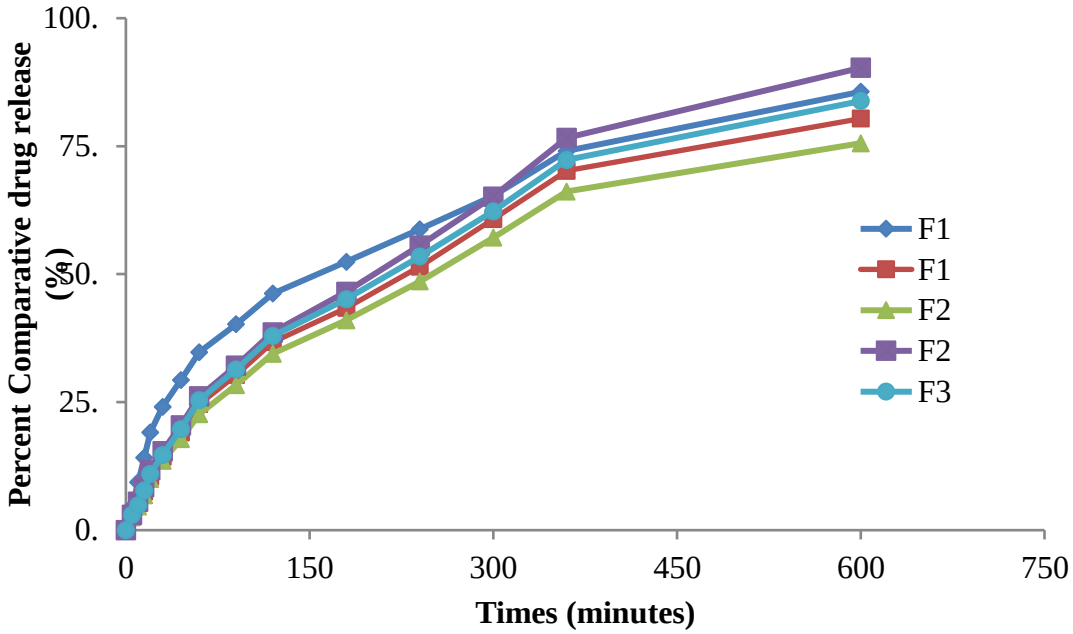

Figure 11: *In-vitro* Comparative drug release fof various nanoformulations (F1-F5).

Journal of Chemical Health Risks
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JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

In-Vitro Anticancer studies

Evaluation of Cell proliferation using MTT Assay

By using the MTT test for 24 hours, 48 hours, and 72 hours, it was possible to measure the cell proliferation of U937 & KG-1 after treatment with the suggested concentration of the drug AZT Nanoemulsion (3 µM to 15 µM).We saw that chemicals had effects that varied with time and dosage. Figure 3 shows that there was no discernible difference between 48 and 72 hours.The synergistic effects of the chosen doses of AZT Nanoemulsion (8 µM for KG-1 and 6 µM for U937) were assessed.According to our research, AZT Nanoemulsion dramatically decreased cell growth in both the U937 & KG-1 cell lines when compared to the control group.

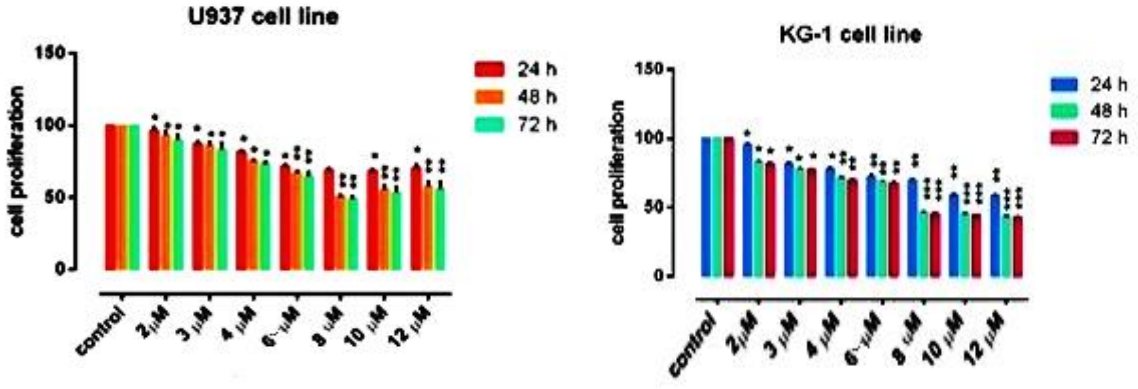
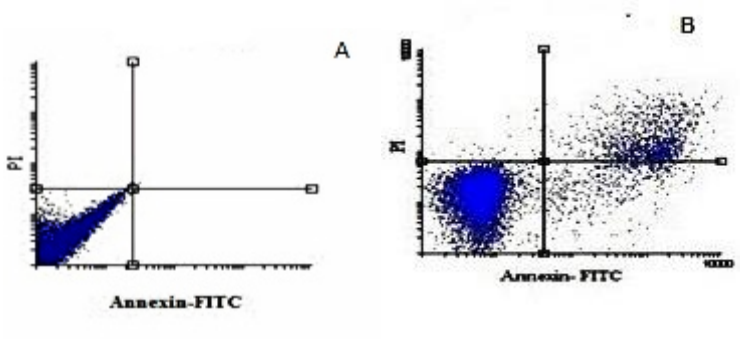


