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Antioxidant and Anticancer Activities of Pomogranate and Custard Apple Extracts

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Abstract: ***Plants are one of the important sources of medicines since the beginning of the human civilization. There is a growing demand for plant based medicines, health products, pharmaceuticals, food supplements, cosmetics, etc. Fruit extracts have antioxidant and anticancer activity. Pomegranate (Punica granatum Linn.) and custard apple (Annona squamosa Linn.) have been used for the prevention and treatment of various diseases. The present study investigated antioxidant and anticancer activity of pomogranate and custard apple using DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay and the potential activity were found to be 69.38% and 36.54% respectively. The total antioxidant capacity was calculated by FRAP (Ferric reducing antioxidant power) assay which was expressed as 1555 µM (pomogranate) and 2350µM (custard apple). Estimation of Catalase, SOD and Nitric oxide assay also was done in this study. Anticancer activity of extracted samples was evaluated based on viability of cells. The optimum cell viability was 58.34% obtained at a concentration of 62.5 (µg/ml) in custard apple and maximum cell viability was 79.00% obtained at a concentration of 7.8 (µg/ml) in pomogranate using MTT assay. The current study showed that pomogranate and custard apple are a potent source of antioxidant and anticancer activity.***
Keywords: Ant oxidative, anticancer, cancer cells, fruit extract, MTT assay.

I. INTRODUCTION

In India, several plants have been inferred for various studies for their medicinal value. Photochemistry reveals various apoptotic, ant proliferative, antioxidant, antimicrobial and anticancer activities (Seeramaet al., 2005). Fruit and vegetable consumption have been shown to reduce cancer risk, hence edible plants are increasingly being considered as sources of anticancer drugs (Fergusonet al.,2004).The antioxidant and anticancer activity of the extracts from medicinal plants and herbs was associated with their components of phenolic compounds. The major types of phenolic compounds include phenolic acids, flavonoids, tannins, coumarins, lignans, quinines, stilbenes and curcuminoids (Cai Y et al.,2004).

There is an increasing epidemiological and pharmacological evidence that plants contain biologically active components(e. g. free radical scavengers)offering health benefits and protection against degenerative diseases(Boeing H et al., 2012).The antioxidants contained in fruits and vegetables such as ascorbic acid, flavonoids and tannins, are supposed to play a vital role in prevention of these diseases(Eleonora Turrini et al.,2015).In fact, oxygen radicals and lipid peroxides have been known for their alleged role in the etiology of manyin vivo pathological reactions such as aging and cancer. In this regard, epidemiological studies have shown that consumption of fruits and vegetables is inversely associated with morbidity and mortality of cardio- and cerbro-vascular diseases and certain types of cancers (Huang and Prior, 2005).

Pomogranate (Punica granatum L.) has been shown to an extent to have anticancer activity, which is generally attributed to its high content of polyphenols including ellagitannins, ellagic acid, and other flavonoids (quercetin, kaempferol, and luteolin glycosides) (Seeram et al.,2005).

The peel extract was a potent veridical agent against gential herpes virus due to tannins. It was also used to treat the infection of male or female sexual organs, mastitis, acne folliculitis, pile, allergic dermatitis and dysentery(Hu,1997).Pomogranate inhibited the proliferation and induced apoptosis of human prostate cancer cells(Dorato and Engelhard, 2005).

Custard apple (Sitaphal) is a fruit from a small tree named Annona squamosa which belongs to the family Annonaceae of the order Magnoliales(RajsekharSaha,2011).Annona squamosa has also been extensively used as a traditional medicine for different ailments(Fujimoto et al.,1988).The leaves of the plants have been used as insecticide, antihelminitic and in the healing of bleeding wounds.

Custard apple requires hot dry climate during flowering and high humidity during fruit setting(Aziz et al.,2003).Flowering happens during hot dry climate and fruit setting takes place with the onset of monsoon. Low humidity is not good for pollination and fertilization. Custard apple withstands drought conditions and cloudy weather(PopenoeWilson,1920). Annona Squamosashowed anticancer, anti-HIV, anti-diabetic properties.It is reported to arrest cancer cells at the G1 –phase and cause cytotoxicity in a Bax

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and caspase 3-related pathway in the case of breast cancer(Johnson et al.,1963).The present study was carried out to analyse the antioxidant and anticancer activity of pomegranate and custard apple extract.

