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In-Vitro Anti Cancer Activity Of N-Benzylidene Aniline

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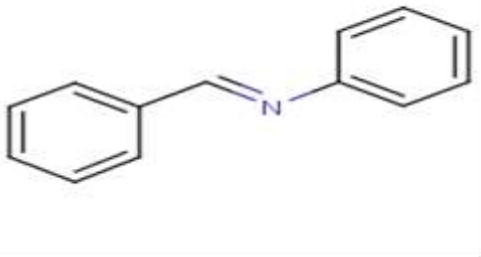
Abstract:
This study aims to describe the synthesis, characterization and determination of anticancer activity of N-Benzylidene aniline. Melting point, IR and UV spectral data were confirm the structure of compound and anticancer activitiy was characterized by the nature of biological activities. Colon cancer remains one of the leading causes of cancer-related deaths worldwide. Despite advancements in treatment modalities, there is a continuous demand for novel therapeutic agents with enhanced efficacy and reduced toxicity. This project aims to explore the potential anticancer properties of N-Benzylidene Aniline (NBA) on the HT-29 colon cancer cell line through in-vitro experiments. NBA, a compound with reported pharmacological activities, will be evaluated for its cytotoxic effects, apoptotic induction, cell cycle modulation, and mechanistic insights on HT-29 cells. The findings from this study could provide valuable insights into the development of novel therapeutic strategies for colon cancer treatment.The anticancer activity of the prepared compound was employed by using the In- vitro MTT Assay method.
Keywords: *N- Benzylidene aniline, anti cancer activity, MTT- Assay method*

INTRODUCTION:

N-Benzylidene aniline (X-CH=N-Y) consists of two phenyl ring such as benzaldehyde (X) and aniline (Y) moiety. N-Benzylidene aniline can be formed the azomethine group(-CH=N-) by the condensation of aniline and benzaldehyde under specific conditions[1,2].The structure of N-Benzylidene aniline is given below,



Structure of N-Benzylidene Aniline

N-Benzylidene aniline like Schiff base having azomethine group((-CH=N-) play an important role in biological, analytical , industrial and pharmacological activity. In synthetic organic chemistry, N-Benzylidene aniline is used in polymer stabilizers and act as intermediates [3-6].

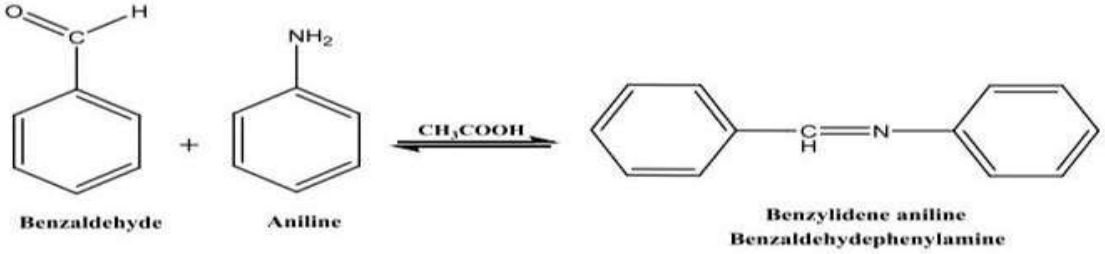
Experimental

Materials:

All the chemicals used are of AnalaR grade. Sodium hydroxide, Ethylalcohol, Benzaldehyde, aniline are used. Water used as a solvent. AnalaR grade reagents are used for the preparation of N-benzylidene aniline. The physical constant of this compound is characterized by Thomas Hoover capillary melting point Instrument. All other chemicals were used as AnalaR grade and purity was checked with comparison of standard physical constants.

