iJRASET
International Journal For Research in Applied Science and Engineering Technology

INTERNATIONAL JOURNAL FOR RESEARCH

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

Volume: 7 **Issue:** IV **Month of publication:** April 2019

DOI: https://doi.org/10.22214/ijraset.2019.4487

www.ijraset.com

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International Journal for Research in Applied Science & Engineering Technology (IJRASET)
ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor: 6.887
Volume 7 Issue IV, Apr 2019- Available at www.ijraset.com

Anti-Cancer Activity of Cantharanthus Roseus in HEp-2 Cell Line

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Abstract: ***Laryngeal cancer may also be called cancer of the larynx or laryngeal carcinoma. Traditional plants are a rich source of novel anticancer agents and still performing better role in health concern. The studied was estimation of the anticancer activity of the of Cantharanthus roseus in HEp-2 cell. The Plants Kingdom is still primary source of medicine. It has been proved to be more efficient to treat many diseases including the world's most terrifying disease tumor with nil or minimum side effects. Distinct studies have been carried out on medicinal plants of India; however, only some medicinal plants attract the interest of scientists. Consequentially with the preparation of medicinally useful compounds from ancient plant remedies there has been an extended interest in studying the pharmacological effect of the plant alkaloids. Cantharanthus roseus has been the presumption of interest for most of the Pharmaceutical scientists for more than two decades. Bearing of thought in mind the need for new therapies, particularly focusing on the tumor microenvironment and the effect of Cantharanthus roseus as an anticancer agent, in this study we evaluated the anticancer activity of Cantharanthus roseus in HEp-2 cell line. Till now many cancer cell lines such as lungs, prostrate, esophageal cancer cell lines are treated with the ethyl acetate extract of Cantharanthus roseus. Till, now no any result observed on HEp2 cell line. Now, we use ethanolic extract of Cantharanthus roseus for the treatment of cancerous HEp2 cell line. Cantharanthus roseus is of importance due to its anti tumor and anti diabetic actions.***
Keyword: MTT Bioassay, NRU Bioassay, HEp-2 Cell line culture, Cell viability, Gel documentation*.*

I. INTRODUCTION

Vinca alkaloids are an important class of antitumor agents, widely used in combination chemotherapy regimens for the remedy of leukemia and solid tumors[1][2]. There have been extremely researches on this class of agents, which at the starting to VBL2 and subsequently somewhat modifications to VBL2 molecule to VCR and VDS which have efficiently different clinical behavior. Nowadays, onto vinblastine chemist grafted amino acid derivative to facilitate transport of these large molecules[3]. This work led to the synthesis of molecules with interesting pharmacological properties, namely vinglycinate and vintryptol, which are structural analogues of vindesine[4] Taking into consideration the possibility of drastically modulating the toxicity and antitumor activity of these compounds by introducing relatively slight structural modifications, we synthesized a series of α aminophosphonate derivative of vinblastine[5][6]. The most active compound of this series, called S 12363, consists of an aminophosphonate, a bioisoster of the amino acid valine, grafted onto the vindoline moiety. In this paper, we present the in vitro cytotoxic activity and in vivo antitumor effect of this new vinca alkaloid derivative S- 12363, which has been tested both according to the usual and the most recent strategy (called disease- oriented strategy) adopted by the NCI. This work points to the high potency and stereo specifiity of this compound compared with its epimer S 12362, which differs only by the absolute configuration of the asymmetric carbon atom of the side chain[7]. Bearing in mind the need for new therapies, specially focusing on the tumor microenvironment and the potential of *Vinca rosea* as an anticancer agent , in this study we evaluated the *In vitro* anticancer activity of *Vinca rosea* in HEp-2 cell line for the treatment of laryngeal cancer[8] .