II. OBJECTIVES

In this study, pomegranate and custard apple samples were extracted from fruits. The present study represents activity of samples against antioxidant and anticancer activity. These samples are also suitable for isolation of active components to be tested against HeLa cell line. This knowledge helps to try it to test antioxidant and anticancer activity of different cancer cell lines in order to measure the potential difference in cytotoxicity which can make their findings even more valuable.

III. MATERIALS REQUIRED

A. Preparation of extract

Pomegranate and custard apple were purchased from Koyambedu market, Chennai, Tamilnadu. The fruits were peeled and cut into pieces. They were washed with distilled water to remove any adherent particles, shade dried and powdered. 25gof each sample was weighed and extracted with 300ml of ethanol by continuous hot percolation with the help of soxhlet apparatus for 10hr. The extracts were filtered and concentrated using rotary evaporator. The concentrates were stored in the refrigerator for further analysis.

IV. PROCEDURE

A. Antioxidant assay

1) *DPPH assay:* DPPH (1,1-diphenyl-2-picrylhydrazyl) is characterised as a stable free radical by virtue of delocalisation. DPPH assay was carried out by adding3.7 ml of absolute methanol was added to the two test tubes marked as T1 andT2and 3.8ml of absolute methanol was kept as a blank T3. 100µl of BHT [Butyalted Hydroxy Toluene] was added to all test tubes including blank. 100µl of pomogranate was added and custard apple samples in T1 and T2 respectively. 200µl of DPPH reagent were added to all the test tubes including blank. All test tubes were included at room temperature in dark condition for 30 minutes. The absorbance was recorded at 517nm.

1) *Frap assay:* Total antioxidant activity was measured by FRAP(Ferric reducing antioxidant power assay). FRAP assay was carried out by adding 100µl of ferulic acid sample and 3 ml of FRAP reagent was added to the two test tubes marked as T1 and T2. 3 ml of FRAP reagent was taken as a blank T3. Absorbance was observed at 593 nmafter vortexing and then the samples were incubated at 37ºC in water bath and absorbance were recorded at 593nm after 4 minutes of incubation. Ascorbic acid was used as the standard. FRAP value of ascorbic acid is 0.2.

2) *Catalase:* The activity of catalase was determined by this antioxidant method. 0.9 mL of phosphate buffer, 0.1 mL of supernatant (sample) and 0.4 mL of hydrogen peroxide were added to two test tubes T1 and T2. The reactions were arrested after 15, 30, 45 and 60 seconds by adding 2.0mL of dichromate-acetic acid to T1 and T2. The tubes were kept in a boiling water bath for 10 min, cooled and the color developed was observed at 620 nm.

3) *SOD assay:* Superoxide dismutase in the liver tissue was assayed by antioxidant method. 0.5 mL of tissue homogenate was diluted to 1.0 mL with water followed by addition of 2.5 mL of ethanol and 1.5 mL of chloroform (chilled reagents were added)to T1and T2. This mixture was shaken for 90 sec at 4 °C and then centrifuged. The enzyme activity in the supernatant (samples) was determined as follows. The assay mixture contained 1.2 mL of sodium pyrophosphate buffer, 0.1 mL of phenazine methosulphate and 0.3 mL of nitro blue tetrazolium and appropriately diluted enzyme preparation in a total volume of 3 mL. The reaction was started by the addition of 0.2 mL NADH (Nicotinamide adenine dinucleotide). After incubation at 30 °C for 90 sec, the reaction was stopped by the addition of 1 mL glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 mL n-butanol. The mixture was allowed to stand for 10 min; centrifuged and n-butanol layer was separated. The color density of the chromogen in n-butanol was measured at 510 nm.

