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Comparative Study of DPPH, ABTS and FRAP Assays for Determination of Antioxidant Activity

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Abstract- Three simple spectrophotometric methods: (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity assay (DPPH), [2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)] free radical scavenging activity assay (ABTS) and Ferric ion Reducing Antioxidant Power assay (FRAP) for determination of antioxidant activity were compared. The antioxidant activity of aqueous extracts of the mycelia of three mushroom species (Ganoderma lucidum, Lentinus edodes and Agaricus bisporus) were compared for this study. A comparative account was made between the three antioxidant assays and their advantages and disadvantages were briefly reported along with the scope of application of each method. DPPH was found to be the most preferred method for the determination of antioxidant activity of mycelia of the three mushroom species because it could be rapidly performed and showed high reproducibility. Results were reported so that it becomes easy to select the right method that can give accurate results for the antioxidants in study.
Keywords- Antioxidant activity, DPPH, ABTS, FRAP, Comparative study.

I. INTRODUCTION

Oxidative stress is caused by an imbalance between ROS (Reactive oxygen species) and the anti-oxidative defence systems and is considered to be the major reason for many diseases such as cancer, cardiovascular diseases, neurodegenerative diseases, rheumatoid arthritis, atherosclerosis, hypertension and AIDS. Antioxidants for the treatment of cellular degenerations are beginning to be considered because they inhibit or delay the oxidative process by blocking both the initiation and propagation of oxidizing chain reactions. Under stress, our bodies end up having more reactive oxygen species than antioxidant species, an imbalance that can lead to damage in cellular components such as lipids, protein and DNA. Cell degradation eventually leads to partial or total functional loss of physiological systems in the body [19].

Even though almost all organisms possess antioxidants and several enzyme systems like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase to protect them from oxidative damage, these systems are inadequate to prevent the damage entirely. Hence antioxidant supplements or foods containing high concentrations of antioxidants are needed which may help scavenge free radicals and reduce oxidative damage. Currently available synthetic antioxidants including Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA), Gallic acid, etc., have been found to cause negative health effects. Hence, their application has been restricted and there is a trend to substitute them with naturally occurring antioxidants [1]. Many edible and non- edible mushrooms have long been used in Asia and Africa for medicinal purposes. In recent years many pharmacologically active compounds from mushrooms have been discovered and isolated which impart antimicrobial, antioxidant, antiviral, antitumour, antiallergic, immunomodulating, anti-inflammatory, hepatoprotective activities [17]. *Ganoderma lucidum, Ganoderma applanatum, Grifola frondosa, Poria cocos, Tremella fuciformis, Lentinus edodes,* etc. are just few of the medicinally important mushrooms that possess therapeutic properties. Thus the antioxidant activity of *Ganoderma lucidum, Lentinus edodes and Agaricus bisporus* was described in this study. The aim of this study was to compare three simple spectrophotometric methods of determination of antioxidant activity. Since several methods exist for the in vitro determination of antioxidant capacity, it becomes necessary to select the right method that can give accurate results for the antioxidants in study. The three spectrophotometric methods selected were (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity assay (DPPH), [2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)] free radical scavenging activity assay (ABTS) and Ferric ion Reducing Antioxidant Power assay (FRAP). An attempt was made in the present study to evaluate the comparative response of the three methods by using three mushroom species (*G. lucidum, L. edodes and A. bisporus).*The results of each of these methods are discussed along with their advantages and limitations.