II. METHODOLOGY

A. Hep2 cell line Culture

The required cell line was acquired from the National centre for cell science, Pune, India. The cells were maintained in a CO_2 incubator with 5% CO_2 and 95% humidity, and supplemented with Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS). Penicillin and streptomycin (PAA) were also added to the medium to 1x final concentration from a 100x stock. Once the cells had attained confluent growth, the cells were trypsinized using trypsin -EDTA (PAA) and the number of cells required for carrying out several assays was seeded into sterile six- well plates and 96- well plates. In each well of the six well plates, a clean, dry, sterile cover slip was placed before the cells were seeded. Then the plates hydrogen peroxide (H_2O_2) at a concentration of 200µm was used as an oxidant. The concentration of plant extract used was 20mg. The cells were treated with the

oxidant, both in the presence and absence of the leaf extracts. The cells were exposed to H_2O_2 for 1 hour at 37ºc. The time points were arrived at by conducting a time related response analysis of each cell type. After treatment, the coverslips from the six-well plates were removed and placed on a glass slide and sealed with Vaseline. These slides were used for various staining techniques; however in 96-well plates, the medium was removed and replaced with fresh medium .These were used for checking the viability status.

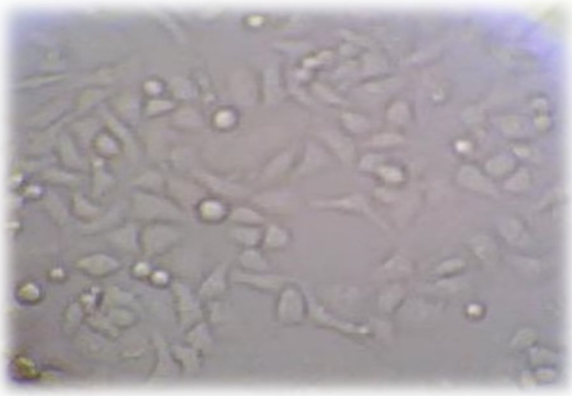

Fig.1. cell culture

B. Reagents

Minimum essential medium, FBS, Trypsin, 3 – (4, 5 – dimethylthiazol – 2- yl) – 2, 5 – diphenyltetrazolium bromide (MTT) , NRU and dimethyl sulfoxide (DMSO).

C. Preparation of Extracts

Plant leafs were collected from Biotech park Lucknow. This leaves dried at room temperature and then grind and powdered. 6gm plant material was loaded in the inner tube of Soxhlet Apparatus and then filtered into a round bottom flask containing 200 ml Ethanol. The solvent boiled gently over a water bath using the adjustable rheostat. The extraction was continue for 8 h and then solvent was removed at the reduced pressure with the help of vaccume pump distillation[9]

III. MTT BIOASSAY

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors[10]. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing[11]. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation. If you are familiar with the procedure and know the cell counts to use in your specific assay. You may follow this basic protocol. The first step of MTT bioassay protocol is plate cells at 1000 to 100,000 per well, after then incubate the cells for 6 to 24 hours. The next step is adding of 10 µL MTT Reagent in the suspension. After then incubate the cells for 2 to 4 hours until purple precipitate is visible. The next step in MTT bioassay adding of 100µl detergent reagent in the cell suspension culture. After then leave it at room temperature in the dark for 2 hours. After then record the absorbance of suspension medium at 570 nm[12].

IV. NRU BIOASSAY

Another method for quantitative estimation of viable cell is NRU (Neutral red uptake) assay. It contain neutral red which is a eurhodin dye which stains lysosomes red[13]. This can be used to as a vital stain. Live cells incorporate neutral red into their lysosomes. It is a weak cationic that diffuses through cell membranes and accumulates in cellular lysosomes. In the acidic environment of the lysosomes NU is oxidized by damaged, dye remain trapped in lysosomes. It is based on the ability of viable cells

