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In vitro Anticancer Activity of Ethanolic Extracts of Azadirachta Indica in HEp2 Cell Line

Shivanee Gupta[1], Vivek Srivastava[2], Ajay Kumar[3]
[1, 2, 3] *Department of Biotechnology, Faculty of Engineering & Technology, Rama University, Uttar Pradesh, Kanpur- 209217, India*

Abstract: ***Cancer is one of the leading causes of death in India as well as world. Scientists have developed various strategies for combating and treating cancer viz. radiotherapy, chemotherapy, surgery as well as plant derived and plant based drugs. Phytotherapeutic agents have no serious side effects and they are better assimilated than their synthetic counterparts. Azadiracta indica (Neem) has antitumor as a therapeutic property. The antineoplastic property of Azadiracta indica is due to its apoptosis-inducing, antiangiogenic, and immunomodulatory effects via several molecular mechanisms. The objective of this study was to determine the cytotoxic effect of Azadirachta indica using Hep2 cells. Azadirachta indica was extracted with 95% ethanol and tested for cytotoxic effects. Anti-proliferative effects on Hep2 cells determined the cytotoxic activity. Cytotoxicity was measured using MTT and NRU assays and apoptosis was analyzed with DNA Fragmentation assay. The results showed that ethanolic extract of neem at 1000 µg/ml concentration exhibited high cytotoxic activity against the Hep2 cell lines and least at 1 µg/ml concentration.***

Keywords: Azadirachta indica, Hep2 cell lines, MTT assay, NRU assay, DNA Fragmentation assay

I. INTRODUCTION

Neem (*Azadirachta indica*) is standout amongst most versatile medicinal plant, widely circulated in the Indian subcontinent and is a rich wellspring of limonoids that are endowed with powerful therapeutic properties predominantly antioxidant, anti-inflammatory and anticancer activities. The active ingredients of neem are found in all parts of the tree but in general, seed, bark, leaves and roots are mostly utilized for extraction purpose. In excess of 300 distinctive dynamic compounds have been reported from various parts of neem tree but the most significant limonoids are azadirachtin, salannin and nimbin [1].

The antineoplastic potential of Azadirachta indica has garnered much attention as well as interest in the last few years [2, 3]. Anti-proliferative potential of aqueous as well as alcoholic extracts of Azadirachta indica has been tested on a number of cancer cell lines in vitro and has the ability to act as a therapeutic agent for combating cancers [4, 5].

Cancer is the second leading cause of death globally, and is responsible for an expected 9.6 million deaths in 2018 [6]. Globally, about 1 in 6 deaths is due to cancer. Roughly, 70% of deaths from cancer occur in low- and middle-income nations. Cancer arises from the transformation of normal cells into tumour cells in a multistage process that generally progresses from a pre-cancerous lesion to a malignant tumour.

Hep2 cell lines are abbreviated for Human epithelial type 2. These cells allow recognition of over 30 different nuclear and cytoplasmic patterns that are given by upwards of 50 different autoantibodies with various autoimmune conditions. These cell lines are thought to be derived from epidermoid carcinoma of the larynx. Cells of these cell lines are found to contain HeLa marker chromosomes, and were derived of via HeLa contamination.

Cells contain keratin and contain papilloma virus [7]. These cell lines are used to detect patients with autoimmune disease. Indirect Immunofluorescence assay (IIFA) is used for detection. In this, patient's serum is incubated with monolayer of HEp2 cells. Antibodies adhere to monolayer that is visualized by antihuman immune-globin reagent that has conjugated to a flourescent tag [8].

MTT assay was used for observing anti-proliferative activity [9]. Measurement of cell Viability and proliferation forms the basis for numerous in vitro assays of a cells population's response to external factors. Another method for quantitative estimation of viable cell is NRU (Neutral red uptake) assay. It contains neutral red eurhodin dye which stains lysosomes red. This can be used to as a vital stain. DNA Fragmentation Assay is a tool to study apoptosis in treated cells. This assay was performed to determine the effect of ethanolic extract of neem in the DNA of the HEp2 cell lines. This assay will highlight the DNA damaging potential of Neem extract.