Figure 12: Cell Proliferation.

Apoptosis Assay

To examine the effects of the suggested drugs on apoptosis, we carried out a flow cytometry test using Annexin V FITC/PI labelling.In both the U937 & KG-1 cell lines, we found that treatment reduced the proportion of necrosis and increased the number of early cells that were apoptotic (Annexin +/PI-), as shown in Figures 13.A considerable increase in apoptotic cells was also seen in the AZT Nanoemulsion (65% in KG-1 & 75% in U937).In comparison to the control groups, the treated KG-1 (AZT Nanoemulsion 8 µM) and U937 (AZT Nanoemulsion 6 µM) cell populations had statistically notablely higher percentages of apoptotic cells.



Journal of Chemical Health Risks
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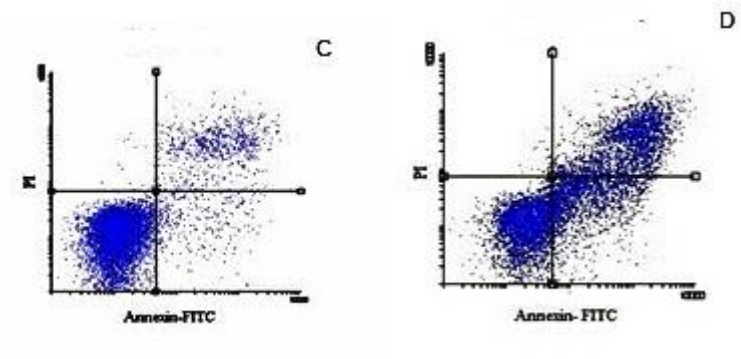

Figure: 13 A: U937 Control, B: U937 AZT Nanoemulsion, C: KG-1 Control, D: KG-1 AZT Nanoemulsion

Cell Cycle Assay

To learn more about cell cycle development, DNA present in KG-1 & U937 cells was assessed throughout the cell cycle.In the current investigation, cells subjected to AZT Nanoemulsion for 48 hours caused a significant rise in the number of cells in the G0/G1 phase after treatment with U937.Our findings further showed that AZT Nanoemulsion raised the hypodiploid partially G0/G1 DNA fraction in a dose-dependent manner (1.97% - 9.1% for KG-1 cells and 8.32% – 16.85% for U937 cells, respectively), indicating an increase in the apoptosis population **(**Figures 14).