to incorporate and bind the supra-vital dye neutral red in the lysosomes. Cells are seeded in 96-well plates and treated for appropriate period. Plates are then incubated for 2h with a medium containing neutral red. The cells are subsequently washed, the dye is extracted in each well and absorbance is read using a spectrophotometer. Assay can be completed in less than 3h. The absorbance values are used to determine the viability of each well by comparing the OD of each test material treated well compared the negative control wells. Harvest suspension cells by centrifugation. Adherent cells should be released from their substrate by trypsinization or scraping. Resuspend cells at 1 x 10^6 per mL Prepare serial dilutions of cells in culture medium from 1 x 10^6 to 1 x 10^5 cells per mL.Plate out, in triplicate, 100µL of the dilutions into wells of a microtiter plate. Include one control well of medium alone to provide the blanks for absorbance readings. Incubate the cells under conditions appropriate for the cell line for 6 to 48 hours (to recover from handling). The time required will vary but 12 hours to overnight is sufficient for most cell types. The plate is then incubated for 2h with a medium containing neutral red. The cells are subsequently washed, the dye is extracted in each well and absorbance is read using a spectrophotometer

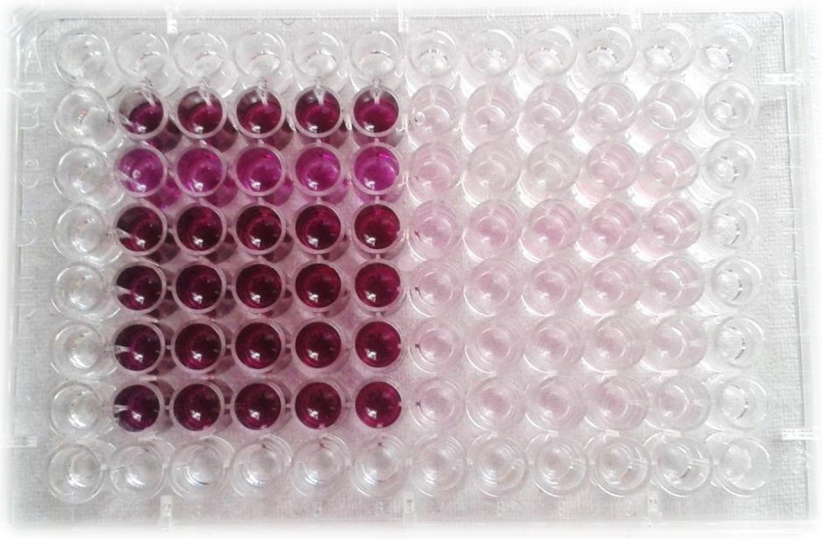

Fig. 2. MTT- NRU Assay

V. DNA FRAGMENTATION ASSAY

The ethanolic extract of vinca rosea which is treated with HEp-2, cell lines was passed to DNA fragmentation technique. A distinctive feature of apoptosis at the biochemical level is DNA fragmentation. This method was used as a semi quantitative method for measuring apoptosis[14][15].

A. DNA Isolation From Adherent cell Line

1) Completely remove all the media from 6 well plates and then add cell 200µl cell lysis buffer.
2) Scrap the cells and collect the cell lysate in a 1.5 ml eppendorf tube.
3) Add 2µl of RNase solution and incubate for 1 hour at 37ºc.
4) Add 2µl of proteinase and incubate for 1hour at 37ºc.
5) After incubation, add 500µl phenol: chloroform: isoamyl alcohol mixture and mix × properly.
6) Centrifuge at 10,000g for 10 min and collect aqueous phase supernatant.
7) Add 50µl, sodium acetate (3M) and 200µl isopropanol in the collected supernatant & incubate for 2 hours to overnight incubation at -20ºc.
8) Centrifuge at 10,000g for 10mins.
9) Wash the pellet in 70% ethanol.
10) Centrifuge at 10,000g for 10mins.
11) Discard the supernatant and air dry the pellet.
12) Dissolve the pellet in 100-200µl TE buffer.
13) Quantify DNA using nanodrop.

For long term storage of DNA storing at -20ºC is recommended

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B. Nanodrop Readings

Distilled water was taken for calibration and TE buffer was used to set as blank. 1µl of sample were taken from each tube and readings were taken in nano- drop. These nano - drop reading will help to estimate the amount of sample to be poured in gel electrophoresis.