II. METHODOLOGY

A. Extract Preparation

Neem extracts was prepared in ethanol solvent. They were milled to fine powder with the aid of a clean electric blender. 6 gm milled neem powder was soaked in 200 ml of ethanol to prepare the ethanolic extract. After removal of water powered neem 6gm was loaded in the inner tube of soxhlet apparatus and then filter into a round bottomed flask containing in 200 ml of 100% ethanol to prepare the ethanolic extract. The extracts of neem were boiled gently over a water bath using adjustable rheostat. The extraction was continued for 8hrs and the solvent was removed at the reduced pressure with the help of vacuum pump distillation unit. Left residual volume was taken in small petri-plate and preserved for several days to let ethanol evaporation from Neem extract. After that, Neem extract is taken for dose preparation.

B. Cell Line Screening

HEp-2 cell lines were derived from epidermoid carcinoma of the larynx. Cells of these cell lines are found to contain HeLa marker chromosomes, and were derived from HeLa contamination. Cells contain keratin and papilloma virus [7].

C. Chemicals and Reagents

EMEM, Trypsin-EDTA, MTT reagent, NRU reagent, Ethanol, PBS (Phosphate Buffer Saline), TAE Buffer, TE Buffer, Lysis Buffer, Fetal Bovine Serum (FBS).

D. Cell Culture

EMEM (Earle's minimal essential medium) is used for the culture of HEp2 cells. Once Hep-2 cells reached approximately 80% confluence on plates, decant media from flask. Add 1.5 ml of 0.25% trypsin-EDTA solution to cells to detach cell layer. Do not agitate the cells during dispersal, either by hitting or by shaking the flask. This may cause clumping of the detached cells. Once Hep-2 cell layers are detached, deactivate trypsin by adding 2 ml of incomplete media in above T-25 flask. Aspirate cells by gently pipetting. Transfer the cells into the 25 ml centrifuge tube. Centrifuge cells in 25 ml eppendroff tube for 5 minutes at 300 RCF. Discard the supernatant and add 6 ml complete media and mix it well with micropipette. Differentiate the cells into three T-25 flasks and make their volume 6ml each with complete media. Store the T-25 Flask at 37.7 ºC in CO_2 incubator with 5% CO_2 supply for 48 h. Cells were counted using haemocytometer (10 µl cells + 10 µl of trypan blue).

E. MTT Assay

The reduction of tetrazolium salts is now mostly 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 intra-cellular purple formazan can be solubilized and quantified by spectrophotometric means. Prepared serial dilutions of cells in culture medium from 1 x 10^6 to 1x 10^5 cells per ml. Plate out, in triplicate, 100 µl of the dilutions into wells of a micro-titer 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 24 hrs. The time required will vary but 12 hrs to overnight is sufficient for most cell types. Added 10 µl of MTT reagent to each well, including controls. Return plate to cell culture incubator for 2 hrs. Periodically view the cells under an inverted microscope for presence of intracellular punctuates purple precipitate. When the purple precipitate is clearly visible under the microscope, added 100µL of detergent reagent to all wells, including controls. Swirl gently. Leave plate with cover in the dark for 2 to 4 hrs or overnight at room temperature. Remove plate cover and measure the absorbance in each well, including the blanks, at 570 nm in a micro-titer plate reader. Absorbance can be read with any filter in the wavelength range of 550 - 600 nm. The reference wavelength was higher than 650 nm. The blanks should give values close to zero (+/- 0.1). If the readings are low return the plate to the dark for longer incubation. Determine the average values from triplicate readings and subtract the average value for the blank. Plot absorbance against number of cells/ml. The number of cells to use in your assay should lie within the linear portion of the plot and yield an absorbance of 0.75-1.25 [10].

F. Dose Preparation

Doses were prepared with 100% ethanol extract of Neem and weighed 50mg/ml and diluted to 20 mg/ml (T_1) concentration and they are further serial diluted in T_2, T_3 and T_4 eppendroff tubes. Doses prepared for treatment are of concentration of 1000 µg/ml, 100 µg/ml, 10 µg/ml and 1 µg/ml. They are 5 µl transferred to each well of 96 well plate as per different column decided for treatment T_1, T_2, T_3 and T_4. 100 µl of 20 mg/ml is taken and added with 900µl-distilled water and further diluted.

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G. NRU Assay

Harvest suspension cells by centrifugation. Adherent cells should be released from their substrate by trypsinization or scraping. Re-suspend 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, 100 µl of the dilutions into wells of a micro-titer 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 h (to recover from handling). The time required will vary but 12 h to overnight is sufficient for most cell types. Suck out media from well plate and add 100µL of the NR. The plates were incubated at 37ºC with 5% CO_2 for further 2 h. After incubation, plates were taken out, NRU is completely poured out and 100µL of NR desorb added per well. Plates were protected from light and well mixed with pipette. Plates were taken to 96-well plate reader and their absorbance is measured in each well at 540nm, 550 nm and 650 nm.

