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Anticancer Activity and Green Synthesized TiO_2 Nanoparticles From Calotropis Gigantea Leaves Extract

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Abstract: ***Aim & Objective: Research the Anticancer Abilities of TiO_2 Nanoparticles Obtain using Calotropis Gigantea by liver cells (HepG2). Materials & Methods: Various plant materials for the synthesis of nanoparticles are considered in green technology. In this present study, reports the synthesis of Tio_2 nanoparticles that were synthesized from Calotropis gigantea leaf, and the formation of Tio_2 nanoparticles was observed with different time intervals. The characterization was recorded from scanning electron microscope, X-ray diffraction, Fourier transfer infrared spectra, DLS, UV-Vis spectrum. The supporting green synthesis and characterization of Tio2 nanoparticles. Result: The result is recorded from MTT assay and ETBR/AO Staining.***

Keywords: Calotropis gigantea, Tio2 Nanoparticles, MTT, ETBR/AO.

I. INTRODUCTION

Cancer are most serious health problems globally. Liver cancer is one of the maximum competitive occurrences of most cancers with a excessive fatality rate global huge[1]. Hepatocellular carcinoma (HCC) is the maximum common form of number one liver cancer, with cholangiocarcinoma accounting for the remainder. *C.gigantea* (Apocynaceae, Asclepedaceae) is usually used globally as a traditional medication for the treatment of several elements. *C.gigantea* plant is extensively grown in many countries in Africa, eastern Asia, and South-east Asia. Extracts from all elements of this plant had been shown to have an expansion of organic activities[2]. The findings of this studies may provide valuable statistics for the improvement of low-chance most cancers remedies based on plant extracts. The blended healing benefits of *C.gigantea* leaves extracts may be beneficial for destiny anticancer treatment regimens.



Plant of Calotropis Gigantea

A. Plant Profile

Plant name: Calotropis gigantea
Family name: Apocynaceae
Synonyms: Calotropis procera, Calotropis acia
Common name: Giant milkweed, Crown flower
Aakh Parts used: leaves.

B. Classification

Kingdom: Plantea

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Subkingdom: Tracheobionata
Superdivision: Spermatophyta
Division: Magnoliopsida
Subclass: Asteridea
Order: Gentianales
Family: Apocynacea
Subfamily: Asclepiadoideae
Genus: Calotropis
Species: gigantea, procera, acia

II. MATERIALS AND METHODS

A. Collection and Authentication of Plant

Calotropis gigantea was collected from Mallasamudram, Namakkal district, TamilNadu, India in the month of August, 2023. The plant material was identified and authenticated by Dr. D.Vijayakumar Assistant Professor, Mahendra Institue of Technology Namakkal, TamilNadu, India.

B. Preparation of Plant Extract

Calotropis gigantea have been washed 3 times with faucet water then double distilled water and dried for 10 days via using a hot air oven at 40^0C. The dried leaf had been grinded right into a excellent powder[3]. In 100ml of conical flasks, 10 grams of leaf powder were mixed with 100 mL of distilled water. The aggregate become boiled at 200℃ for 20 min. The extract turned into filtered via muslin cloth .The filtered extract become stored at 4°C within the refrigerator for further use.

C. *Bulk Synthesis of Tio2NPs Using Calotropis gigantea leaf*

10ml of calotropis gigantea leaf extract was mixed with Tio_2 Solution (90ml) in dark condition for 24hours. A change in the color of the solution from white to light yellow indicated the synthesis of Tio_2.

D. Characterization of synsthesized Tio2 Nanoparticles

The biosysthesized Tio_2NPs have been characterized consistent with the technique. The form and length of Tio_2 nanoparticles were determined by using Scanning electron microscopy (SEM)[4]. X-ray diffraction (XRD) is the main method for crystallographic characterization for bulk, nano and thin film materials[5]. Fourier transform infrared spectroscopy (FTIR) turned into used to signify the adjustments and the composition at the floor of the synthesized nanoparticles. The particle size have been measured by means of dynamic light scattering (DLS) the usage of a Zetasizer Nano ZS90[6]. UV-via spectra were measured using a UV-2450.

E. MTT Assay

1) *Principle:* MTT (3-4, 5 dimethylthiazol-2yl-2, 5-diphenyl tetrazolium bromide) assay, is based totally on the capability of a mitochondrial dehydrogenase enzyme of viable cells to cleave the tetrazolium rings of the light yellow MTT and form a dark blue colored formazan crystal which is essentially impermeable to cell membranes, therefore resulting in its accumulation within healthy cells[7]. Solubilization of cells with the addition of detergents (DMSO) results in the liberation of crystals which might be solubilized. The number of surviving cells is directly proportional to the level of formazan product created[8]. The color may be quantified using a multi-well plate reader.
2) *Materials Required:* Fetal Bovine Serum (FBS) and antibiotic solution have been from Gibco (USA), DMSO (Dimethyl sulfoxide) and MTT (3-4,5 dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide) (5 mg/ml) were from Sigma, (USA), DMEM medium, 1X PBS, (India)[9]. 96 well tissue culture plate and wash beaker have been from Tarson (India)[10].
3) *Procedure*

a) *Cell Culture:* Change liver cellular line have been purchased from NCCS, Pune and have been cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37^oC**.**
b) *MTT assay:* The Test sample was tested for *in vitro* cytotoxicity, using Change liver cells by MTT assay. Briefly, the cultured Change liver cells were harvested by trypsinization and pooled in a 15 ml tube[11].

Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (200 µL) into the 96-well tissue culture plate in DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with the Test sample in a serum-free DMEM medium[12]. Every sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After incubation, MTT (10 µL of 5 mg/ml) was added to each well and the cells were incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope[13]. Later, the medium together with MTT (220 µL) was aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate changed into shaken for 5 min[14]. The absorbance for each well have measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA) and IC50 value were calculated using Graph Pad Prism 6.0 software (USA).

Formula Cell viability % = Test OD/Control OD X 100

F. ETBR/AO STAINING

1) *Principle:* Fluorescent dyes with aromatic amino or guanidine groups, such as acridine orange (AO), interact with nucleotides to emit fluorescence. EtBr molecules intercalate inside the DNA double helix[15]. AO can molecule intercalate in double-stranded DNA or single-stranded DNA and RNA. One molecule of AO can also interact with one phosphate group of DNA or RNA to form an aggregated, or stacked, structure that emits pink fluorescence with the maximum wavelength at 650 nm. This fluorescent dye is impermeable through the cell membranes of viable cells and can be used as fluorescent indicators of dead cells. Acridine orange is a vital dye and will stain both live and dead cells[16]. Necrotic cells stain orange however have a nuclear morphology such that of viable cells, and not using a condensed chromatin[17]. Ethidium bromide (EtBr) is only taken up by cells when cytoplasmic membrane integrity is lost and stains the nucleus red. EtBr also dominates over AO. For that reason live cells have a normal green nucleus; early apoptotic cells have a bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus[18]. Ethidium re-emits there energy in the colour of yellow/orange light centered at 590 nm. The fluorescence of ethidium bromide in an aqueous solution is considerably lower than that of the intercalated dye.
2) *Materials required:* DMEM medium, Penicillin/Streptomycin antibiotic solution, Trypsin-EDTA have been purchased from Gibco (USA), EtBr, and Acridine orange also purchased from Sigma Aldrich (USA), Fluorescent Imaging System, (ZOE, Bio-Rad, USA).
3) *Procedure:*

a) Cell culture

Change liver cell line have been purchased from NCCS, Pune and was cultured in liquid medium (DMEM) supplemented for 10% Fetal Bovine Serum (FBS), 100 u/ml penicillin, and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37°C[19].

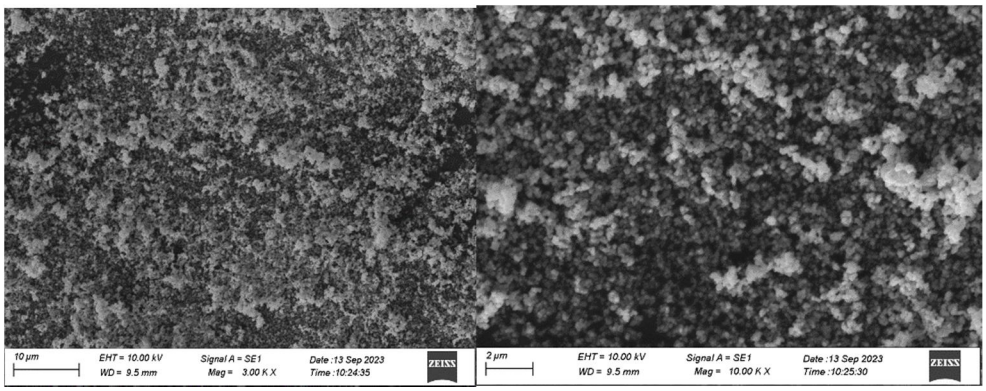
b) EtBr/AO staining

Briefly, 5 x 10^5 cells/ml of Chang liver cells were plated into a 96 well tissue culture plate and incubated for 24 hr in a DMEM growth medium. After incubation, the cells were treated with 44.85 µg/ml of TiO_2 sample in a serum-free DMEM medium[20]. The plate was incubated at 37°C at a 5% Co2 incubator for 24 hours[24]. After incubation, 10 µl of 1 mg/ml acridine orange and ethidium bromide were added to the wells and mixed gently[21]. Finally, the plate was centrifuged at 800 rpm for 2 minutes and evaluated immediately within an hour, and examined at least 100 cells by a Fluorescent Imaging System, (ZOE, Bio-Rad, USA).