1) Reading were taken as follow

	Concentration	Absorbance	260 nm	280 nm	260/280	260/230
C	26	0.286	0.521	0.271	1.92	1.82
T1	36.2	0.460	0.724	0.437	1.66	1.571
T2	24.2	0.264	0.483	0.267	1.81	1.83
T3	33.5	0.373	0.670	0.359	1.87	1.80
T4	124.1	1.421	2.483	1.531	1.62	1.75

Table1. Nano-drop reading

2) Calculation for pouring sample in wells of gel electrophoresis

	(C)Concentration observed in nano-drop (ng/µl)	(A) Volume of sample (µl) for 400 ng [(1/c)*400]	(B) Volume of dye (µl)[A/10]	Total volume [A+B]
C	26	15	1.5	16.5
T1	36.2	11	1	12
T2	24.2	16	1.6	17.6
T3	33.5	12	1.2	13.2
T4	124.1	3	0.3	3.3

Table2. Calculation

C. Agarose gel Electrophoresis

Gel is prepared by adding 1% agarose in 100 ml of 1X TAE buffer, buffer, then sample were loaded in a calculated fashion and then gel was run under 80V electric current . Then gel is placed under gel documentation unit to observe DNA fragment.[16]

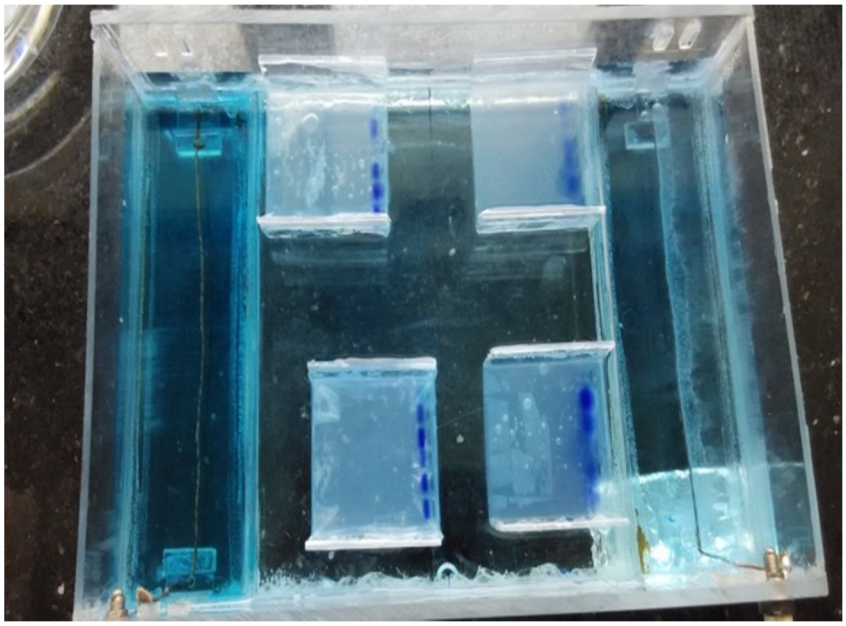

Fig .3. Agarose Gel electrophoresis

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VI. RESULT

A. MTT Bioassay

HEp-2 cells were treated with different concentrations of vinca extract (1, 10,100 and 1000µl per ml) and the viability was measured by the MTT reduction assay. Amoung the three concentrations, the 1000µg, showed the maximum growth inhibition. The cytotoxic effect of vinca extract on HEp-2 cells was analysed on an Optical microscope and presented below;