Preparation of N- benzylidene aniline:[7]

Benzylidene aniline is prepared by refluxing equimolar quantities of benzaldehyde and aniline in ethanol for about 2-3 h.The resulting mixture is cooled to room temperature and stream into ice-cooled water with constant stirring.The precipitate is filtered off, soaked with ethanol and then dried. The product is recrystallized with ethanol.The purity of benzylidene aniline is verified by determining its melting point(53°C) and comparing the literature melting point (53°C)



Characterization of N- benzylidene aniline:

The synthesized compound have been characterized by UV, IR H^1 NMR and C^{13} NMR Spectral analysis. The UV –Visible Spectrum were obtained by Perkin Elmer LAMBDA 365+ double-beam UV/Vis spectrometer. The FT-IR spectra were obtained using the BRUKER ALPHAFT-IR MB 102 spectrophotometer, in the 4000-400 cm^{-1} region on KBr pellets. The ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were performed on the BRUKER ADVANCE spectrometer, using dimethylsulfoxide ($(CD_3)_2SO$).

Anti cancer Activity of N- benzylidene aniline:

The anticancer activity of N-Benzylidene Aniline (NBA) was evaluated by *in-vitro* MTT Assay Method from the Royal BioTech laboratory, Chennai. MTT Assay Method, investigated the Colon Cancer (HT -29) Cell line activity.

In-Vitro Anticancer Activity (Colon Cancer (Ht29) Cell Line) of MTT assay Method:

(i) Preparation of Minimal Essential Media:(MEM)

Minimal Essential Media(MEM) was prepared by the weighing of 9.5 grams of MEM dissolved in 950ml of pre-sterilized double distilled water and mixed well. After, 2.2 grams sodium hydrogen carbonate is dissolved in 50ml of pre-sterilized double distilled water mixed well. Both these mixtures were sterilized at 15 lbs, 121 °C, for 15 min. and allow to cool down at room temperature. Then, 0.3grams of L-glutamine was weighed and dissolved in 10ml of pre-sterilized double distilled water and poured in to the above mixture. About 1mg of Antibiotics – streptomycin, pencillin G and amphotericin B was weighed. Each antibiotic was dissolved separately in 1ml of pre-sterilized double distilled water. pH was checked and adjusted to 7.2 - 7.4 with Diluted HCL. All the above contents were filtered in syringe and stored at 4^0C.

(ii) ***Preparation of 10% Fetal Calf Serum (FCS)***

10ml of ***Fetal Calf Serum*** FCS was filtered in syringe and added 90 ml of MEM media. One aliquot of the prepared MEM is kept for 2 days at 37 °C and checked for sterility, pH drop and floating particles. Check the contamination and store at 4 °C.

(iii) Preparations of Trypsin, phosphate buffer saline(PBS), Versene, Glucose solution : (TPVG solution)

a) Preparation of Trypsin:

0.14 g of trypsin was accurately weighed and dissolved in 10 ml sterile distilled water for half and hour and then filtered through membrane filter. After Stored at -20 °C.

b) Preparation of PBS (phosphate buffer saline)

8 grams sodium Chloride, 0.2grams potassium chloride, 1.15grams disodium Hydrogen Phosphate and 0.2grams potassium dihydrogen phosphate were dissolved in 1000ml of double distilled. In this solution, pH was maintain at 7.2.Finally PBS solution was stored at room temperature.

b) Preparation of 0.2% EDTA (Versene)

200 mg of EDTA was accurately weighed and dissolved in 10 ml sterile distilled water. Then the soution was autoclaved at 15 lbs / 15 min.

c) **Preparation of *Glucose***

0.5g of Glucose was accurately weighed and dissolved in 5 ml of sterile distilled water. Then

the soution was filter through Whatman filter paper and the soution was autoclaved at 15 lbs / 15 min.

TPVG solution make up to 100mL

84 ml of PBS,10 ml of trypsin, 10 ml of 0.2% EDTA, 5 ml of glucose and 0.1 ml of Penicillin & Streptomycin were added and add antibiotics streptomycin (1mg/ml stock). In this solution distributed in 5mL aliquots and Store at -20 °C.

MTT

MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide =5mg/ml in 1XPBS

SUB CULTURING OF CELL LINES

Bring the (MEM)medium and TPVG to room temperature. Observe the tissue culture flask for growth, cell degeneration, pH & turbidity. Select the flask for splitting.

The following procedure is followed in sequence.