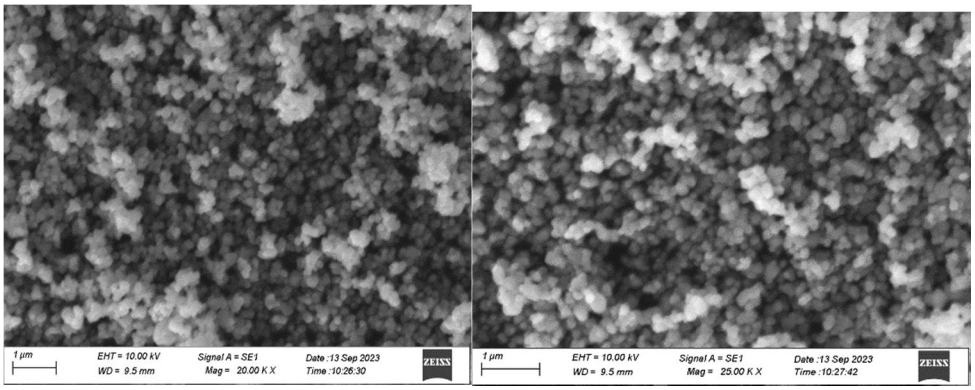
III. RESULTS & DISCUSSION

A. Characterization of synsthesized Tio2 Nanoparticles:

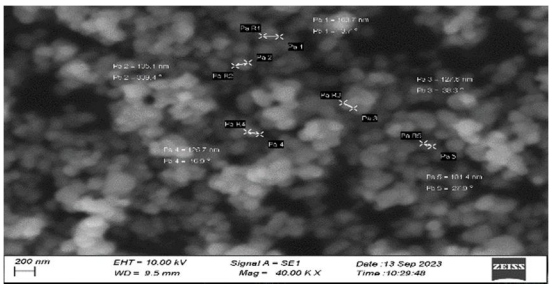
1) *Scanning Electron Microscopy (SEM):* In the SEM Pictures, a microscope is used to qualitatively identify the microstructural developments within the matrix of the stabilized soil specimens[21]. The SEM photos of clays are shown (Unique magnifications); thus, the microstructure is easily located because the pictures can be enlarged[22].



(a) ×3,000 magnification (b) ×10,000 magnification



(c) ×20,000 magnification (d) ×25,000 magnification



(e) ×40,000 magnification

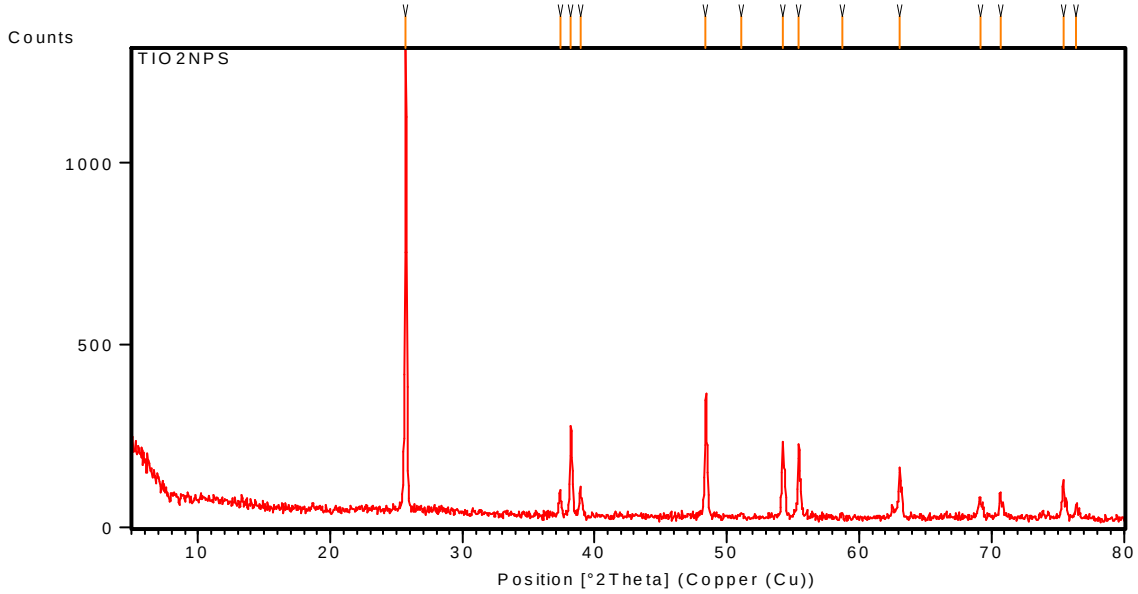
2) X-ray diffraction (XRD):

X-ray diffraction is used most frequently to investigate the structure of biocomposites with embedded nanostructure.