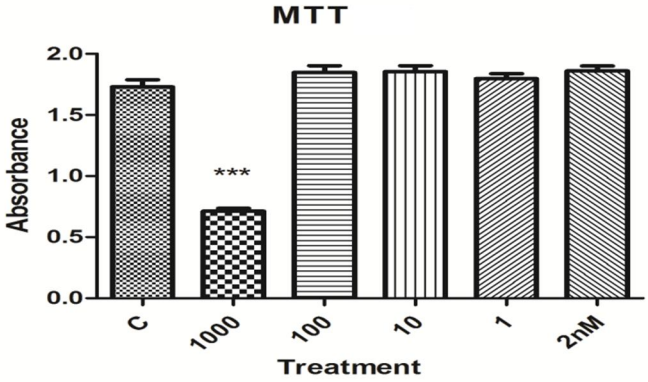

Fig.3. MTT Bioassay

B. NRU Bioassay

HEp-2 cell line was again treated with different concentration of vinca extract at different Concentrations and the viability was measured by the NRU reduction assay. There is no any cytotoxic effect was found on HEp-2 cell lines in NRU assay. The effect of NRU reagent are visualized in the following bar graps.

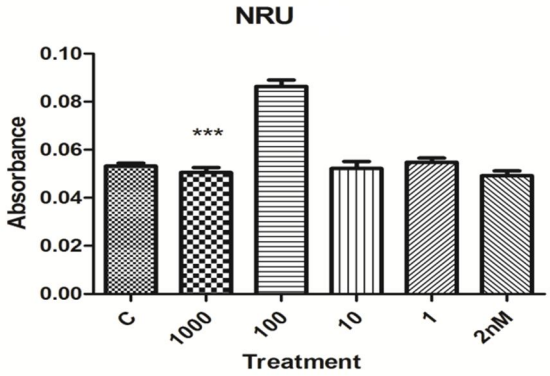

Fig.4. NRU Bioassay

C. DNA Fragmentation Assay

DNA fragmentation assay which show fragments of DNA which was breaks by the apoptosis process. The bands of fragments of DNA can be visualized by Gel- documentation system. The fragments of DNA visualized in Gel- documentation are given below.

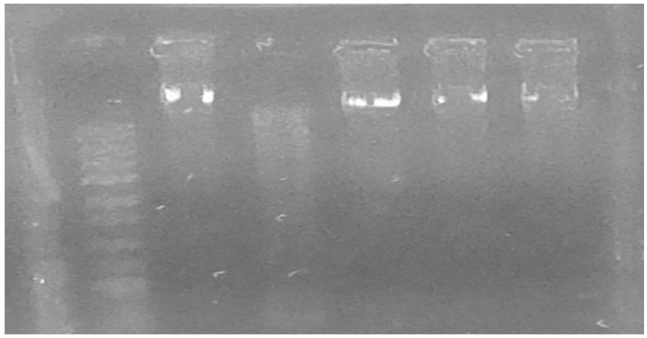

Fig.5. DNA Fragmentation Assay

VII. DISCUSSION

The alkaloids of vinca rosea result from the linkage of alkaloids a derivative of catharanthine and vindoline. From a chemical point of view , transformation carried out on the catharanthine moiety have usually led to compounds devoid of antitumor activity apart from dehydration derivatives of the tertiary alcohol on the C4' atom (vinorelbine) . In contrast, modification of the vindoline moiety led to the formation of more active compounds which are more easily synthesized, using vinblastine as starting material. The region modified in these latter compounds is located around the indolic nitrogen atom. The chemical modifications in this part of the molecule can have at least two important consequences: the interaction of the functional group with biological molecules and the modification of the physicochemical properties (polarity and partition coefficient). Ethanolic extract also had same effect as like the ethyl acetate extract induces apoptosis on cell line. As like the lung cancer and other cancer has been inhibited by vinca extracts, the ethanolic extract shows inhibitory effect on the HEp2 line.

VIII. CONCLUSION

The aim of anticancer agents is to trigger the apoptosis signaling system in these cancer cells while disturbing their proliferation. Plants have many photochemical with various bioactivities, including antioxidant, anti inflammatory and anticancer activities. Therefore, many plants have been examined to identify new and effective antioxidants and anticancer compounds, as well as to elucidate the mechanisms of cancer prevention and apoptosis. Our findings suggest that the *vinca rosea* leaf extracts most likely have anticancer properties. The qualitative analysis revealed the presence of vinblastine and vincristine alkaloids in *vinca* leaves, which may contribute to their antitumor forming properties.