(i) Wipe the mouth of the flask with cotton soaked in spirit.
(ii) Discard the medium and wash the cells with MEM medium for twice
(iii) Add 4ml of TPVG (pre-warmed to 37°C) over the cells.
(iv) Allow TPVG to act for 1-2 minutes
(v) Discard the TPVG and add 5ml of 10% MEM
(vi) Break off the cell clusters by gently pipetting back and forth with pipette (Passaging the cells).
(vii) Add 20ml of growth medium to tissue culture flask and transfer the cells into 96 well plates

RESULTS AND DISCUSSION:

The synthesized N-Benzylidene Aniline (NBA) was characterized by the following analytical assays

- Electronic spectroscopy
- FT –IR Spectroscopy
- H^1 NMR Spectrocopy &
- C^{13} NMR Spectroscopy

Electronic Spectroscopy:

The electronic spectra of N- benzylidene aniline in UV visible region was obtained in DMSO using a spectrometer in the range of 200-800 nm .In Figure 1the electronic spectra of benzylidene aniline show absorption bands at 211,228 and 276 nm. These bands explain the n→π* and π→ π* transitions of the present azomethines , chromophore group and aromatic ring. An extra absorption band was observed above 400 nm in the electronic spectra of the benzylidene aniline.

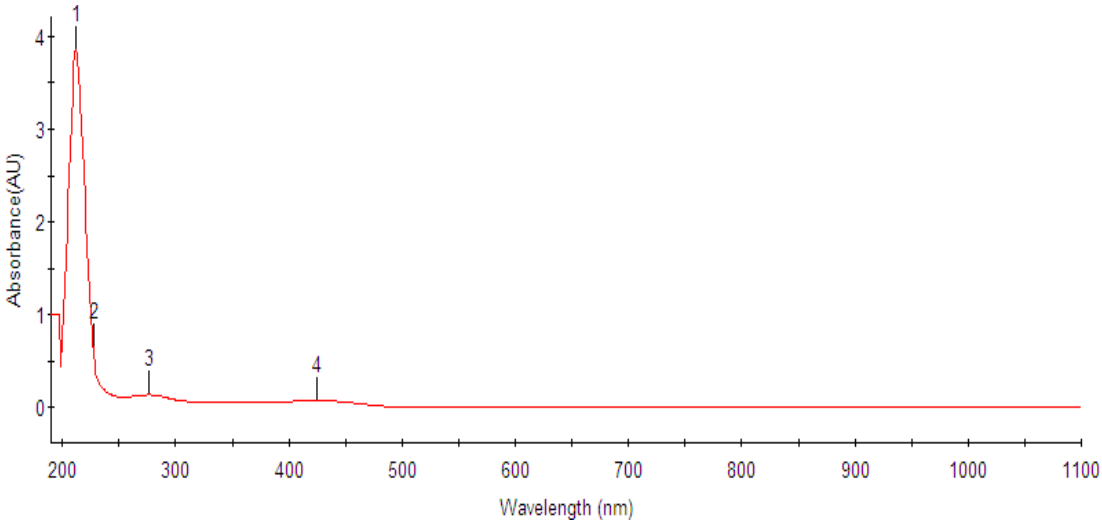


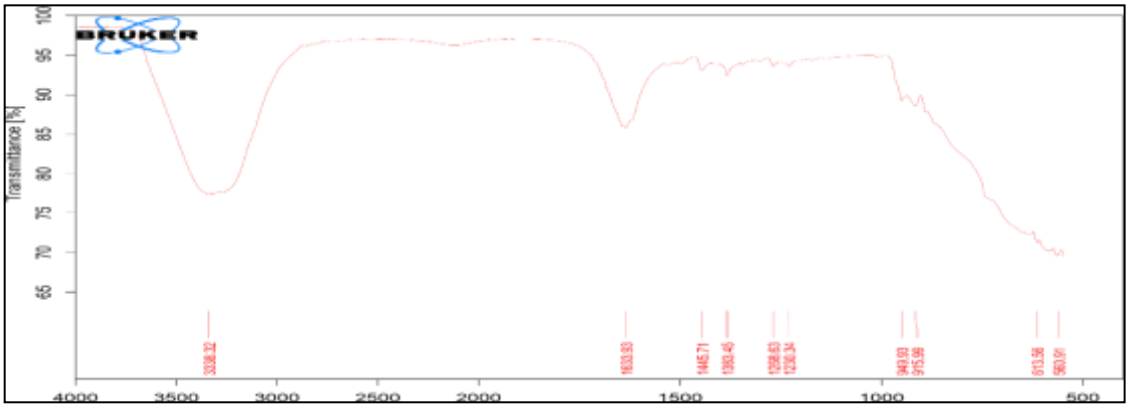
Figure-1-Electronic Spectrum of N-Benzylidene Aniline (NBA)