Measurement Conditions:

Dataset Name	TIO2NPS
Raw Data Origin	BRUKER-binary V4 (.RAW)
Scan Axis	Gonio
Start Position [°2Th.]	5.0000
End Position [°2Th.]	80.1600
Step Size [°2Th.]	0.0400
Scan Step Time [s]	13.4400
Scan Type	Pre-set time
Offset [°2Th.]	0.0000
Divergence Slit Type	Fixed
Divergence Slit Size [°]	9999.0000
Specimen Length [mm]	10.00

Receiving Slit Size [mm]	0.1000
Measurement Temperature [°C]	25.00
Anode Material	Cu
K-Alpha1 [Å]	1.54060
K-Alpha2 [Å]	1.54443
K-Beta [Å]	1.39225
K-A2 / K-A1 Ratio	0.50000
Generator Settings	30 mA, 40 kV
Diffractometer Type	Theta/Theta
Diffractometer Number	0
Goniometer Radius [mm]	240.00
Dist. Focus-Diverg. Slit [mm]	91.00
Incident Beam Monochromator	No
Spinning	No



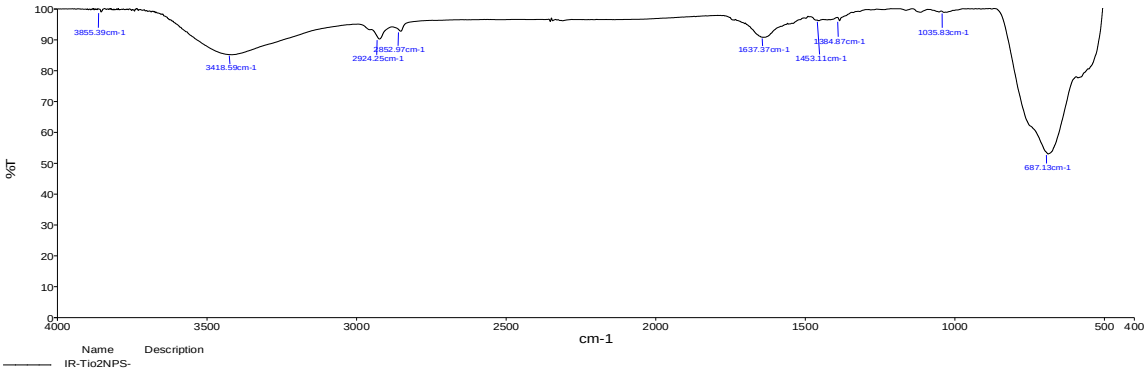
3) Peak List of XRD:

Pos. [°2Th.]	Height [cts]	FWHM Left [°2Th.]	d-spacing [Å]	Rel. Int. [%]
25.7039	1180.84	0.1181	3.46594	100.00
37.3352	68.06	0.1574	2.40859	5.76
38.1740	252.27	0.1574	2.35758	21.36
38.9627	79.99	0.1968	2.31165	6.77
48.4084	333.81	0.1968	1.88038	28.27
51.0752	10.33	0.4723	1.78829	0.87
54.2616	219.02	0.1574	1.69056	18.55
55.4423	202.34	0.1968	1.65733	17.14
58.7508	11.95	0.2362	1.57164	1.01
63.0669	139.36	0.1181	1.47408	11.80
69.1823	51.24	0.3149	1.35796	4.34
70.6650	75.06	0.1181	1.33306	6.36
75.4360	107.32	0.1181	1.26016	9.09
76.4292	40.18	0.3149	1.24624	3.40

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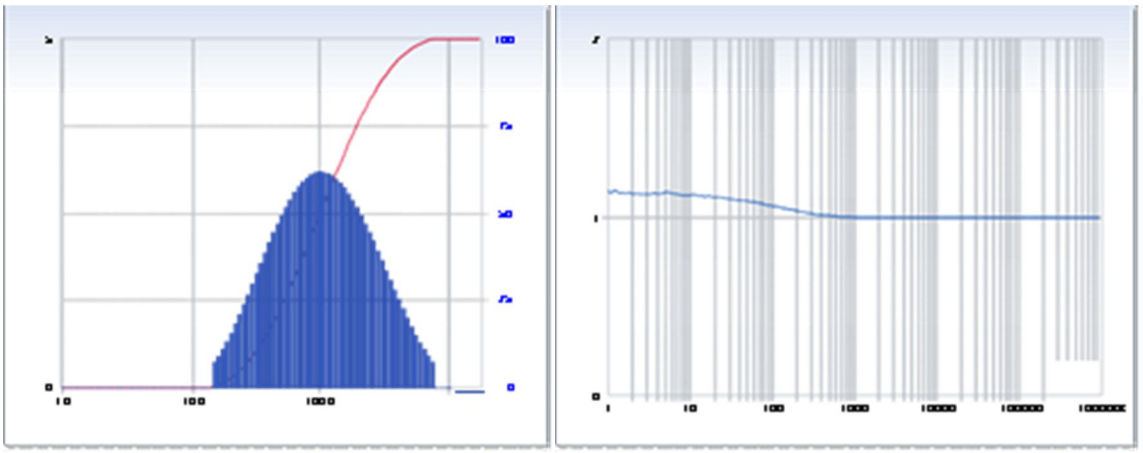
4) Fourier Transform Infrared Spectroscopy (FTIR):

FTIR spectroscopy is a very powerful tool with many applications, however data interpretation is not straightforward[23]. By nature, the total spectrum generated is a series function of absorbed energy response (hence the Fourier Transform portion of the name).