4) *Nitric oxide assay:* Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates Nitrite oxide which interacts with oxygen to produce Nitrite ions. Sodium nitroprusside (5mM) in standard phosphate buffer saline (0.025M, pH 7.4) was incubated with 100 mg/ml in T1and T2 and are incubated at 29ºC for 3 hours. The experiment was controlled without the test compounds but with equivalent amount of buffer was conducted in an identical manner. After 3 hours, incubated samples were diluted with 1 ml of Griess reagent. The absorbance of the colour developed during diazotization of Nitrite with sulphanilamide and its subsequent coupling with Napthylethylenediamine hydrochloride were recorded at 550nm on a spectrophotometer.

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B. Anticaner activity

The anticancer activity of ferulic acid was studied against HeLa cell line. HeLa cell line was obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO2 at 37 °C. The cells were grown in 24-well plates and incubated at 37°C with 5% CO2. Once the cell reached the confluence, the ferulic acid samples were added to the cells at different concentrations and incubated for 24 hrs. After incubation, the samples were removed from the well and washed with phosphate-buffered saline (PBS) (pH 7.4). 100 µl/well of 0.5% of (5mg/ml) 3-(4, 5-dimethyl2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. 1ml of DMSO was added in all the wells after incubation.The absorbance was measured with UV- Spectrophotometer at 570 nm using DMSO as the blank. Graph was plotted using the % of Cell Viability on Y-axis and concentration of the sample on Xaxis.

V. RESULTS

A. Antioxidant activity

1) *DPPH assay:* The antioxidant activities of standard and extracted samples were calculated using DPPH Assay to delocalize the stable free radical. The antioxidant potential of the sample wasshown in Table1.In DPPH assay custard apple showed maximum antioxidant activity of 69.38% when compared to the pomegranate sample 36.54 %.

TABLE 1: DPPH ASSAY

Sl.No	Sample	Concentration (µg/ml)	O.D values	DPPH activity (%)
1	CUSTARDAPPLE	1000	0.331	69.38
2	POMOGRANATE	1000	0.686	36.54

The antioxidant activity was determined by the formula

$$\% \text{ Antioxidant activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test}) \times 100}{(\text{Absorbance at blank})}$$

2) *Frap assay:* The total antioxidant activity of sample and standard weredetermined usingFRAP Assay and their values are showed in Table 2. In FRAP assay pomegranate showed maximum antioxidant activity of 2350µM when compared to the pomegranate sample 1555µM.

TABLE 2: FRAP ASSAY

Sl.No	Sample	FRAP(µM)
1	CUSTARD APPLE	1555
2	POMOGRANATE	2350

FRAP value of the sample was calculated by the formula

FRAP value of sample (µM) = (Change in absorbance of sample from 0 to 4 minute / change in absorbance of standard from 0 to 4 minutes) x FRAP value of standard (1000 µM)

3) *Catalase***:** The antioxidant activity of samples and standard were estimated using catalase and their values are shown in Table 3. In catalase, pomogranate showed maximum antioxidant activity of 1.8U/mg protein when compared to custard apple1.4U/mg protein.

TABLE 3: Catalase

Sl.No.	Sample	CAT (U/mg protein)
1.	CUSTARD APPLE	1.4
2.	POMOGRANATE	1.8

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4) *Superoxide dismutase assay:* The antioxidant activity of samples and standard were assayed by thisSOD method and their values are shown in Table 4. InSOD, pomogranate showed maximum antioxidant activity of 93.78U/mg protein when compared to custard apple 76.98 U/mg protein.

TABLE 4: SOD ASSAY

Sl. No.	Sample	SOD(U/mg Protein)
1	CUSTARD APPLE	93.78
2	POMOGRANATE	76.98

5) *Nitric oxide assay:* The antioxidant activity of samples and standard were assayed by this nitricoxide assay and their values are shown in Table 5. In Nitric oxide pomegranate showed maximum inhibition activity of 25.90% when compared to custard apple 18.34 %.