II. MATERIALS AND METHODS

A. Microorganism and Growth Of Mycelia

Mycelia obtained from three mushroom species (*Ganoderma lucidum, Lentinus edodes* and *Agaricus bisporus*) were collected

from Waghai village (20.7667°N, 73.4833°E), district Dang of Gujarat (India). All the three cultures were maintained in potato dextrose agar plates at 25°C for 9 days and were periodically transferred onto new PDA medium. The strains were maintained and stored at 4°C. Three pieces of 5 mm diameter of each of these actively growing cultures from agar plates (9 days old) were transferred with the help of 5mm cork borer into 250 mL Erlenmeyer flasks containing 100 mL of the seed culture for 10 days at 150 rpm and 25°C. The seed culture media consisted of the following components dissolved in 100 mL Double distilled water (DDW): 1.5g glucose, 0.2g yeast powder, 0.1g KH_2PO_4, 0.1g K_2HPO_4, 0.15g $MgSO_4.7H_2O$, and 0.25g peptone.

The mycelia of all the three cultures were harvested according to the method of Suberu *et al.*, 2013 [6]. After the incubation period of 10 days, the culture media were decanted and then filtered using Whatman #1 filter paper to get the fresh mycelia after washing with DDW twice. The mycelia were oven dried at 60°C until a constant dry weight was obtained. The dried mycelia were weighed in milligrams (mg) and recorded.

B. Preparation of Extracts

The extraction method of Modi *et al.*, 2014 [5] was followed. The dried mycelia were ground to a fine powder using a domestic blender. For preparing the extracts, double distilled water was used as solvent to obtain the pharmacologically active compounds from the mushrooms. For every1 gram (g) of powder, 50 milliliter (mL) of solvent was used and was subjected to extraction using a reflux apparatus. After the completion of extraction, the supernatant was filtered through Whatman #1 filter paper. The solvent extracted fractions were evaporated to dryness to obtain residues. The residues were reconstituted using double distilled water to obtain stock solutions of the aqueous extracts having concentration of 10 mg/mL. The aqueous extracts were stored at 4ºC in air tight containers. The antioxidant activities of the different extracts were carried out by preparing working solutions of different concentrations (0.5 mg/mL, 1.0 mg/mL, 2 mg/mL and 5 mg/mL) from the stock solution.

C. Determination Of Antioxidant Capacity

1) DPPH (1, 1-Diphenyl-2-Picrylhydrazyl) Free Radical Scavenging Activity Assay: The assay was carried out according to the modified method of Blois (1958) [13]. 1mL of 0.1mM solution of DPPH (Sigma Aldrich, India) in methanol was mixed with 2mL of the aqueous extracts at different concentrations (0.5-5.0 mg/mL). The mixture was then incubated at room temperature for 30 min in the dark. The control was prepared by mixing 1 mL of DPPH solution with double distilled water. The absorbance was measured against a blank at 517 nm using spectrophotometer (Systronics Visiscan 167). Lower absorbance of the reaction mixture indicates higher DPPH free radical scavenging activity. Ascorbic acid (Merck, India) was used as the standard. Samples were prepared and measured in triplicates. The percentage of scavenging activity of each extract on DPPH radical was calculated as %inhibition of DPPH (I%) using the following equation:

$$I\% = [(A_o - A_s)/A_o] \times 100$$

Where A_o is the absorption of control and A_s is the absorption of the tested extract solution.

2) ABTS [2,2'-Azinobis(3-Ethylbenzothiazoline-6-Sulphonic Acid)] Free Radical Scavenging Activity Assay: This assay was carried out using the modified method of Re *et al.*, (1999) [14].

The $ABTS^{\bullet+}$ stock solution was prepared by reacting ABTS (Sigma Aldrich, India) aqueous solution (7 mM) with 2.45 mM aqueous solution of potassium persulfate (Merck, India) in equal quantities; the mixture was allowed to stand in the dark at room temperature for 12-16 hrs before use. The working solution of $ABTS^{\bullet+}$ was obtained by diluting the stock solution in methanol to give an absorbance of 0.70 ± 0.02 at 734 nm. Then, 2.0 mL of $ABTS^{\bullet+}$ solution was mixed with 1 mL of the aqueous extracts at different concentrations (0.5-5.0 mg/mL). The mixture was then incubated at room temperature for exactly 10 min in the dark. The control was prepared by mixing 2.0 mL of $ABTS^{\bullet+}$ solution with 1 mL of double distilled water. The absorbance was measured against a blank at 734 nm using spectrophotometer (Systronics Visiscan 167). BHT (Merck, India) was used as the standard. Samples were prepared and measured in triplicates. The percentage of scavenging activity of each extract on $ABTS^{\bullet+}$ was calculated as % inhibition (I%) using the following equation:

$$I\% = [(A_o - A_s)/ A_o] \times 100$$

Where A_o is the absorption of control and A_s is the absorption of the tested extract solution.

3) Determination Of Ferric Reducing Antioxidant Power (FRAP): This method is based on the ability of the sample to reduce Fe^{3+} to Fe^{2+} ions. At low pH, in the presence of TPTZ (Sigma Aldrich, India), ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}-TPTZ) form with the formation of an intense blue colour having an absorption maximum at 593

nm. The method described by Benzie and strain (1996) [7] was followed. 2.3mL of the FRAP reagent was mixed with 0.7 mL of the aqueous extracts at different concentrations (0.5-5.0 mg/mL). The mixture was then incubated at 37ºC for 30 min in the dark. The absorbance was measured at 593 nm against a blank having all the reagents excluding the sample using spectrophotometer (Systronics Visiscan 167). Increased absorbance of the reaction mixture indicates an increase of reduction capability. Samples were measured in triplicates. Ascorbic acid (Merck, India) was used as the standard. Standard curve of ascorbic acid solution was prepared using the similar procedure from which the regression formula was derived. Results were expressed in mg of ascorbic acid equivalents (AAE)/mL of extract.

D. Statistical Analysis

All determinations of antioxidant capacity by DPPH, ABTS and FRAP assay were conducted in triplicates. Experimental data were expressed as mean ± SD. Analysis of variance and significant difference among means were tested by one way ANOVA. Statistical significance was accepted at a level of 5%. $P < 0.05$ was regarded as significant and $p < 0.01$ was very significant.

III. RESULTS AND DISCUSSION

A. DPPH Free Radical Scavenging Activity Assay

Results from the graph (Fig. 1) clearly show that among the three mushrooms studied, *G. lucidum* had the highest % scavenging activity for all the concentrations (0.5-5.0 mg/mL) of the aqueous extract followed by *L. edodes* and *A. bisporus.* At 5 mg/mL, G. lucidum showed highest % scavenging activity of 68.10±3.66% followed by *L. Edodes* (41.16±0.81%) and *A. Bisporus* (35.12±3.26%). Significant difference ($p<0.01$) between all the concentrations of the aqueous extracts of the three mushroom species was observed. The results obtained in the present study gave better scavenging activity when compared to the results obtained by F. Kalyoncu *et al.*, 2010 [4]. The water extracts of *G. lucidum, L. edodes* and *A. bresadolanus* at 1 mg/mL showed % DPPH scavenging values of 21.51%, 13.66% and 24.70% respectively, whereas in the present study %DPPH scavenging values of water extracts of *G. lucidum, L. edodes* and *A. bisporus* at 1 mg/mL were found to be 54.14%, 32.60% and 27.08% respectively.