H. DNA Fragmentation Assay

Culture HEp2 cells in 6-well plate. Incubate for 24 h. Take culture plate, add 200 µl of lysis buffer in every well, and then scrap cells with the help of tip (backside of tip). Transfer cells in the fresh eppendroff tube marked with C, T_1, T_2, T_3 and T_4 for control, cell treated with 1000 µg/ml, 100 µg/ml, 10µg/ml and 1µg/ml respectively. Add 2 µl of RNase in the tubes, incubate for 1 h and give invert mixing at half an hour. After 1 h add 2 µl proteinase K in tubes and incubate for 1 h. Then add 500 µl of solution of phenol, chloroform and iso-amylalcohol in ratio of 25:24:1 in control and 200 µl in other tubes. Perform invert mixing and centrifuge tubes at 10,000 RPM for 10 mins. Collect the upper aqueous phase, having DNA, in fresh eppendroff tubes. Add 200µl isopropanol and 50µl sodium acetate. Then centrifuge at 10,000 rpm for 10 mins. Decant supernatant and add 1 ml 70% ethanol and again centrifuge the tubes. Decant supernatant and allow ethanol to evaporate completely under horizontal LAF. Then add 50 µl of TE buffer in tubes to preserve DNA.

III. RESULTS AND DISCUSSION

A. Cell Culture

HEp2 Cell growth is shown in figure 1.

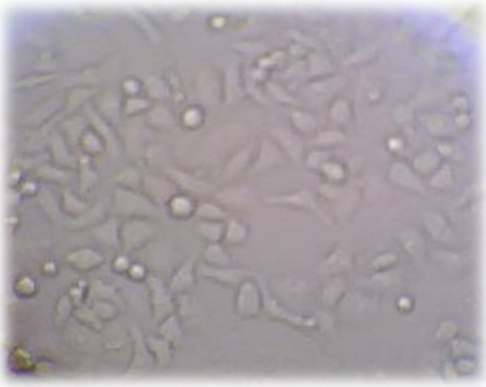

Figure 1: HEp2 cell line

MTT treatment on 96 Well plates is shown in fig. 2.

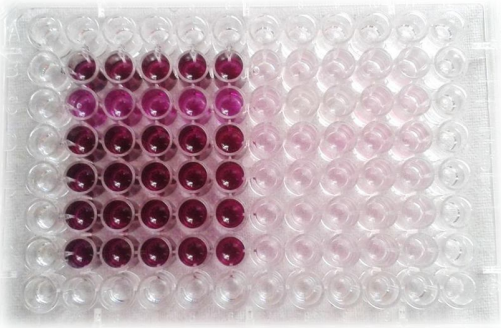

Figure 2: MTT Assay 96-well

Graph (fig.3) of MTT Assay compares the result of each treatment with the control. This shows that 1000 µg/ml of dose had drastic effect on the cancerous cell lines. Other does not show any effect on the cancerous cells. However, high amount of decrement in the cell line shows that about 300 µg/ml and above will show cytotoxicity in the HEp2 cell lines. Here 3 stars show 99.9% chances of same result in the case of experiment done in the same way.

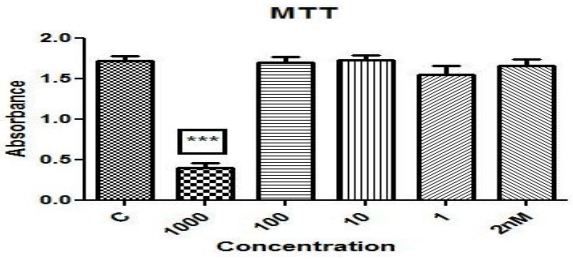

Figure 3: MTT Assay for Neem

NRU treatment on 96 Well plates is shown in fig. 4.

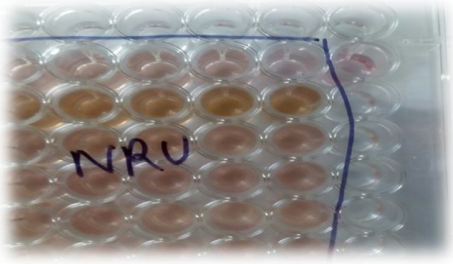

Figure 4: NRU Assay 96-well

Graph (fig.5) of NRU Assay shows apoptosis in the 1000 µg/ml of dose, other dose does not show any proper effect on the cancerous cell line. This confirms that the Neem have cytotoxicity in the HEp2 cell lines.