TABLE 5: NITRIC OXIDE ASSAY

Sl.NO.	Sample	Inhibition %
1.	CUSTARD APPLE	18.34
2.	POMOGRANATE	25.90

B. Anticancer Activity

The anticancer activity of extracted was expressed in percentage of cell viability using MTT assay in HeLa cells. The cell viability with different concentrations was shown in Table 3. The % cell viability was calculated using the following formula:

% Cell viability = A570 of treated cells / A570 of control cells × 100

TABLE 6: ANTICANCER EFFECT OF CUSTARD APPLE *ON HELA* CELL LINE

Sl.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.145	12.22
2	500	1:1	0.242	20.40
3	250	1:2	0.324	27.31
4	125	1:4	0.411	34.65
5	62.5	1:8	0.488	41.14
6	31.2	1:16	0.587	49.49
7	15.6	1:32	0.665	56.07
8	7.8	1:64	0.764	64.41
9	Cell control	-	1.186	100

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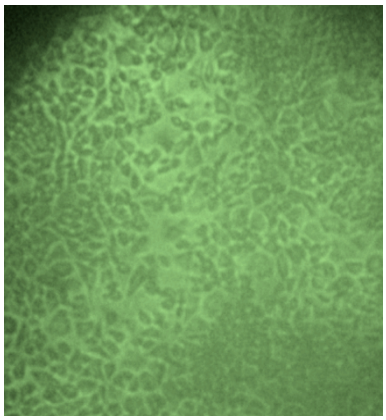
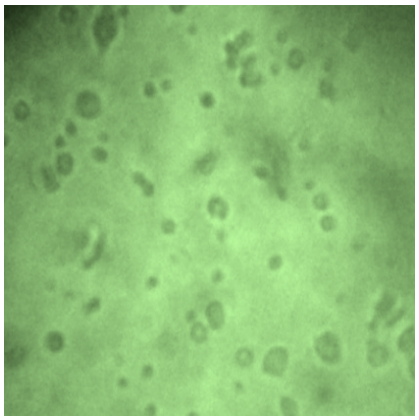
TABLE 7: ANTICANCER EFFECT OF POMOGRANATE *ON HELA* CELL LINE

Sl.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.345	29.08
2	500	1:1	0.432	36.42
3	250	1:2	0.501	42.24
4	125	1:4	0.596	50.25
5	62.5	1:8	0.692	58.34
6	31.2	1:16	0.774	65.26
7	15.6	1:32	0.874	71.58
8	7.8	1:64	0.937	79.00
9	Cell control	-	1.186	100

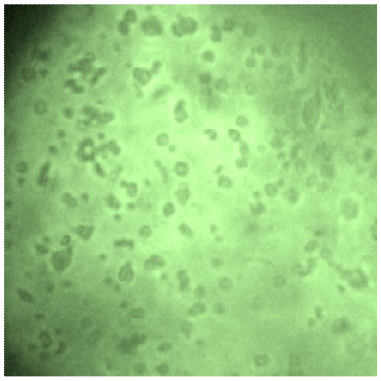
Graphs are plotted using the % of Cell Viability on the Y-axis and concentration of the sample on the X-axis. The optimum cell viability was 58.34% at a concentration of 62.5 µg/ml in custard apple. The maximum cell viability was 79.00%and wasobtained at a concentration of 7.8 µg/ml in pomogranate.