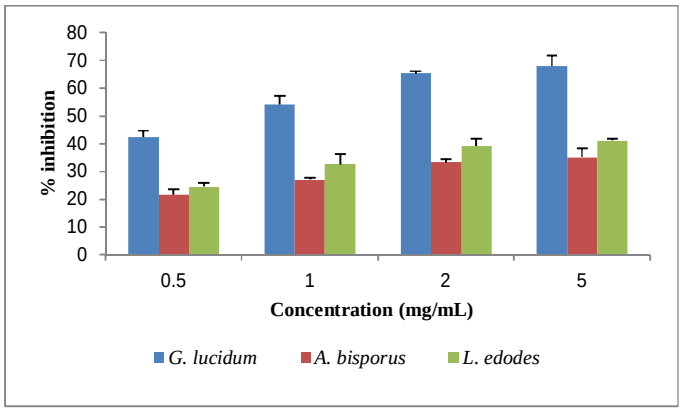

Fig.1: % inhibition of DPPH (I%) by the extracts

B. ABTS Free Radical Scavenging Activity Assay

Results from the graph (Fig. 2) show that among the three mushrooms studied, *G. lucidum* again had the highest % scavenging activity for all the concentrations (0.5-5.0 mg/mL) of the aqueous extract followed by *L. edodes* and *A. bisporus*. Thus, *G. lucidum* showed the highest ABTS radical scavenging activity. At 5 mg/mL, *G. lucidum* showed highest % scavenging activity of 72.22±1.96% followed by *L. Edodes* (51.5±3.96%) and *A. Bisporus* (49.38±3.9%). Significant difference ($p<0.05$) between all the concentrations of the aqueous extracts of *G. lucidum* and *L. edodes* was observed. Results of present study were found to be lower when compared to the results obtained by F. Kalyoncu *et al.*, 2010 [4]. The water extracts of *G. lucidum, L. edodes* and *A. bresadolanus* at 1 mg/mL showed % ABTS scavenging values of 70.71%, .28.23% and 75.98% respectively, whereas in the present study %ABTS scavenging values of water extracts of *G. lucidum, L. edodes* and *A. bisporus* at 1 mg/mL were found to be 46.72%, 23.32% and 21.66% respectively.

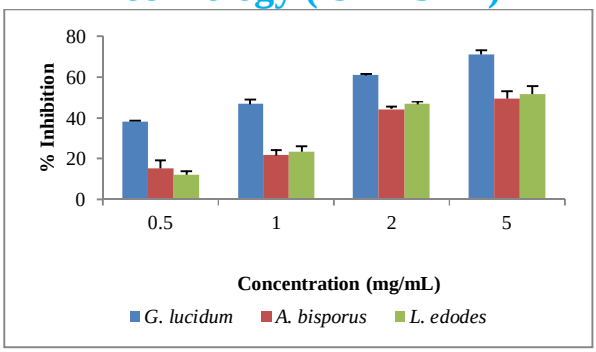

Fig.2: % inhibition of $ABTS^{\bullet+}$ (I%) by the extracts

C. Determination Of Ferric Reducing Antioxidant Power (FRAP)

The reducing ability of the aqueous extracts of the three mushrooms is expressed in mg of ascorbic acid equivalents (AAE)/mL of extract and is shown in Fig. 3. *G.lucidum* at different concentrations exhibited strong antioxidant activity. All values increased with the increasing concentrations (0.5-5.0 mg/mL) for all the three mushrooms. The reducing ability of *G. lucidum* was found to be the highest among the three species. Significant difference ($p<0.05$) was observed in all the concentrations of aqueous extracts between *G. lucidum* and *L. edodes*. Also significant difference ($p<0.01$) was observed in all the concentrations between *L. edodes* and *A. bisporus*.

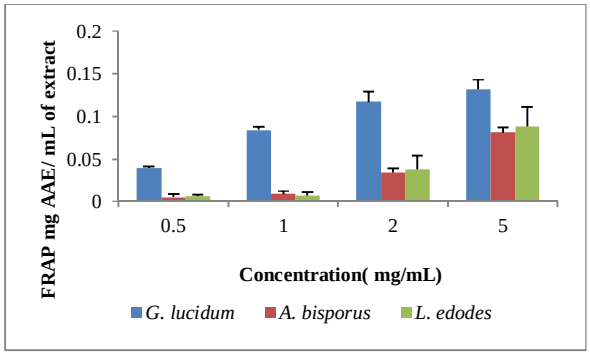

Fig. 3: Ferric Reducing Antioxidant Power (FRAP) of the extracts

D. Comparison between Antioxidant Assays

All the three methods used (DPPH, ABTS, FRAP) for the evaluation of antioxidant activity of aqueous extract of the three mushroom species were spectrophotometric methods.