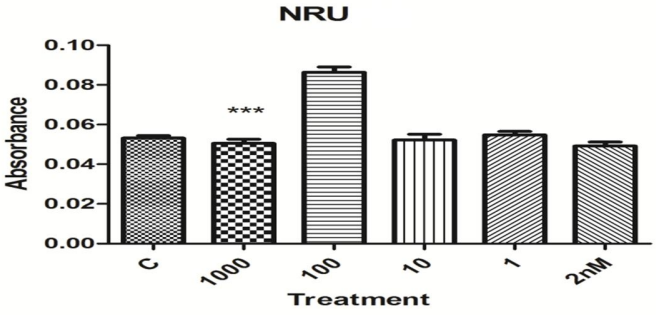

Figure 5: NRU Assay for Neem

DNA Fragmentation Assay on 96 Well plates is shown in fig. 6.

Figure 6: DNA Fragmentation Assay

DNA Fragmentation Assay perfectly determines the cytotoxicity of the Neem on the HEp2 cell line. Decreasing the concentration of the Neem extract in every well of the cell plate has some cytotoxic effect on HEp2 cell line. DNA got fragmented and separates in electrophoresis gel. Long lines in the gel represent the high concentration of fragmented DNA. Control has very less DNA fragments and others have DNA fragments. T_1 have more DNA fragments than T_2, T_3 and T_4. In the following way, there is the increase in the order of DNA fragments: C < T_4< T_3< T_2< T_1. Ethanolic extract had been used in various Anti-cancerous experiments.

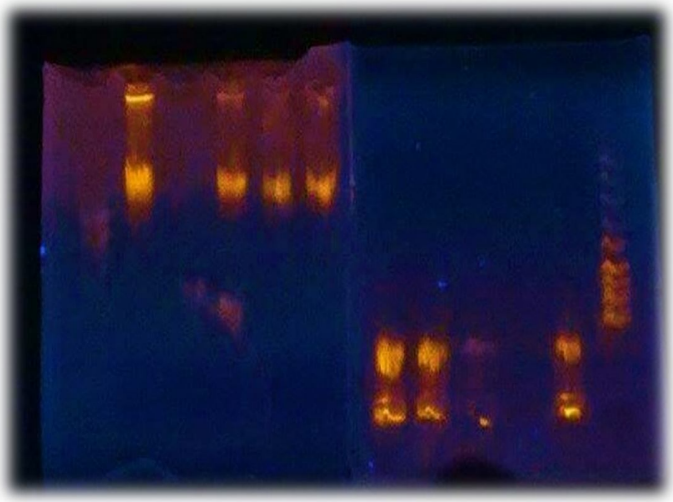

Figure 7: DNA Fragmented Assay of Neem

Water, ethanol and oil extract were taken from neem, which show different activity on different cell line. Oil extract shows maximal cytotoxicity. Morphological analysis and DAPI staining showed cytotoxicity to be a result of cell disruption with subsequent membrane rupture. Maximum cell death and apoptosis occur in TE cell within 24 h whereas minimal death is in DU-145 cells [11]. Neem ethyl acetate extract (EAEC) is also beneficial for anti-cancerous activity. Mainly molecular changes are associated with it, Oleanolic acid (OA), a component of EAEC is responsible for anti-cancerous activity, EAEC is superior to OA. EAEC promoted G0/G1 cell cycle arrests and induces apoptosis in dose wise manner. Thus, in the same way, we took ethanolic extract of Neem and it induces the apoptosis in the cell in a dose wise manner high concentration shows high amount of apoptosis. Ethanolic extract also had same effect as like the EAEC induces apoptosis on cell line. As like the lung cancer and other cancer has been inhibited by Neem extracts, the ethanolic extract shows inhibitory effect on the HEp2 line.