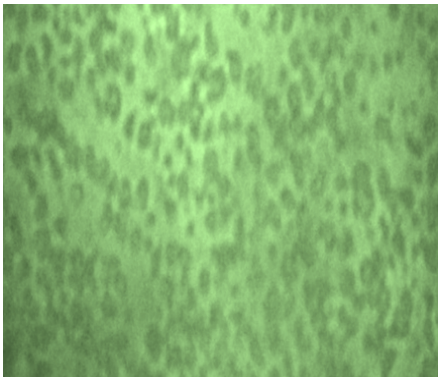
a.Normal cell line



b.Toxicity-1000µg/ml



c.Toxicity-31.2 µg/ml



d. Toxicity-7.8µg/ml

Fig. 1 Anticancer effect of pomegranate on HeLa cell line

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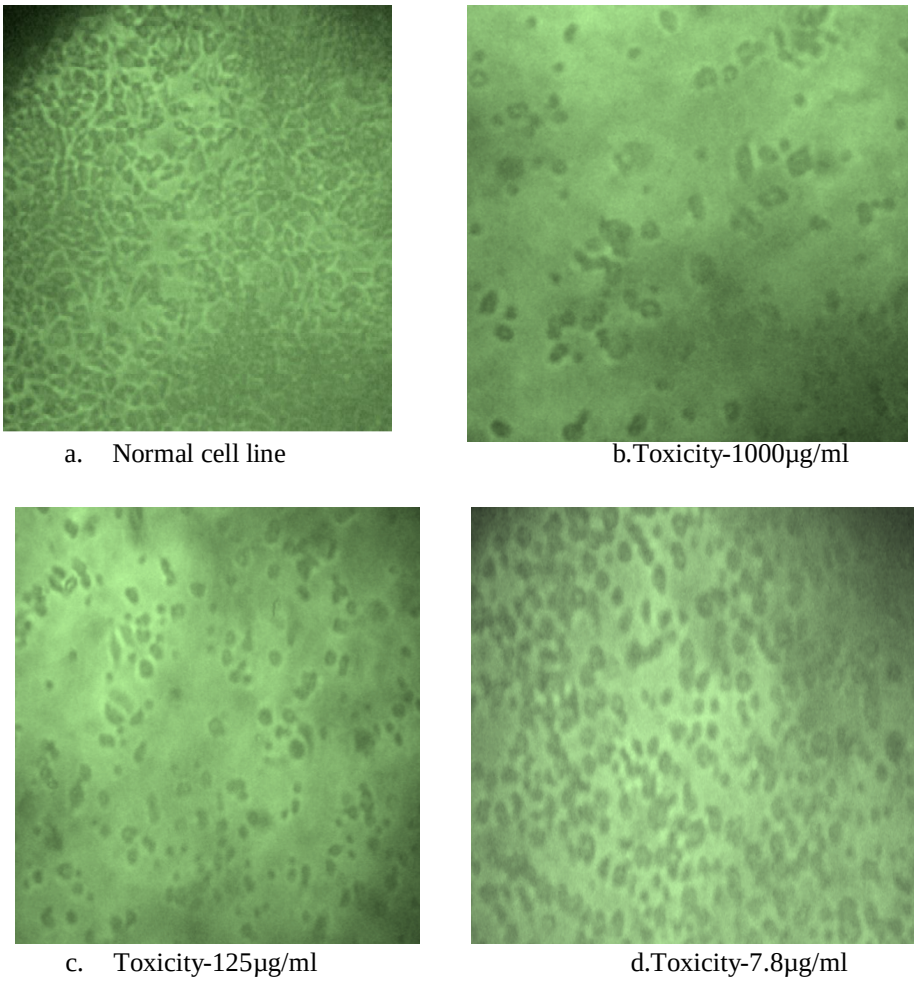

a. Normal cell line b.Toxicity-1000µg/ml

c. Toxicity-125µg/ml d.Toxicity-7.8µg/ml

Fig. 2Anticancer effect of custard apple on HeLa cell line

VI. DISCUSSION

The study of anticancer agents from natural sources has been going on worldwide. Active constituents that have been isolated presently are used to treat human diseases. There is an increasing epidemiological and pharmacological evidence that plants contain biologically active components (e.g. free radical scavengers) offering health benefits and protection against degenerative diseases (Boeing H *et al.*, 2012). The antioxidants contained in fruits and vegetables such as ascorbic acid, flavonoids and tannins, are supposed to play a vital role in prevention of these diseases (Eleonora Turrini *et al.*,2015). The extracts showed anticancer and antioxidant activities based on concentrations.

VII. CONCLUSION

In conclusion the extracts of pomogranate and custard apple a potential source of antioxidants and could be used as natural antioxidants agents. The extracts have high anticancer potential compared to other fruits. *Pomogranate and custard apple* extract has explicit antioxidant and anticancer activity which is helpful for therapeutic and pharmaceutical purposes.