IR spectroscopy:

In Figure 2 explain the infrared (IR) spectrum, the compound contains several functional groups. First, there is a strong peak at 3338.32 cm^{-1}, indicate the nitrogen-hydrogen (N-H) bond. Additionally, a peak at 1633.93 cm^{-1} suggests the presence of a carbon-nitrogen double bond (C=N).Further the peaks at 1445.71 cm^{-1} for C-C bonds in an aromatic ring and at 1268.63 cm^{-1} for stretching vibrations of C-C bonds[8-11] (Table .1)

Table:1 FT-IR Spectral data of N-Benzylidene Aniline (NBA)

Functional groups	IR Frequency (cm^{-1})
N-H	3338.32
C=N	1633.93
C-C (aromatic)	1445.71
C-C (stretching)	1268.63
Ar-H (bending)	613.56



Wavenumber cm-1

Figure-2-FT-IR Spectrum of N-Benzylidene Aniline (NBA)

***H^1 NMR spectroscopy*:**

The N- benzylidene aniline proton NMR in Figure 3 shows highly de-shielded singlet absorption at δ (8.617 ppm 1H) which can be attributed to the azomethine proton (HC=N-). The region of δ (7.239–7.968 ppm, 10 H) with multiple signal absorption were assigned to chemically non equivalent aromatic protons (Ar-H).(Table -2)

Table:2 H^1 NMR Spectral Data of N-Benzylidene Aniline (NBA)

Proton Environment	Chemical Shift (*ppm*)	Splitting Pattern	Integration
azomethine proton(HC=N-).	8.617	Singlet	1 H
Ar-H (benzene ring)	7.239 – 7.698	Multiplet	10H

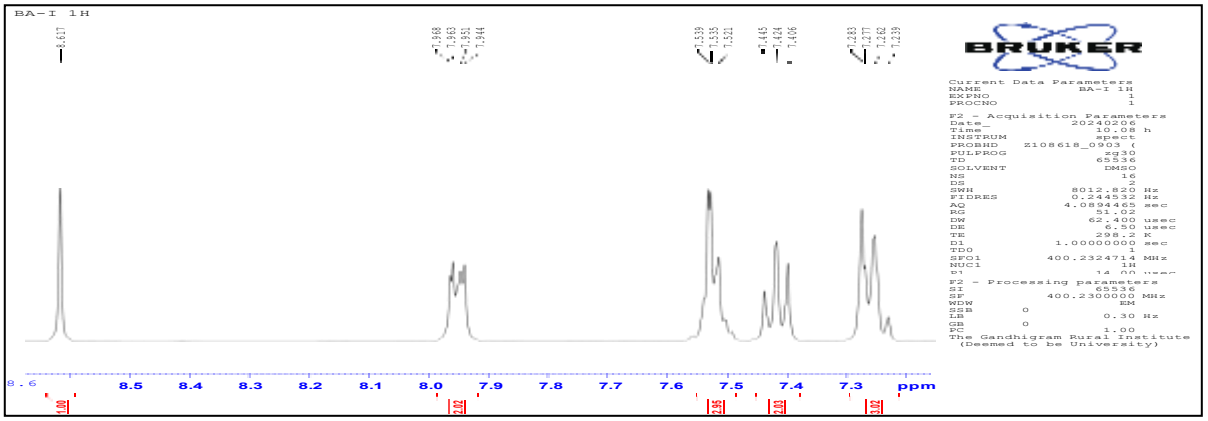


Figure – 3- H^1 NMR Spectrum of N-Benzylidene Aniline (NBA)

***C^{13} NMR spectroscopy*:**

In Figure 4, explain the the carbon atoms within the benzene ring were observed in the range of δ 114.36-136.49 *ppm*, reflecting their chemical environment within the aromatic system.(Table.3).