IV. CONCLUSION

MTT, NRU and DNA fragmentation assays were proved the cytotoxicity of ethanolic extract of Neem on HEp2 cell line. MTT Assay at 1000 µg/ml shows drastic effect on the cell line but others low concentration do not show that effect on HEp2 cell line. NRU assay also shows cytotoxic effects of ethanolic extract of neem on the HEp2 cell line at 1000 µg/ml concentration but other concentration do not show any effect on cell line. The more proper way of determining is DNA Fragmentation assay, which is indicator of apoptosis, since apoptosis can be seen with different concentration of ethanolic extract of neem thus we can conclude the cytotoxic effect of ethanolic extract on HEp2 cell line. Here by DNA Fragmentation assay, we observe the effect of different concentration on cancerous cell line at 1000µg/ml, 100 µg/ml, 10µg/ml and 1µg/ml induces decreased effect on HEp2 cell line. Highest effect is of 1000µg/ml and least is of 1 µg/ml ethanolic extract of neem.

V. ACKNOWLEDGEMENT

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VI. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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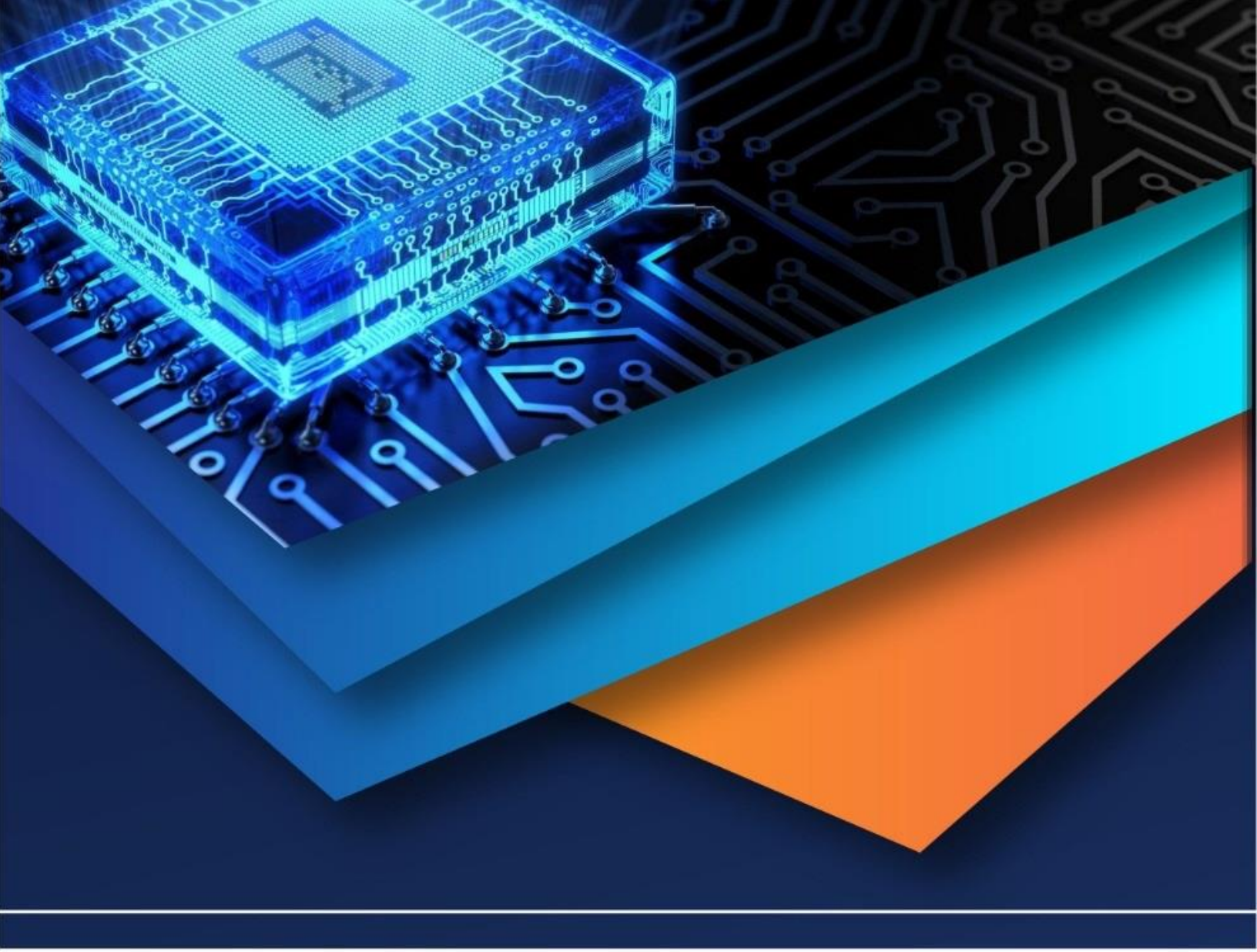

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