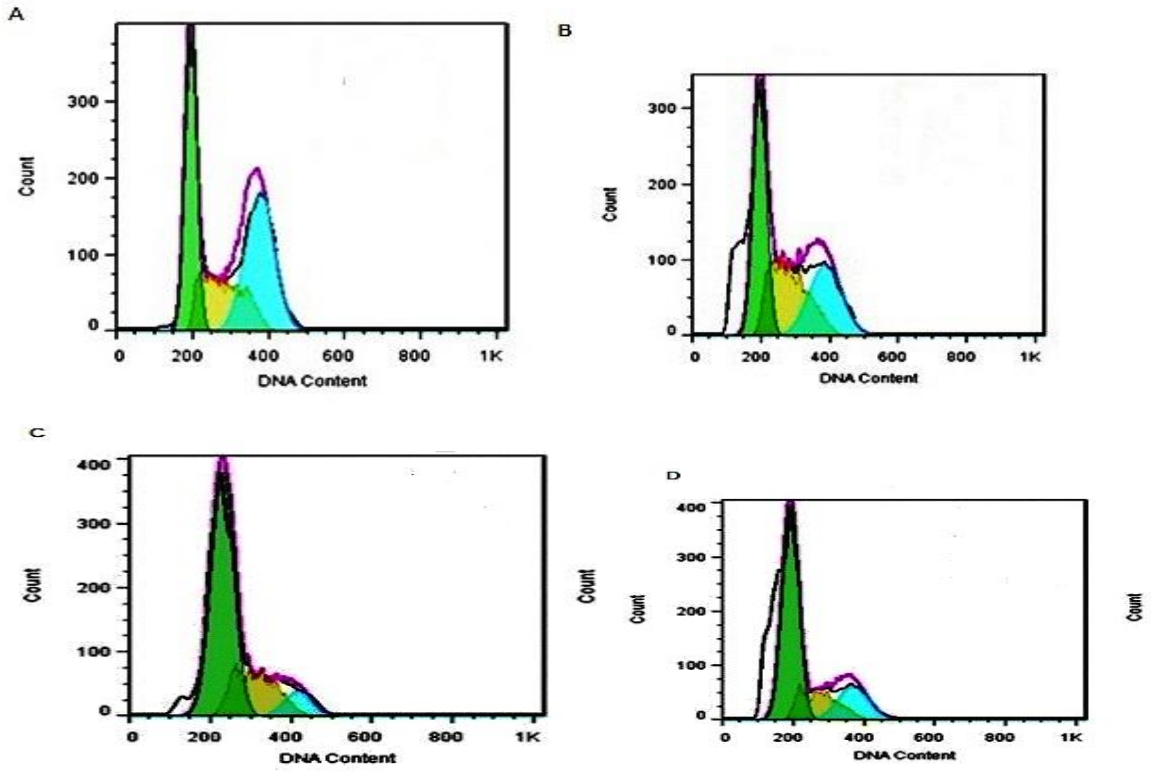

Figure: 14 A: KG-1 Control, B: KG-1 AZT Nanoemulsion, C: U937 Control, U937 AZT Nanoemulsion

Journal of Chemical Health Risks
www.jchr.org
JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

Stability study

Mechanical stress study

Through centrifugation at 2000 revolutions per minute under mechanical stress Under certain circumstances, the long-term stability of the various nanoemulsion compositions was evaluated.The investigations showed that after approximately 60 minutes of centrifugation at 2000 rpm, the compositions remained unchanged

Table 15: Stability study by mechanical stress method.

Centrifugation Time (Minutes)	Phase separationupon centrifugation				
	F1	F2	F3	F4	F5
10	---	---	---	---	---
20	---	---	---	---	---
30	---	---	---	---	----
40	---	---	---	---	---
50	---	---	---	---	---
60	---	---	---	---	---

(---): No significant change

Stability study

The formulations were stored at 4±1°C, 25±1°C, and 40±1°C for 1, 10, 20, and 30 days, respectively, to determine the extent of separating the phases or any physical modification, as part of an increased temperature study to evaluate the durability of the compositions at various temperatures.

Table: Stability study

Temperature/ Days	Phase separation %				
	F1	F2	F3	F4	F5
5±1°C					
1	---	---	---	---	---

Journal of Chemical Health Risks
www.jchr.org
JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

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

Conclusion:

According to the results of the current study, a high-speed homogenization process was effectively used to create a Azacitidine Nano emulsion formulation for solubility augmentation.Nowadays, nano emulsion is increasingly commonly used as a drug delivery system's carrier system.A promising carrier system that included polysorbate 80, oleic acid, and transcutol P (co-surfactant) was employed to include azacitidine. Polysobate 80 and transcutol P work well together and aid in the solubilization of the medication during the manufacturing process ofthenanoemulsion.An analysis revealed a 100% drug content. Zeta potential data showed that the final formulation included globules that ranged in size from 13.89 nm and had high stability. In-vitro drug release data showed that the final formulation was 90.30 % and the stability of the nanoemulsion formulation was very good.

In the current in vitro investigation, we looked at how AZT Nanoemulsion affected leukemia cell lines and reported for the first time their synergistic anti-oncogenesis in hematologic myeloid neoplasms.First,

Journal of Chemical Health Risks
www.jchr.org
JCHR (2024) 14(2), 3536-3554 | ISSN:2251-6727

using the myeloid leukemia cell lines U-937 and KG-1, we looked at how AZT Nanoemulsion affected cell growth.According to earlier research, AZA inhibited KG-1 and U-937 cells in a concentration- and time-dependent manner.Last but not least, similar to solid tumours, the AZA Nanoemulsion provides important benefits in growth suppression over the usage of either medication.In the apoptosis investigation, we found that AZT nanoemulsion therapy significantly raised the levels of apoptosis. In addition, Given that it has been shown that AZT nanoemulsion may also function to block histone deacetylases, we propose that it may one day be employed as a therapeutic option in myeloid leukaemia cell lines.

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