Table:3 C^{13}NMR Spectral Data of N-Benzylidene Aniline (NBA)

Carbon Environment	Chemical Shift (*ppm*)
Ar-C (benzene ring)	114.36-136.49

The ^{13}C spectra recorded on dimethyl sulphoxide (DMSO) in Figure 4 showed a signal was assigned to the azomethine carbon atom (CH=N). The chemically non equivalent aromatic ring carbon atom of the N benzylidene anline furnished resonance peaks at chemical shift of 114.36, 121.45,126.45,129.15,129.28 129.67,131.94,136.49,151.94 & 161.14 ppm and quaternary carbon atoms which were parts of the rings and adjacent to the azomethine carbon and nitrogen as well, provided low intensity signals at δ of 136.49 and 151.94 ppm.

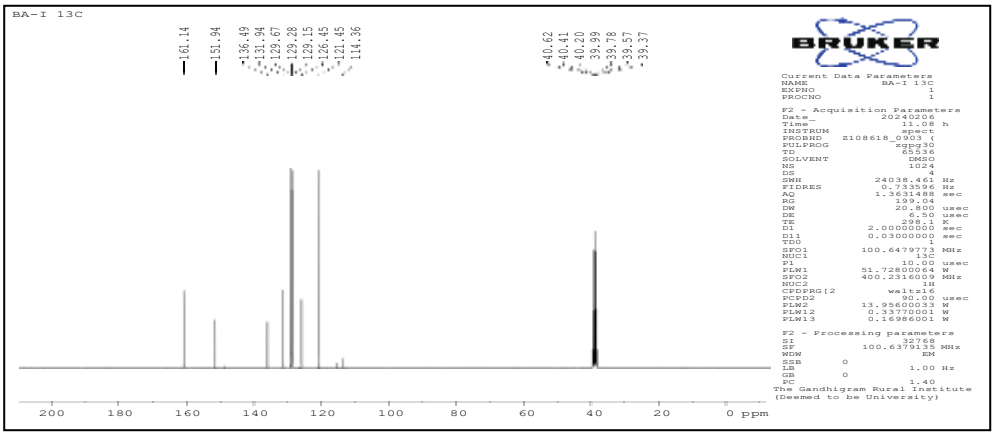


Figure -4- C^{13}NMR Spectrum of N-Benzylidene Aniline (NBA)

Biological Activity of N- Benzylidene aniline:

Anticancer activity of N-Benzylidene Aniline (NBA):

MTT assay

An MTT assay was performed to determine the anticancer activity of N-Benzylidene Aniline. Prominent morphological aberrations were clearly noticed, and this is indicative of cancer cell growth inhibition and cell death after 48 h of N-Benzylidene Aniline when compared to the untreated (control) cell.