5) DLS (Dynamic Light Scattering):

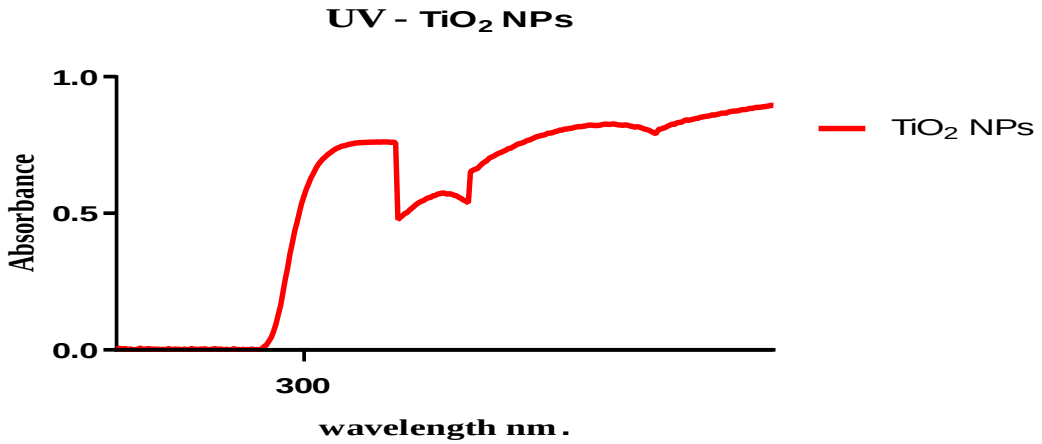
Dynamic Light Scattering (DLS) analysis allows us to confidently measure the size distribution profiles of particles in the sub-micron range.



(a) Intensity Distribution (b) ACF

6) Ultra Voilet (UV):

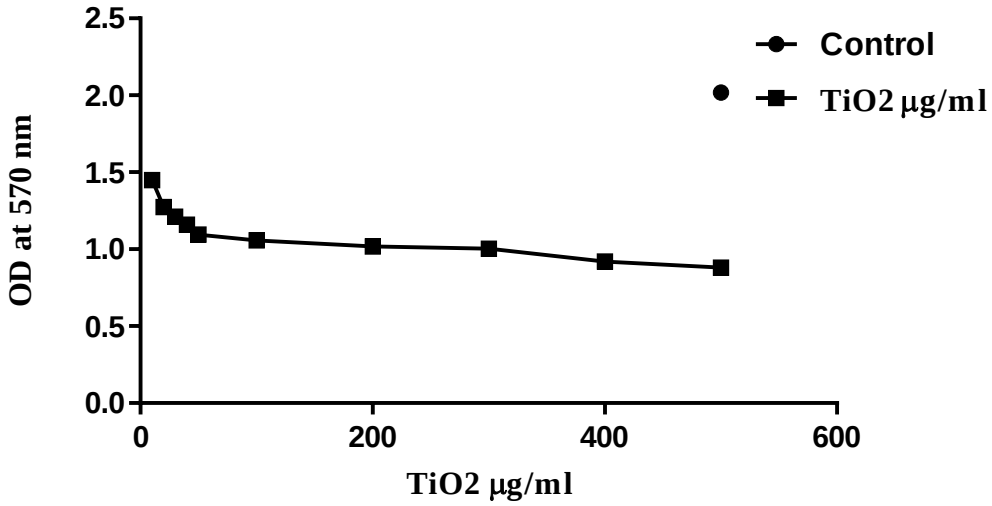
Titanium oxide nanoparticles produced diagnostic UV-Vis peaks at 350-365nm [25].



B. MTT ASSAY IN HEPG2 CELLS

1) OD Value at 570 nm

S. No.	Tested sample concentration (µg/ml)	OD value at 570 nm (in triplicates)		
1	Control	2.012	2.019	2.021
2	500 µg/ml	0.876	0.877	0.887
3	400 µg/ml	0.901	0.929	0.929
4	300 µg/ml	0.998	1.004	1.007
5	200 µg/ml	1.013	1.017	1.02
6	100 µg/ml	1.043	1.051	1.08
7	80 µg/ml	1.091	1.094	1.1
8	60 µg/ml	1.133	1.171	1.173
9	40 µg/ml	1.176	1.216	1.242
10	20 µg/ml	1.244	1.289	1.289
11	10 µg/ml	1.421	1.441	1.482