1) DPPH Assay: DPPH is one of the few stable and commercially available organic nitrogen radicals [2]. It is one of the most widely reported methods [3, 8, 9,10,15,16, 20] for the determination of antioxidant activity. DPPH is not a very tedious assay in terms of preparation of chemicals and also in terms of performing the assay and hence can be used for its operational simplicity. In the present study, the antioxidant activity of each sample was measured three times to test the reproducibility of the assay. DPPH showed high reproducibility. The only disadvantage of this assay is that it is not very cost effective and is not suitable for measuring the antioxidant capacity of plasma, because proteins are precipitated in the alcoholic reaction medium [12].

2) ABTS Assay: The ABTS assay uses ABTS radicals preformed by oxidation of ABTS with potassium persulphate. Thus, this assay becomes time consuming in terms of waiting for the ABTS radicals to be generated as it takes around 12-16 hours for the reaction of ABTS with potassium persulphate, unlike the DPPH assay where one does not have to wait for it to be generated. However, once the radicals are generated, it is a very simple assay in terms of performing the assay. The ABTS radical is soluble in water and organic solvents, enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compounds/samples [12]. One major drawback of this assay is that the radicals formed are not very stable and the results are not reproducible. Nevertheless, this assay is also widely reported for the measurement of antioxidant activity.

3) FRAP Assay: The FRAP assay is more tedious and time consuming in terms of preparing the chemicals of the working solution. It is a simple and inexpensive method and does not require the use of any exclusive chemicals. The results obtained in

FRAP are found to be reproducible for all the concentrations. Hence, FRAP is a suitable method for the determination of antioxidant activity. In one study done by Katalinic *et al*., in 2006 [18], they suggested that from the methodological point of view, the DPPH method is easy and accurate with regard to measuring the antioxidant activity of the extracts and also the results are highly reproducible and comparable to other antioxidant methods such as ABTS. In another study done by Thaipong *et al*., in 2006 [11] for estimating antioxidant activity from guava fruit extracts, they concluded that the FRAP assay showed high reproducibility, was simple and could be rapidly performed.

IV. CONCLUSION

The aim of the study was to compare three simple spectrophotometric methods (DPPH, ABTS and FRAP) of determination of antioxidant activity. Hence, the antioxidant activity of aqueous extracts of three mushroom species (*Ganoderma lucidum, Lentinus edodes and Agaricus bisporus)* was studied. For the DPPH method, the highest antioxidant activity was observed by the aqueous extracts of *G. lucidum* for all the concentrations (0.5-5.0 mg/mL). Similarly *G. lucidum* also showed high antioxidant activity for ABTS and FRAP assays thus proving it to be a potent antioxidant. *A. bisporus* and *L. edodes* also showed moderate antioxidant activities, but less than that of *G. lucidum.*Various methods are used in different laboratories for the evaluation of total antioxidant capacity. As seen from the present study, the results of various assays of the same material can differ significantly. The differences may be due to various kinds of antioxidants present in the samples which react differently with the radicals used. Every method has its own advantages and limitations in terms of cost, availability of chemicals, tediousness, preparation time, reproducibility, etc. In the present study, DPPH was found to be the most suitable method for the determination of antioxidant activity of mycelia of the three mushroom species because it could be rapidly performed and showed high reproducibility. In the DPPH method, the radicals did not have to be generated before the assay which turned out to be the biggest disadvantage of the ABTS method. The only disadvantage of the FRAP method was that it was time consuming in terms of preparation of chemicals. An attempt was made in this study to compare the different antioxidant assays. However, before selecting any particular assay, it is necessary to understand the mechanism underlying each method. It is important to realise which method will be best suited for the samples under study considering all the advantages and limitations available.