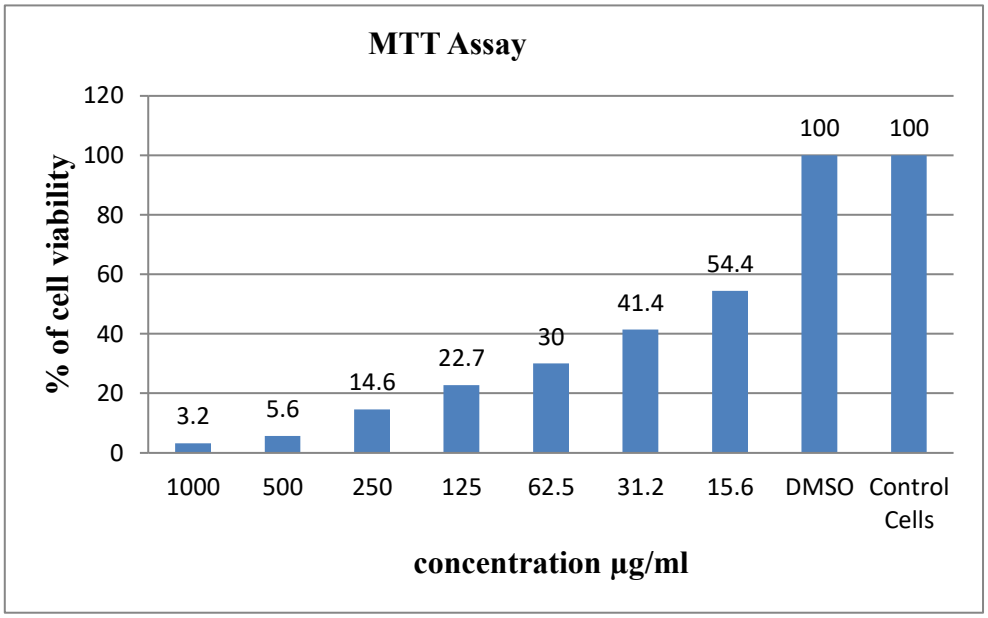
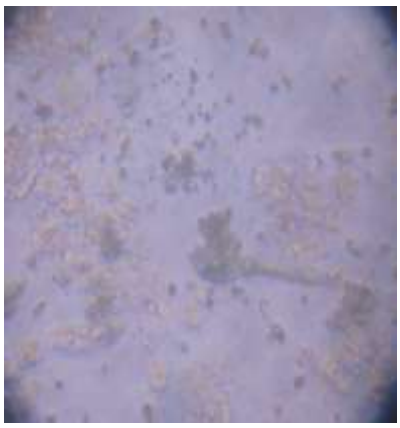
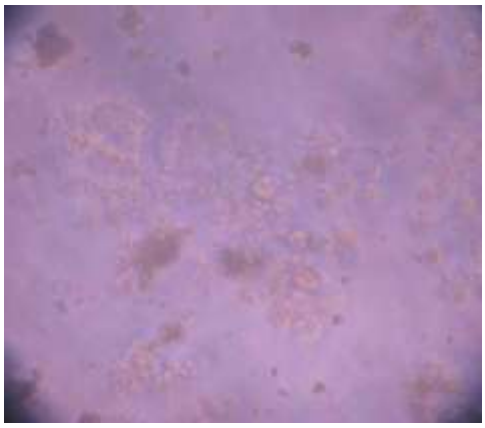


Figure -5-Anticancer activity of NBA by MTT assay Method

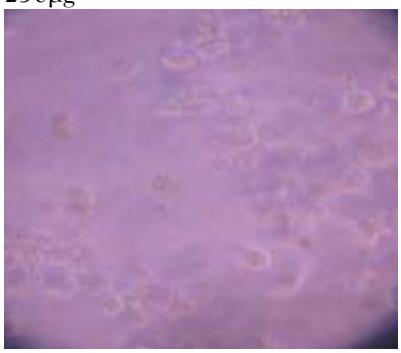
The cancer activity of samples on HT29 cells were determined by the MTT assay[12].(Figure .5) Cells (1 × 10^5/well) were plated in 0.2 ml of medium/well in 96-well plates. Incubate at 5 % CO_2 incubator for 72 hours. Then, added various concentrations of the samples in 0.1% DMSO for 48 hrs at 5 % CO_2 incubator. View the images under Inverted microscope 40X and take the photos. After removal of the sample solution and 20µl/well MTT reagent was added. Incubate at dark for 4to 6 hrs. After incubation add 1 ml of DMSO.(Figure .6) Viable cells were determined by the absorbance at 540nm. 50% inhibition of cell viability (IC50) value was determined graphically.