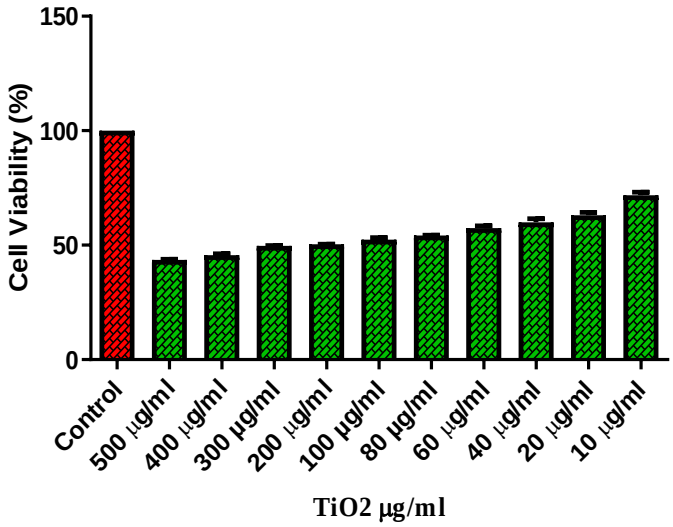


2) Cell Viability (%)

S. No.	Tested sample concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
1	Control	100	100	100	100
2	500 µg/ml	43.5388	43.4373	43.8892	43.621759
3	400 µg/ml	44.7813	46.0129	45.9673	45.587178
4	300 µg/ml	49.6024	49.7276	49.8268	49.718931

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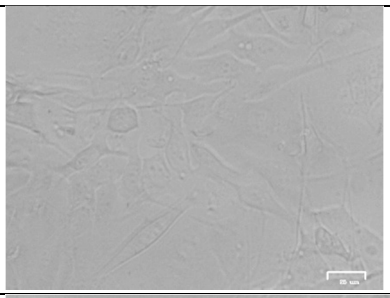
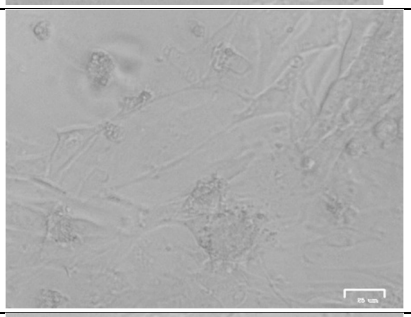
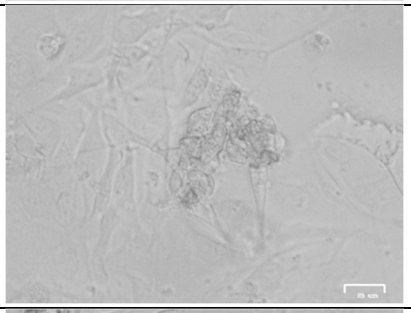
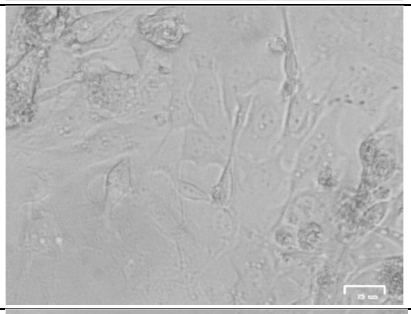
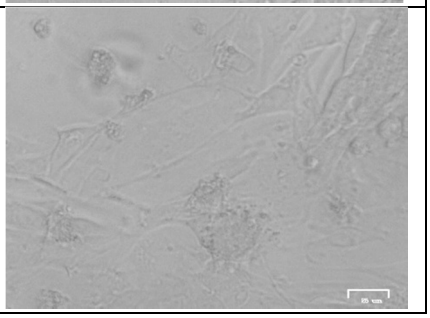
5	200 µg/ml	50.3479	50.3715	50.4701	50.396483
6	100 µg/ml	51.839	52.0555	53.4389	52.444444
7	80 µg/ml	54.2247	54.1852	54.4285	54.279464
8	60 µg/ml	56.3121	57.999	58.0406	57.45057
9	40 µg/ml	58.4493	60.2278	61.4547	60.043955
10	20 µg/ml	61.829	63.8435	63.7803	63.15094
11	10 µg/ml	70.6262	71.372	73.33	71.776081

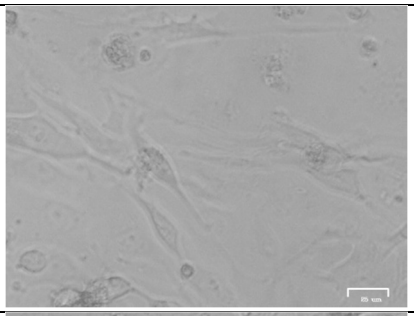
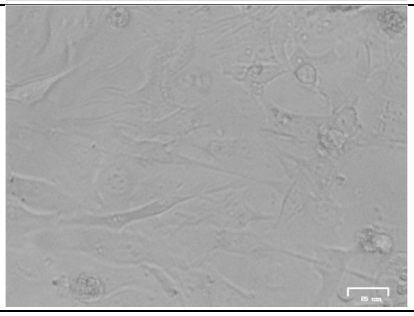