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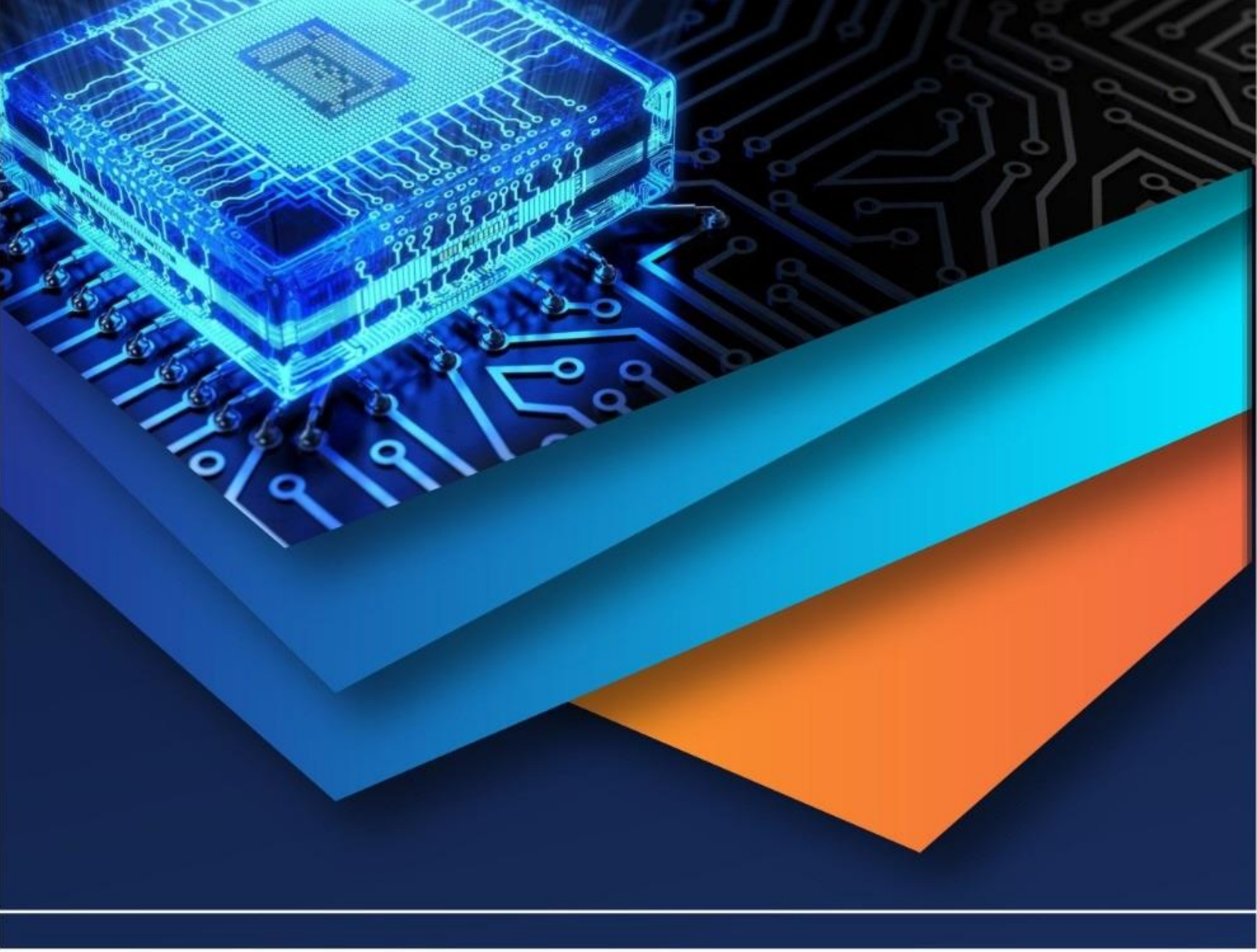

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