1000µg 500µg

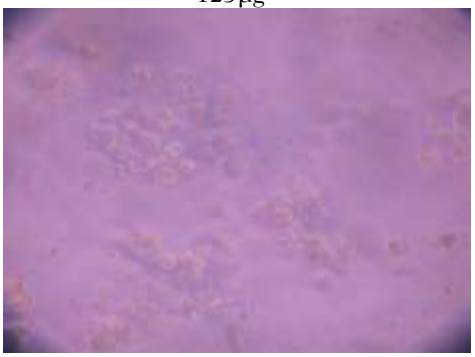




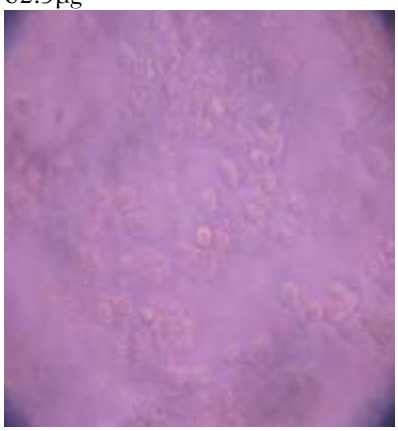
250µg



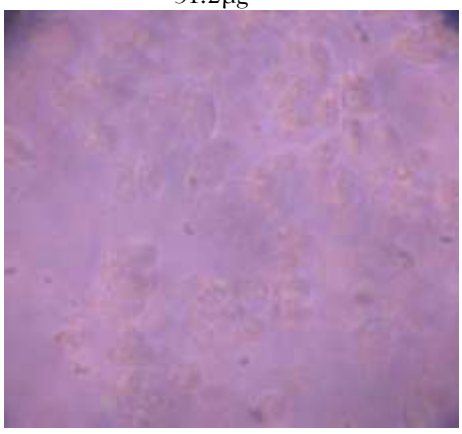
125µg



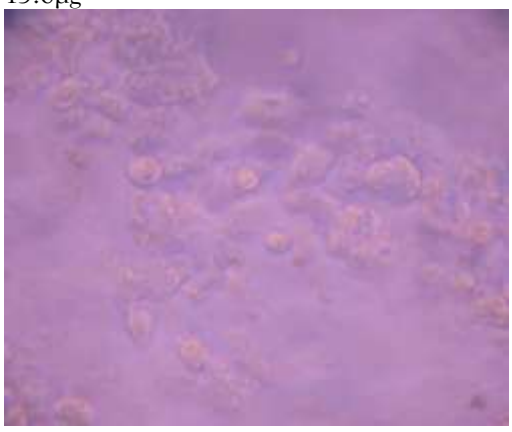
62.5µg



31.2µg



15.6µg



DMSO

CONTROL

Figure -6-Morphological frame work of anticancer activity of NBA by different concentration

The effect of the samples on the proliferation of HT29 cells was expressed as the % cell viability, using the following formula:

Calculation

% cell viability = A540 of treated cells / A540 of control cells × 100%

In Table 4 explain the % inhibition of cell viability of N-Benzylidene Aniline. Concentration decreases % inhibition of cell viability of N-Benzylidene Aniline increases.(Table .4)

Table:4 % cell Viability Data of N-Benzylidene Aniline (NBA)

S.No	Concentration µg/ml	Absorbance 540nm	% cell Viability
1	1000	0.04	3.2
2	500	0.07	5.6
3	250	0.18	14.6
4	125	0.28	22.7
5	62.5	0.37	30
6	31.2	0.51	41.4
7	15.6	0.67	54.4
8	DMSO	1.23	100
9	Control Cells	1.23	100

Based on the results presented above, there was a clear dose-dependent response to N-Benzylidene Aniline. It is evident that N-Benzylidene Aniline is more effective in inducing cytotoxicity in HT29 colon cancer cells compared to the untreated control cells, with an IC50 concentration of 20.28µg

IC50 value of N-Benzylidene Aniline =20.28µg

CONCLUSION:

The synthesized compound was characterized using various analytical techniques including UV, FT-IR, H^1 NMR, and C^{13} NMR spectroscopy and Further evaluated for *in vitro* activity against colon cancer HT-29 cell line., this study lays the groundwork for future research aimed at harnessing N-Benzylidene anticancer properties for the benefit of patients with colon cancer.N-Benzylidene aniline demonstrates dose-dependent cytotoxic effects on HT-29 colon cancer cells.The compound induces apoptosis in HT-29 cells, suggesting its potential as an anti-cancer agent.

Acknowledgement

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