3) IC50 Value of tested sample is 44.85 µg/ml

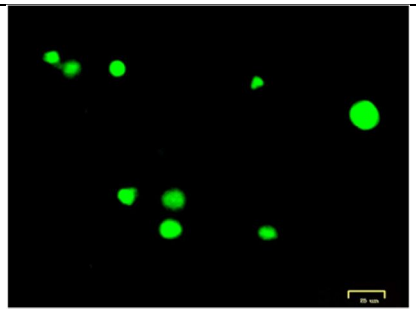
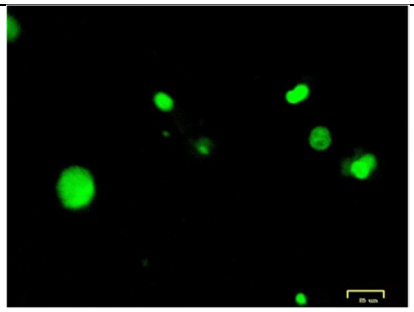
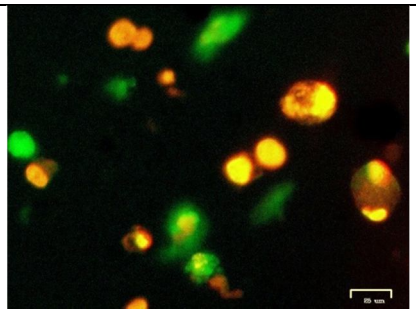
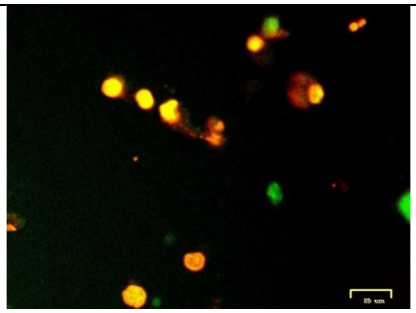
log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	1.652
HillSlope	-1.107
IC50	44.85
Std. Error	
LogIC50	0.03408
HillSlope	0.1028
95% CI (asymptotic)	
LogIC50	1.582 to 1.722
HillSlope	-1.318 to -0.8966
IC50	38.19 to 52.67
Goodness of Fit	
Degrees of Freedom	28
R squared	0.9189
Sum of Squares	2025
Sy.x	8.505
Number of points	
# of X values	30
# Y values analysed	30

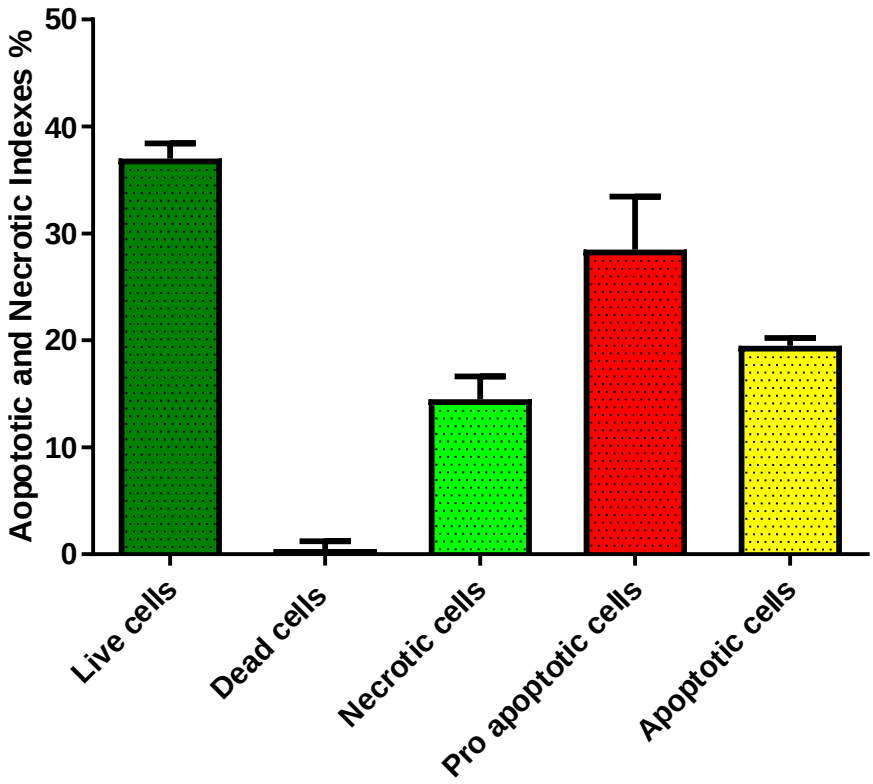
4) Images of control cells and treated cells.