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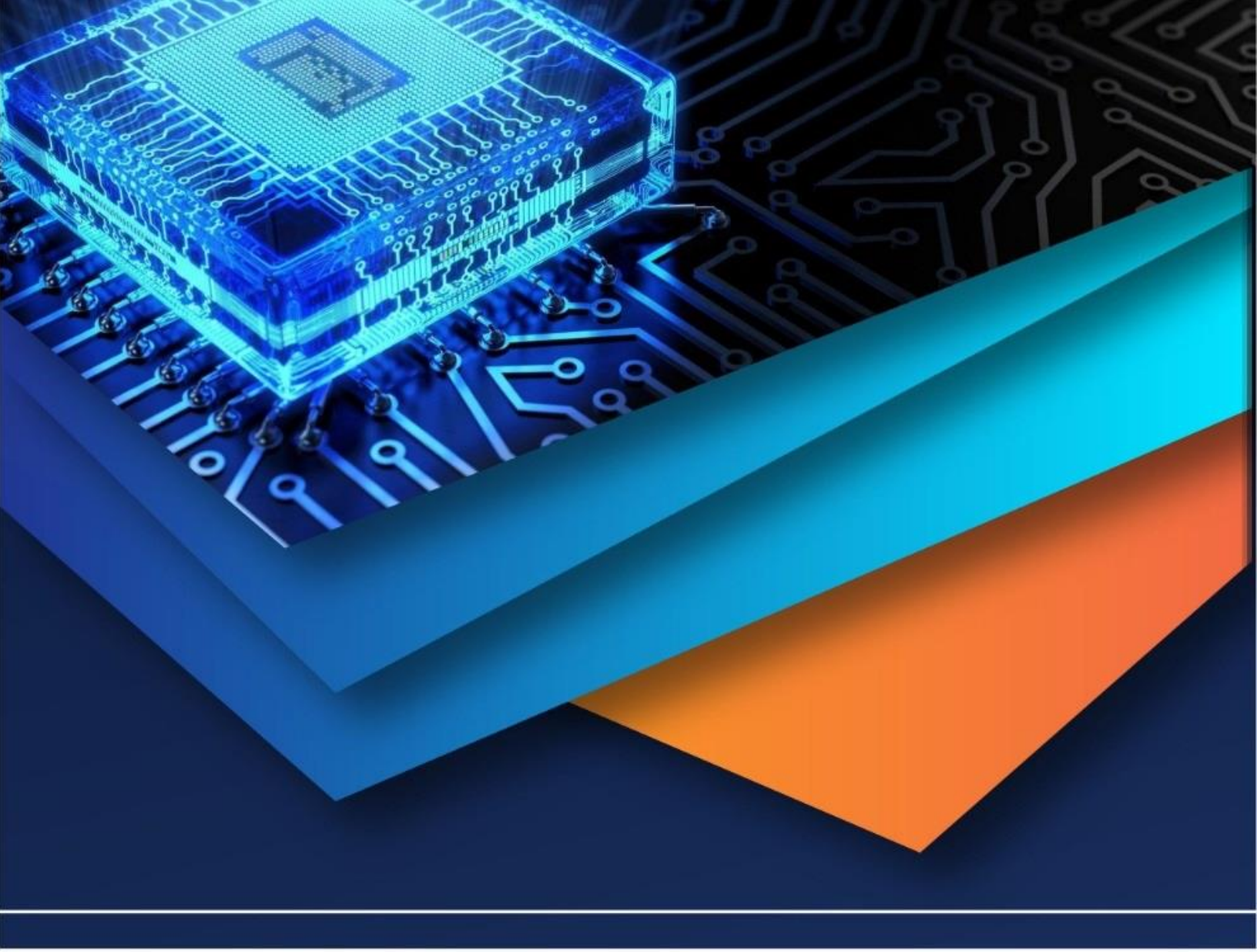

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