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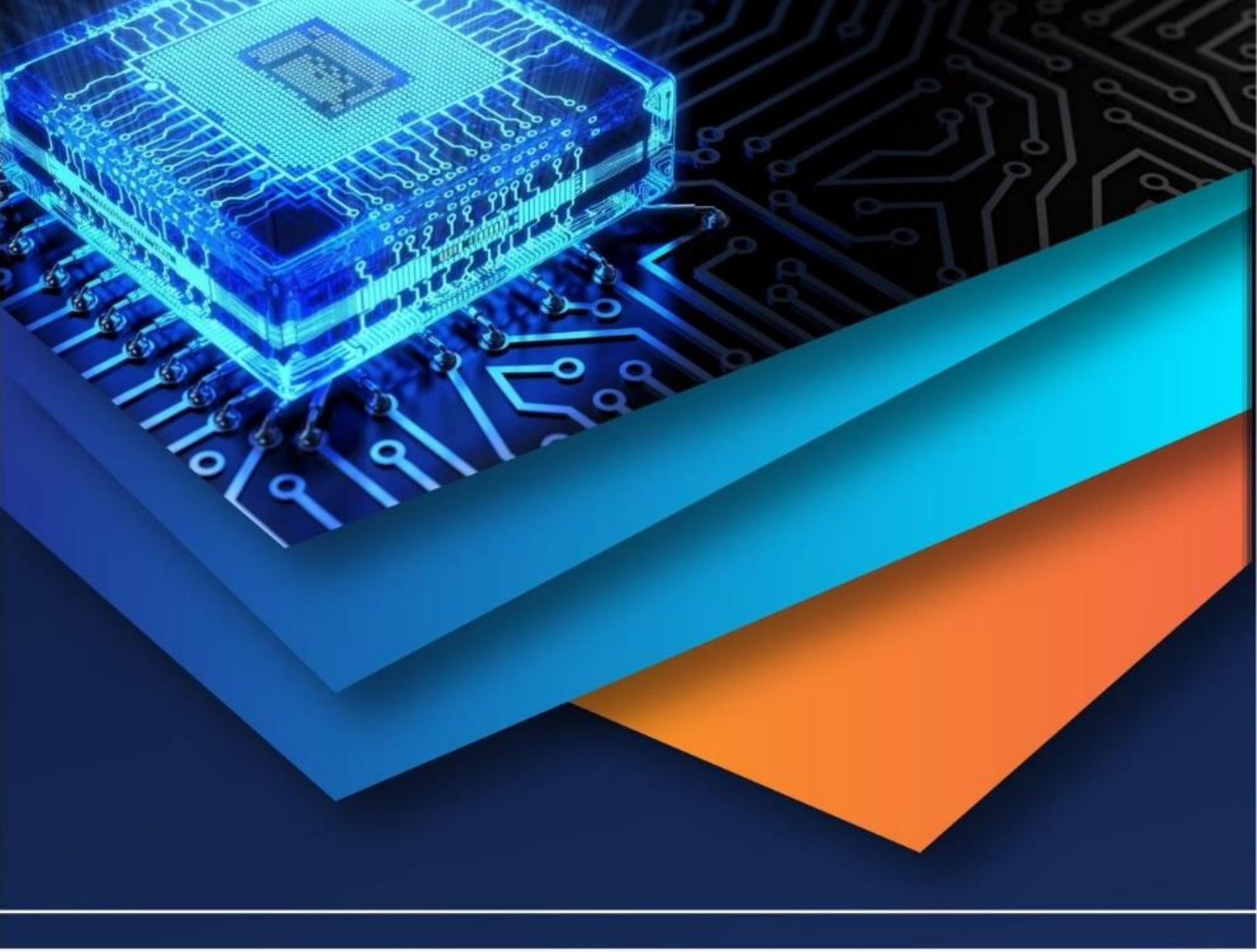

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