Control cells	
TiO2 500 µg/ml	
TiO2 300 µg/ml	
TiO2 100 µg/ml	
TiO2 60 µg/ml	

TiO2 20 µg/ml	
TiO2 10 µg/ml	

C. ETBR/AO STAINING:

Samples	Fluorescent images	Fluorescent images
Control		
Treated with 44.85 µg/ml of TiO_2		



S.No	Dead cells	Necrotic cells	Pro-Apoptotic cells	Apoptotic cells	Live cells
1.	1	16	25	20	38
2.	0	13	32	19	36

IV. CONCLUSION

The present study discover that the extracts of Calotropis gigantea exhibited anticancer activity on HepG2 cellular lines. Thus the result further studies are needed to investigate and isolate its energetic compounds and also to assess its anticancer potential on other cellular lines.

V. ACKNOWLEDGEMENT

We acknowledge TRI-BIOTECH (Trichy Research Center) for characterization studies.

VI. CONFLICT OF INTEREST

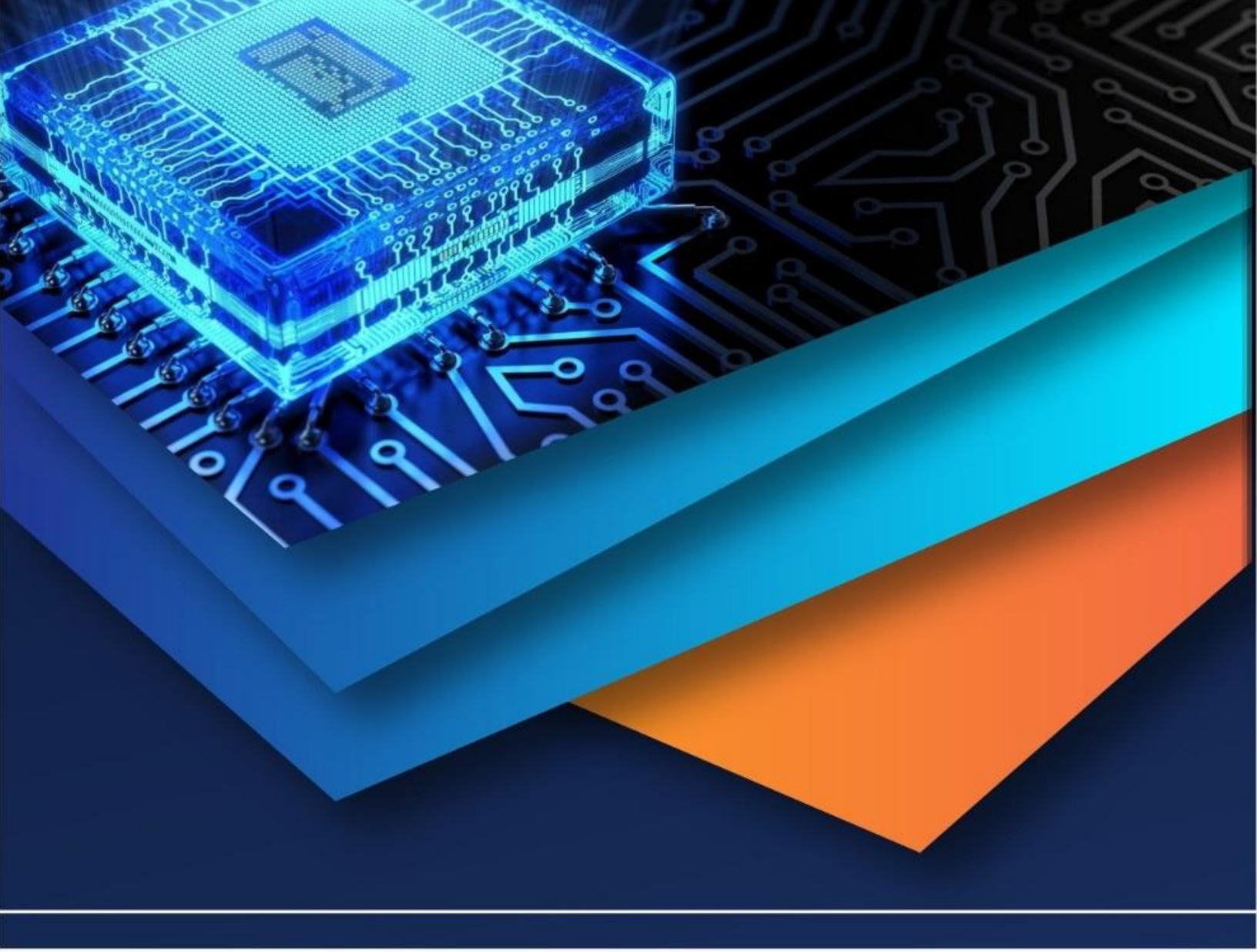
The authors declare that they have“ No Conflict of Interest”.

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